THE MECHANISM OF ACETAMINOPHEN-INDUCED METHEMOGLOBINEMIA IN DOGS AND CATS

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Abstract

Acetaminophen (APAP) overdose in most species is associated with hepatotoxicity due to covalent binding of the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) to hepatocellular proteins. Dogs and cats are unique in that acetaminophen overdose primarily causes methemoglobinemia and hemolysis. It has been proposed that NAPQI is the responsible reactive intermediate in dogs and cats but it does not have the chemical or pharmacokinetic characteristics that would favor methemoglobin formation. Our hypothesis is that the deficiency of *N*-acetyltransferase (NAT) activity in dogs and cats allows accumulation of another acetaminophen metabolite, *para*-aminophenol (PAP) that then induces methemoglobinemia. We have demonstrated that dogs, cats, mice and rats can all deacetylate APAP to PAP *in vitro* with no significant difference between species. Dogs were unable to acetylate PAP to APAP *in vitro* while feline acetylation of PAP *in vitro* was significantly less than rats and mice (feline V_{max}=0.088 ±0.004 nmol/mg prot/min, apparent rat V_{max}=0.63 ± 0.027 nmol/mg prot/min and apparent wildtype mouse V_{max}=1.42 ± 0.1 nmol/mg prot/min; P<0.001).

Erythrocytes of healthy dogs, cats and rats were exposed *in vitro* to APAP, NAPQI and PAP. The only compound that caused significant methemoglobin formation at physiologically relevant concentrations was PAP. The 500 μM PAP-induction of methemoglobin at 60 minutes was greater in feline and canine erythrocytes than in rat and mouse erythrocytes (60.9 ± 2.0%, 67.3 ± 1.9 %, 27.1 ± 2.5% and 28.5 ± 1.1% respectively, n=4 in each species) (P<0.01). Methemoglobin induction was significantly higher in erythrocytes from

NAT1/NAT2 double knockout mice than wildtype C57BL/6 mice (35.8 ± 1.2% versus 28.5 ± 1.1%; $P<0.05$). There was significantly more methemoglobin in the lysed erythrocytes of C57BL/6 mice and rats, most likely due to the loss of methemoglobin reductase activity. In dogs and cats, there was significantly more methemoglobin in the intact erythrocytes, likely reflecting prolonged PAP and oxyhemoglobin redox cycling due to lower NAT activity in these species. The *NAT1/NAT2* double knockout mice showed no significant difference in methemoglobin production between intact and lysed erythrocytes, possibly due to their high methemoglobin reductase activity but minimal acetylation. These results support our hypothesis that PAP contributes to APAP-induced methemoglobinemia and demonstrates a species difference in the sensitivity to methemoglobin induction. They also support that the decreased ability to re-acetylate PAP to APAP in dogs and cats is one contributing factor to the species sensitivity to APAP-induced methemoglobinemia.

The *in vivo* response of C57BL/6 wildtype and C57BL/6 *NAT1/NAT2* double knockout mice to hepatotoxic doses of APAP was compared. Both groups had markedly elevated liver enzymes and evidence of centrilobular hepatic necrosis. There were no significant changes in the erythrocyte morphology. One group of knockout mice treated with 250 mg/kg APAP intra-peritoneally (ip) did have a significantly lower hematocrit at 48 hours than wildtype mice treated with the same dose of APAP. However, this was not a consistent change across multiple experiments. These results suggest that deficient *N*-acetylation alone is usually insufficient to create susceptibility to methemoglobinemia and hemolytic anemia and that it must be accompanied by either a relative deficiency in the

ability to reverse methemoglobinemia or another defect.

There was significantly higher methemoglobin induction in knockout mice compared to wildtype mice treated with 400 mg/kg PAP (P=0.008). This was due to the significantly higher methemoglobinemia in female knockout mice compared to female wildtype mice (P=0.0047). There was no significant difference in methemoglobinemia in male knockout and wildtype mice. This again supports that a deficiency in *N*-acetylation would contribute to differing species sensitivity towards methemoglobin induction by the APAP metabolite PAP.

The H_2 blocker cimetidine has been proposed as a treatment for feline and canine APAP toxicity. However, it has been shown that cimetidine inhibits *N*-acetylation in rats. We examined the *in vitro* effect of cimetidine on feline APAP *N*-acetylation. There was a significant inhibition of acetylation activity, suggesting the possibility that cimetidine could increase the risk of methemoglobinemia.

Our findings support that the hypothesis that PAP is the metabolite responsible for APAP hematotoxicity and that deficient *N*-acetylation in dogs and cats is a contributing factor to their high species sensitivity to PAP-induced methemoglobinemia. Furthermore, we have shown that cimetidine inhibits feline *N*-acetylation of PAP and therefore should not be used in the therapy of feline APAP toxicity.

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First and foremost, I would like to thank my supervisor, Alastair Cribb for his patience and guidance throughout my PhD. Alastair's insight, logic and integrity has been truly amazing. His commitment to my program throughout was constant, despite the unforeseen distance and distractions during the last two years.

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Thank you to my supervisory committee, Shelley Burton, Cathy Chan, Cindy Gaskill, Luis Bates and Scott Grossman. Shelley and Cindy, you have truly been supportive friends throughout. Thank you to Denis Grant for the donation of *NAT1/NAT2* knockout mice.

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Finally, I would like to thank the Atlantic Veterinary College, the Canadian Institutes of Health Research and the Canada Research Chairs program. None of this endeavor would have been possible without the generous financial support of these organizations.

I'll finish with some wisdom from the most amazing 95-year-old woman I have ever met.

"In simplicity there is contentment."

Doris McCarthy, 2002.

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ABBREVIATIONS

AcCOA	Acetyl Coenzyme A
ADR	Adverse drug reaction
ALT	alanine transferase
ANOVA	one-way analysis of variance
AST	aspartate transaminase
APAP:	acetaminophen
ATP	adenosine tri-phosphate
BHT	2,6-Di-*tert*-butyl-4-methylphenol
BPAP	4-hydroxybutyranilide
BQI	benzoquinoneimine
BSA	bovine serum albumin
CAR	constitutive androstane receptor
Cb_5R	cytochrome b_5 reductase
$\cdot CCl_3$	trichloromethyl free radical
CV	coefficient of variation
CYP	cytochrome P-450 enzyme
DMSO	dimethyl sulfoxide (Me_2SO)
DTT	DL-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
G6PD	Glucose-6-phosphate dehydrogenase deficiency
GSH	glutathione

GST	glutathione transferase
HCT	hematocrit
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IP	intraperitoneal
KCl	potassium chloride
mdr1	multi-drug resistance gene
NacPABA	*N*-acetylated *para*-aminobenzoic acid
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NAPQI:	*N*-acetyl-*p*-benzoquinoneimine
NAT:	*N*-acetyltransferase
PABA	*para*-aminobenzoic acid
PAP-BA	*para*-aminophenol-butyric acid
PAP:	*para*-aminophenol
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PAS	para-aminosalicylic acid
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
Prot	protein
PXR	pregnane X receptor
SAM	S-adenosylmethionine

SULT	sulfotransferase
TEA	triethylamine
TEDK	buffer containing TEA EDTA, DTT and KCl
UDGPA	uridine diphosphoglucuronic acid
UGT	UDP glucuronosyl transferase
UV	ultraviolet
v/v	volume/volume

1. GENERAL INTRODUCTION

1.1 Scope of thesis

This thesis describes a series of studies aimed at understanding the role of species differences in drug metabolism in the development of acetaminophen-induced hematotoxicity in dogs and cats.

Acetaminophen (*N*-acetyl-*para*-aminophenol, APAP) is one of the most common analgesics and antipyretics used in humans worldwide. Although usually considered safe with minimal adverse effects, APAP overdose in humans is associated with hepatotoxicity [1]. In fact, APAP is the drug toxicity most commonly reported to human poison control centres in Britain and North America due to both accidental intoxications and intentional overdoses [2-4].

Human hepatotoxicity is characterized by a centrolobular necrosis that occurs following the binding of an APAP reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) to hepatocellular proteins [5]. Many other species also develop hepatotoxicity following APAP intoxication, but the sensitivities vary with the quantity of NAPQI produced [6].

Acetaminophen toxicity is also a common occurrence in dogs and cats and is frequently reported to animal poison control centres [7-9]. Dogs and cats, however, develop hematotoxicity characterized by both hemolysis and methemoglobinemia [10-13]. Dogs who survive the initial hematotoxicity can develop a hepatotoxicity similar to that of humans [14,15].

The reactive metabolite for canine and feline APAP-induced hematotoxicity

has been presumed to be NAPQI, the same reactive metabolite that causes hepatotoxicity in other species [16]. However, NAPQI has never been shown to induce methemoglobinemia or hemolysis. Another reactive metabolite of APAP that is produced by a minor metabolic pathway is *para*-aminophenol (PAP). This metabolite is a known inducer of methemoglobinemia in rats, mice and dogs [17-19]. Dogs and cats are known to be deficient in the *N*-acetyltransferase (NAT) enzymes that detoxify PAP [20,21]. This could predispose them to APAP-induced methemoglobinemia and hemolytic anemia.

Current treatment protocols for APAP toxicity in dogs and cats are based on the premise that the underlying toxic metabolite is NAPQI. Treatment is approximately 65% successful in cats diagnosed immediately and treated using current recommendations [10]. If another metabolite such as PAP is shown to be involved, then it may be possible to modify the therapy and improve the outcome.

Understanding species differences in adverse drug reactions (ADR) is also relevant for human medicine, both for the development of new drugs as well as the use of appropriate animal models. It is essential during preclinical trials that new drugs be thoroughly tested to ensure safety for future patients. This involves the use of laboratory animals. A thorough understanding of species differences in the metabolism of drugs and the underlying mechanisms for ADR allows us to better test future medication. It also allows us to know when to

appropriately extrapolate an ADR from a laboratory species to humans, and when to avoid rejection of a harmless drug by inappropriate extrapolation.

The intent of the work presented in this thesis is to explore the metabolic basis of acetaminophen toxicity in dogs and cats in order to elucidate the mechanism of this species-specific toxicity. This knowledge has the potential to be applied to veterinary medicine by way of improving the treatment of affected dogs and cats, and to human medicine through better understanding of species differences in adverse drug reactions.

1.2 Drug Metabolism

A drug is any chemical compound that can be used in animals or humans to help in the diagnosis, treatment, cure, mitigation or prevention of disease or other abnormal condition. This typically occurs by interaction of the drug or one of its metabolites with a specific receptor in the body. The extent of the pharmacological effect is usually dependent on the concentration of the drug or its metabolites at that receptor. The drug concentration in turn is dependent on the pharmacokinetics, or movement of the drug through the body, including absorption, distribution and elimination. Elimination of drugs includes both biotransformation (metabolism) and excretion from the body [22-24]

The majority of drugs are excreted to some extent in the urine [23]. Less common routes include biliary, pulmonary, milk and placental excretion [23,24]. Urine is produced by the filtration of blood in the glomerular capillaries[23]. The

glomerular filtrate is modified as it passes through the kidney tubules by active secretion of ionized substances in the proximal tubules and by resorption of water and solutes in both the proximal and distal tubules[23]. Polar (ionized) drugs are excreted largely unchanged in urine as they can pass into the glomerular filtrate or be actively excreted in the tubules[23,24]. Non-polar lipophilic drugs must be metabolized or biotransformed to facilitate their elimination in the urine [23,24]. Non-polar drugs are largely passively reabsorbed in the tubules because they are able to cross cell membranes, and are unsuitable for active secretion because they are unionized [23]. Drug metabolism typically produces a more polar metabolite, thereby decreasing tubular resorption and possibly increasing active tubular secretion [23].

Biotransformation of drugs is typically categorized into two phases. Phase I or functionalization reactions involve the exposure or introduction of functional groups to a drug, while phase II or conjugation reactions involve the conjugation of endogenous molecules to functional groups on drugs or drug metabolites [23,24]. Phase I reactions usually occur before phase II reactions.

The majority of biotransformation occurs in hepatocytes [23]. The liver is well perfused, receiving blood directly from the intestines via the portal vein and from the heart by the hepatic artery [25]. The extensive hepatic sinusoidal system lacks a basement membrane which ensures that parenchymal cells are directly in contact with mixed blood from both the portal vein and hepatic artery [26]. Lipophilic drugs diffuse easily through hepatocyte cell membranes while

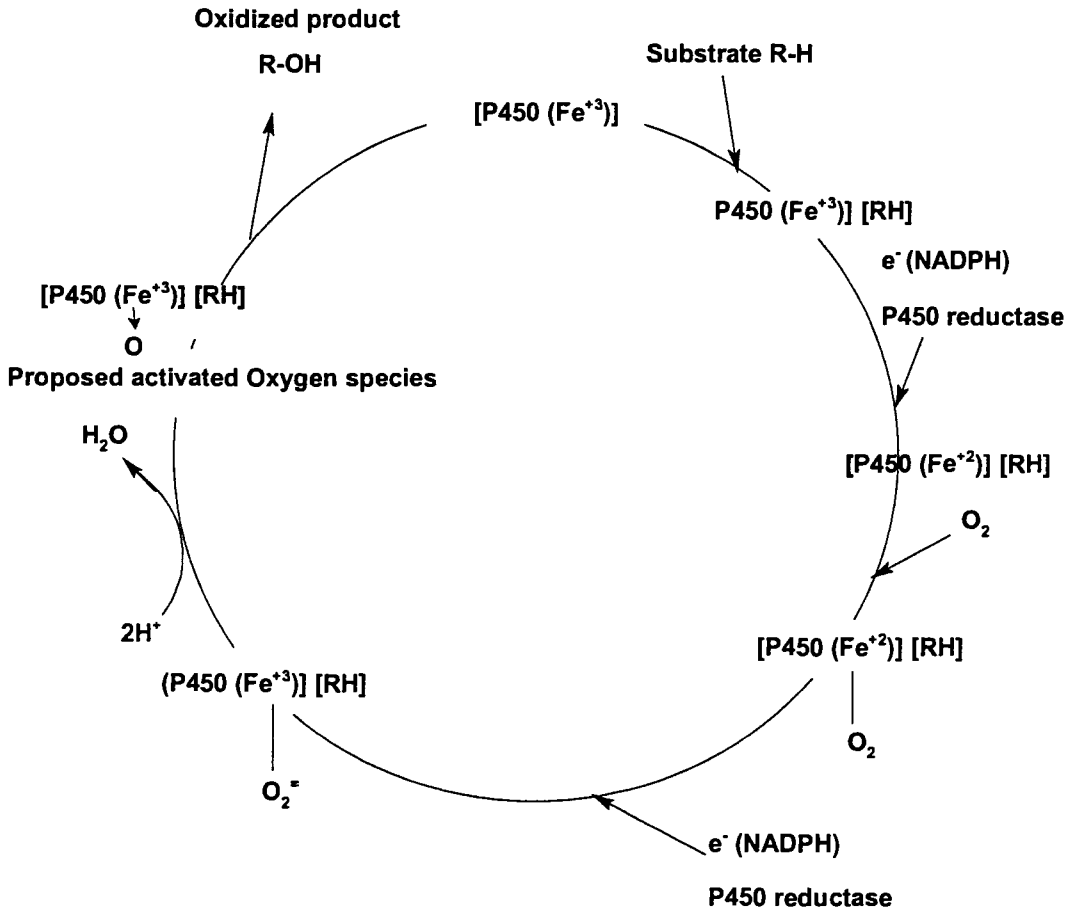
numerous active transporters aid the entry of the non-lipophilic drugs [23,25]. The metabolism of drugs is catalyzed by numerous enzymes, each with particular subcellular locations, cofactor needs, substrate affinities, tissue concentrations and efficacy [23,24].

Phase I (functionalization) reactions include oxidation, reduction and hydrolysis [24]. The primary phase I enzymes are the hemoprotein-containing cytochrome P450 enzymes (CYP enzymes) which are also called mixed function oxidases or microsomal monooxygenases [23,24]. These enzymes are involved in the biotransformation of endogenous substrates such as steroids, prostaglandins and fatty acids as well as xenobiotics [24]. Most CYP enzymes are located in the wall of the smooth endoplasmic reticulum, mainly in hepatocytes, but also in other sites such as the Clara cells in the lung and in intestinal epithelial cells [24]. They are primarily involved in oxidation reactions and require the cofactor nicotinamide adenine dinucleotide-phosphate (NADPH) and an intermediary enzyme, P450 reductase. The CYP enzymes are unable to accommodate two electrons simultaneously from NADPH. The NADPH first donates two electrons to the flavoprotein, P450 reductase. The electrons then pass to the iron in the CYP enzymes and from there to molecular oxygen. The oxygen is then inserted onto a drug (Figure 1.1) [24]. Although CYP enzymes are usually involved in oxidative reactions, they can also catalyze reduction reactions [27].

Over 1,000 CYP genes have been identified [24]. These genes are

Figure 1.1: Oxidative reaction by CYP enzymes

Oxidation of xenobiotics by cytochrome CYP enzymes requires cofactor NADPH for the donation of 2 electrons to P450 reductase. P450 reductase passes the electrons on oxygen via the CYP enzymes. Oxygen is then inserted on to a drug. (Modified from a Short Course on the Metabolism of Drugs given by Dr. Tom Baillie, University of Washington, 1993.)



classified by amino acid sequence into families and subfamilies [24,27]. The classification does not reflect substrate preference or enzyme location. The three families CYP1-3 contain the main CYP enzymes involved in drug metabolism [24]. These enzymes vary with species, individuals (polymorphism), gender, environment, age and disease status [27]. Some of the CYP enzymes are constitutive while others are inducible and not expressed unless exposed to a particular substrate [27].

Other enzyme systems in addition to CYP enzymes can catalyze Phase I reactions. These include flavin-containing mixed function mono-oxidases, cytosolic alcohol and aldehyde dehydrogenases and mitochondrial mono- and diamine oxidases [24,27].

Phase I reduction reactions are less common than oxidation reactions [24]. Reduction reactions convert compounds containing a nitro or azo group to amines[27]. They can occur in the liver or be catalyzed by intestinal microflora [27]. Compounds containing nitro groups can be reduced by NADPH-cytochrome P450 reductase in the smooth endoplasmic reticulum or by nitro reductase in the cytoplasm [27]. Nitro-compounds can also be reduced by hemoglobin, leading to the production of methemoglobin [27]. The latter will be covered in depth in the discussion of co-oxidation of drugs and oxyhemoglobin.

Hydrolysis is the final type of Phase I reaction. There are several families of hydrolases including non-specific esterases, and amidases as well as epoxide hydrolases [27]. The hydrolytic enzymes are present in the plasma, liver, kidney

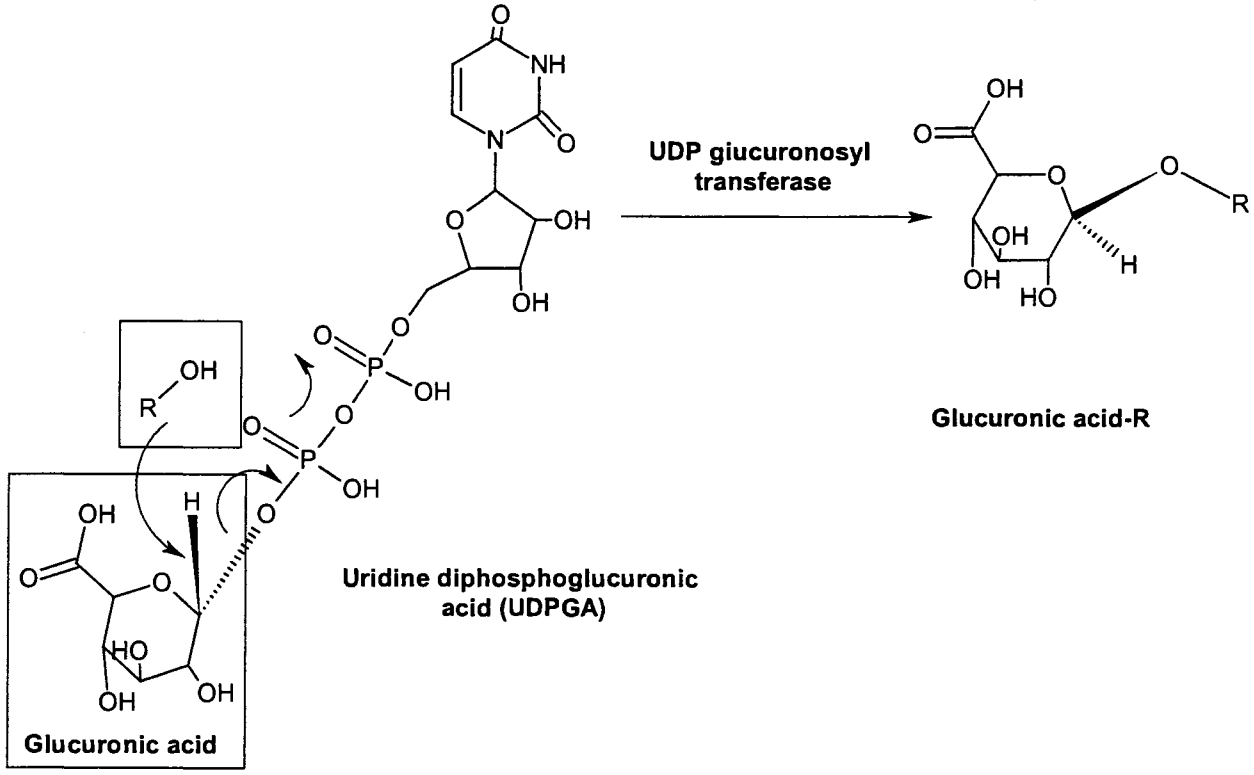
and intestine [27].

Phase 2 reactions are anabolic reactions that increase the water solubility of a drug or metabolite by conjugation with an endogenous substrate such as glutathione (GSH), glucuronide, sulphate or a methyl group [24,27,28]. The conjugation often occurs at the functional group resulting from Phase I reactions and frequently detoxifies reactive metabolites produced during the functionalization phase [24,27,28].

The most common conjugation reaction is glucuronidation (Figure 1.2). This reaction is catalyzed by a family of endoplasmic reticulum enzymes called uridine diphosphate-glucuronosyl transferases (UGT) [27]. The UGT enzymes are categorized into two families and various subfamilies based on the amino acid sequence [27]. The UGT enzymes transfer glucuronic acid from uridine diphosphoglucuronic acid (UDPGA) which is derived from the breakdown of glycogen [27]. This pathway is difficult to saturate as the cellular stores of glycogen tend to be high. The acceptor molecules include phenols, alcohols, aromatic amines and carboxylic acids [27]. There are species differences in the ability to conjugate with glucuronides. Cats have a pseudo gene for UGT1A6, the enzyme responsible for glucuronidating phenols such as acetaminophen [29]. This results in markedly decreased glucuronidation activity and prolonged clearance for drugs such as acetaminophen, chloramphenicol and salicylic acid. Feline glucuronidation of endogenous substrates such as bilirubin and testosterone is normal. This is because feline glucuronide transferases are

Figure 1.2: Xenobiotic glucuronidation

Transfer of glucuronic acid from uridine diphosphoglucuronic acid to a xenobiotic (R) is catalyzed by UDP glucuronosyl transferase. (Modified from a Short Course on the Metabolism of Drugs given by Dr. Tom Baillie, University of Washington,1993)



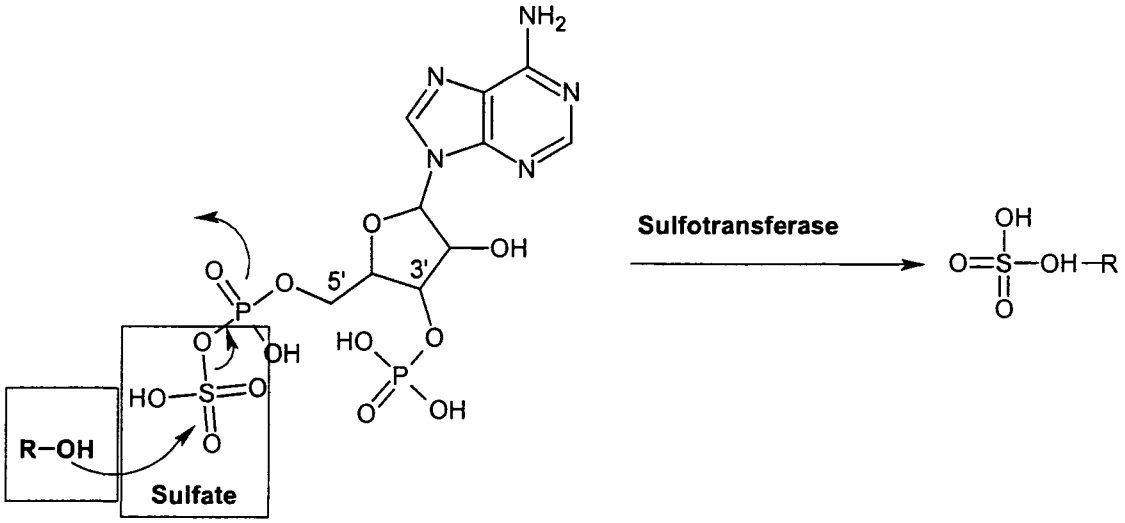
unremarkable except for fewer UGT1A isoforms, which is likely an evolutionary development [29]. Cats are obligate carnivores and therefore are exposed to fewer plant chemicals that require glucuronidation [29]. Gunn rats are a mutant strain of Wistar rats that have no UGT1A1 due to a genetic mutation. They cannot glucuronidate bilirubin or certain drugs and are used as a model for human Crigler Najjar disease [30].

Sulfation is catalyzed by steroid and phenol sulfotransferases (SULTs) (Figure 1.3)[27]. Both groups are cytosolic enzymes that use 3'phosphoadenosine-5'phosphosulfate (PAPS) as a cofactor for the transfer of sulfonate (SO_3^-) [27]. The steroid sulfotransferases are responsible for sulfating mainly endogenous steroids while the phenol transferases catalyze the sulfation of many drugs [27]. This pathway can become saturated because the supply of sulfate is limited [27]. As with all other metabolic enzymes, there are species and individual differences. For example, sulfotransferase activity in rats varies with age and gender [27,31].

Conjugation with GSH is catalyzed by glutathione-S transferases (GST) (Figure 1.4). These enzymes are divided into 4 families: alpha, mu, pi and theta [27]. Most GST enzymes are located in the cytosol and make up > 10% of the cytosolic protein [27]. They are most plentiful in the liver and kidney [27]. Conjugation with GSH is important for the removal of many of the reactive metabolites produced during Phase I reactions. Glutathione can react with electrophiles by either nucleophilic displacement at an electrophilic site or

Figure 1.3: Xenobiotic Sulfation

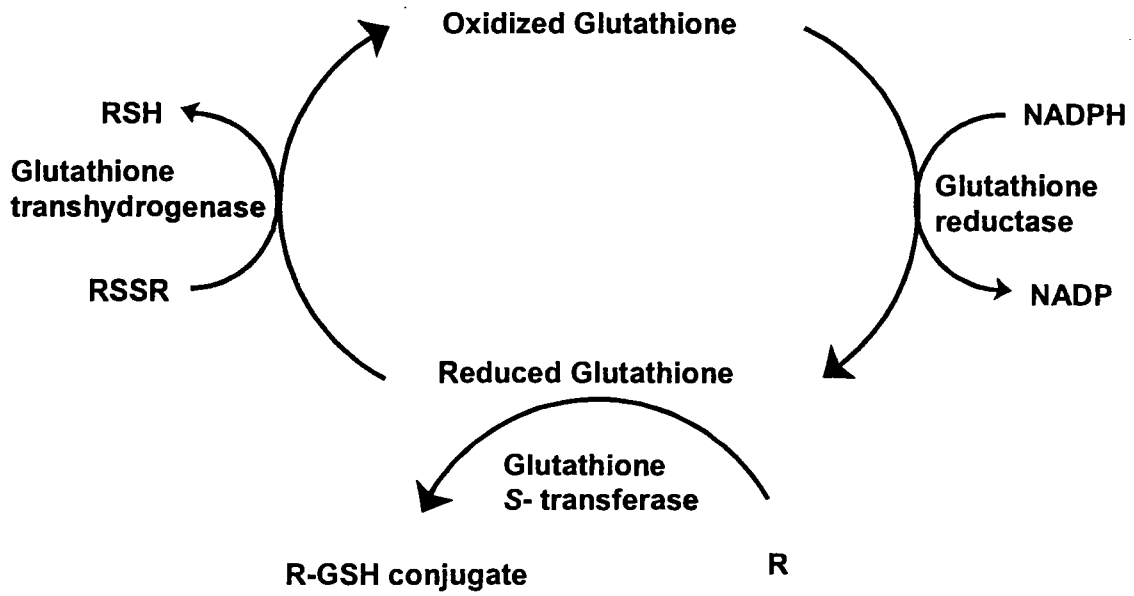
Transfer of sulphate from 3'-phosphoadenosine-5'phosphosulfate (PAPS) to xenobiotics is catalyzed by sulfotransferases. (Modified from a Short Course on the Metabolism of Drugs given by Dr. Tom Baillie, University of Washington,1993).



3'-phosphoadenosine-5'-phosphosulfate (PAPS)

Figure 1.4: Conjugation with glutathione

Reduced glutathione (GSH) is transferred to a nucleophilic xenobiotic (R) by glutathione transferase. Glutathione can also donate an electron to an electron deficient double bond.



nucleophilic addition to an electron deficient double bond [27]. The quantity of GSH available for reaction varies with the nutritional status [27]. Other phase II conjugation reactions include methylation, acetylation and amino acid conjugation. Acetylation will be covered in detail later in the introduction.

Methylation is a minor conjugation pathway. *N, O, S*-methyltransferases catalyze the conjugation of a methyl group to nitrogen, sulfur or oxygen with the cofactor *S*-adenosylmethionine (SAM) and often decrease the water solubility of compounds. Methylation may mask functional groups from other phase II reactions [27]. Methyltranserases typically attach a methyl group to phenols, catechols and aliphatic or aromatic amines [27].

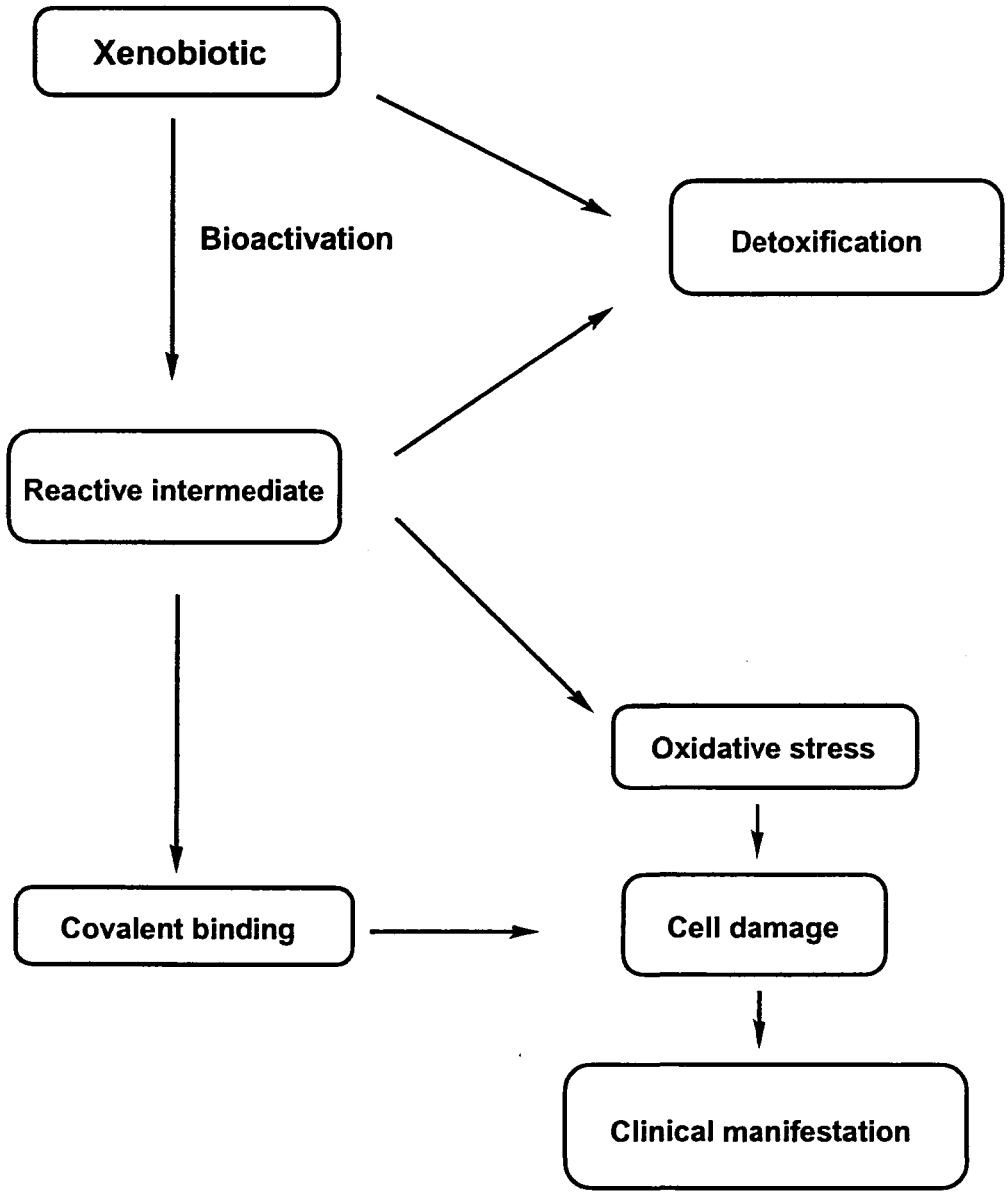
The chemical and pharmacological characteristics of a drug change during biotransformation. Some drugs, such as phenobarbital, lose their pharmacological activity following metabolism [32]. Other drugs, such as diazepam, retain their pharmacologic activity [32]. Some metabolites have stronger pharmacological action than the parent compound. For example, morphine-6- glucuronide is more potent than parent compound morphine [33]. Some drugs are initially inactive prodrugs. They may be given this way to overcome difficulties with gastrointestinal irritability, unpalatability, poor intestinal absorption, or first pass metabolism of the active drug [23,24]. Some drugs are activated to reactive metabolites that may be electrophiles, free radicals, nucleophiles or redox-active reactants that react indiscriminately with nearby endogenous molecules containing susceptible functional groups [23,24,27].

Reactive metabolites can also have physicochemical properties that adversely affect the local microenvironment such as changing the pH [23]. At non-toxic doses, reactive intermediates are typically detoxified to non-reactive metabolites. However, if production of the reactive metabolites increases and overwhelms the detoxification mechanisms, or if the individual or species is deficient in the detoxification processes, then toxicity occurs [27] (Figure 1.5).

1.3 Comparative drug disposition

The disposition or pharmacokinetics of foreign substances (xenobiotics) varies between species and individuals due to differences in anatomy, physiology, metabolism, gender, age, and health [23,24]. Even species that appear similar, such as dogs and cats, or rats and mice, can be markedly different. For example, dogs have a greater blood volume (90 ml/kg) than cats (70 ml/kg) and therefore drugs with a low volume of distribution will have a higher plasma concentration in cats than dogs if given at the same dosage[23]. The bioavailability or absorption may vary markedly between species. For example, the antibiotic metronidazole is metabolized by reduction. Metronidazole has a high bioavailability of 50-100% in dogs, cats and horses but only 2-10% bioavailability in ruminants because of reduction by rumen microflora. An example of a drug varying within a species is the prolonged clinical effect of

Figure 1.5: Bioactivation and detoxification of xenobiotics



barbiturates in Greyhounds compared to other dog breeds due to the low fat to lean ratio of sight hounds [34].

Comparative pharmacogenetics examines the genetic differences between individuals and species that affect the pharmacokinetics of drugs [35-38]. This includes polymorphisms for receptors, metabolic enzymes, transport proteins and cofactors [35-39]. The variations may have no clinical effect or may drastically alter the pharmacologic effect or toxic threshold [39]. An example of a transport protein that can show genetic variation is the multi-drug resistance protein, P-glycoprotein. This protein contributes to the blood-brain barrier by pumping drugs such as ivermectin out of the blood-brain barrier back to the blood. Some collies have a mutation of the multi-drug resistance gene, *mdr1,* that results in a lack of P-glycoprotein [40]. These dogs have a low tolerance to ivermectin because more is able to cross the blood brain barrier, resulting in tremors, ataxia and coma [40,41].

Cytochrome P450 enzymes provide many examples of differences in metabolism between individuals and species. Celecoxib is metabolized by CYP2C9 in humans and most likely by CYP2D15 in dogs [42]. Tributyltin is dealkylated by both CYP2C11 and CYP2C6 in male rats and by only CYP2C6 in female rats because the females lack CYP2C11 activity. Greyhounds have prolonged propofol induced anesthesia due to lower activity of the particular CYP enzymes that metabolize propofol in dogs [43]

Some enzymes show polymorphisms within species due to genetic

mutations, resulting in altered transcription or protein stability [38]. For example, CYP2C9 is responsible for metabolism of losartan to its more potent metabolite E3174 in humans [44,45]. Some people produce less E3174 because of a polymorphism of CYP2C9 that results in decreased CYP2C9 activity [44,45]. People who have the slow polymorphism for *N*-acetylase 2 (slow acetylators) are at increased risk to develop hypersensitivity reactions to sulfonamides [46]. Some polymorphisms are potentially clinically important only if combined with another polymorphism. For example, the drug thiazolsulfone is associated with hemolytic anemia in people who are both slow acetylators and deficient in glucose-6-phophate dehydrogenase (G6PD) [47].

Drug metabolism can also vary following induction or down-regulation of enzyme transcription by xenobiotics or endogenous compounds such as steroids. For example, phenobarbital binds to intranuclear receptors such as CAR (constitutive androstane receptor) and PXR (pregnane X receptor) that then bind to response elements on the DNA, leading to increased transcription of CYP enzymes [48,49]. Phenobarbital consequently increases CYP activity and can alter the metabolism of other drugs such as phenylbutazone, glucocorticoids and even itself [48,49].

Some drugs act as competitive or non-competitive inhibitors of the CYP enzymes. For example, chloramphenicol is an irreversible suicide inhibitor of CYP2B11, a canine CYP enzyme that is normally induced by phenobarbital[50]. Cimetidine is a non-competitive inhibitor of several CYP enzymes[51].

1.4 Adverse Drug Reactions

The World Health Organization defines adverse drug reactions (ADR) as noxious or unintended effects of a drug that occur at an appropriate dose used for prophylaxis, diagnosis, or therapy [52]. Unfortunately, adverse drug reactions are not uncommon. A 2002 prospective analysis of hospital admissions in the UK found approximately 6.5% of all admissions were due to ADR [53]. This was in agreement with an earlier meta-analysis showing that 6.7% of all hospital admissions in the United States were due to ADR [54]. The most common targets during an ADR are macromolecules such as nucleic acids (especially DNA) and proteins or smaller molecules such as membrane lipids [55]. The target must be exposed to a sufficient concentration of the reactive metabolite or toxicant and thus is often in the immediate vicinity of reactive metabolite production [55]. The target must also have the reactivity or steric conformation to enter into either covalent or non-covalent reactions with the toxic chemical [55].

Adverse drug reactions can be classified as either "dose-dependent" (type A) or "dose-independent" (type B or idiosyncratic) [56,57]. Most ADR are predictable, dose-dependent Type A reactions: the higher the drug dose, the more patients affected and the more severe the reactions [57]. This type of reaction can usually be identified during preclinical testing of a drug [57]. Patients with concurrent disease, concurrent medication administration, or genetic susceptibility can be hyper-susceptible to dose-dependent ADR due to changes to the drug pharmacokinetics or pharmacodynamics [57]. Type A reactions can

be subcategorized by mechanism of action into pharmacological and chemical-based [56,57]. Pharmacological based reactions arise through an undesired pharmacologic effect of a drug acting on a specific target or receptor [56,57]. These reactions can involve an exaggerated primary response or an unavoidable secondary effect [56,57]. An example of a pharmacological reaction is non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulceration caused by prostaglandin inhibition. This is the most common cause of ADR admittance to human hospitals in the UK [53,58]. Chemical-based dose-dependent ADR occur due to intrinsic chemical properties of a drug and its metabolites [57]. These reactions involve non-specific binding of a drug or its metabolites to nearby proteins or nucleic acids or disruption of cell membranes [57]. The target reflects the site of detoxification and toxin accumulation as well as the susceptibility of the cell type [56,57]. For example, the reactive metabolite NAPQI is produced during biotransformation of acetaminophen by hepatic P450 enzymes [59]. If a sufficient quantity of NAPQI is produced, it binds to hepatic proteins, disrupting cellular function and leading to hepatocyte necrosis[60,61]. Clinical signs of a pharmacological based ADR are dependent on the pharmacological effect of a drug while the clinical signs of a chemical based ADR are dependent on the target organ [57].

Idiosyncratic reactions are dose-independent, unpredictable ADR that occur at drug concentrations within the therapeutic reference range [56,57]. These reactions are dependent on characteristics of the individual and cannot be

reproduced experimentally [57]. Although idiosyncratic ADR are usually uncommon, they can occur frequently in a particular population with a common attribute such as a genetic polymorphism [56]. The idiosyncratic ADR in these individuals are dose-dependent. Idiosyncratic drug reactions occur due to the chemical characteristics of a drug [57]. The clinical appearance of a reaction varies with the underlying mechanism and target organ. Most are associated with an immune-mediated process. For example, the clinical signs of the immune-mediated reactions seen with sulfonamide toxicity in dogs vary from a nephropathy, dermatopathy or hepatotopathy to blood dyscrasias such as agranulocytosis, eosinophilia, thrombocytopenia or aplastic anemia [62].

Drug molecules are usually too small to be immunogenic but some drugs can serve as haptens or modify proteins, making them into autoantigens, with the protein-drug complex becoming the target of the immune system [63].

1.5 Hepatotoxicity

The liver is a common target of drug toxicity because of its location and function as the major detoxifying organ. The liver receives blood from the intestinal tract via the portal vein and from the body by the hepatic artery and is the first organ to receive absorbed nutrients and potential toxins. The architecture of the liver, with its marked vascularization and lack of sinusoidal basement membranes, is designed to maximize the uptake of nutrients and potential toxins [25]. Numerous active transporters in the hepatocyte cell

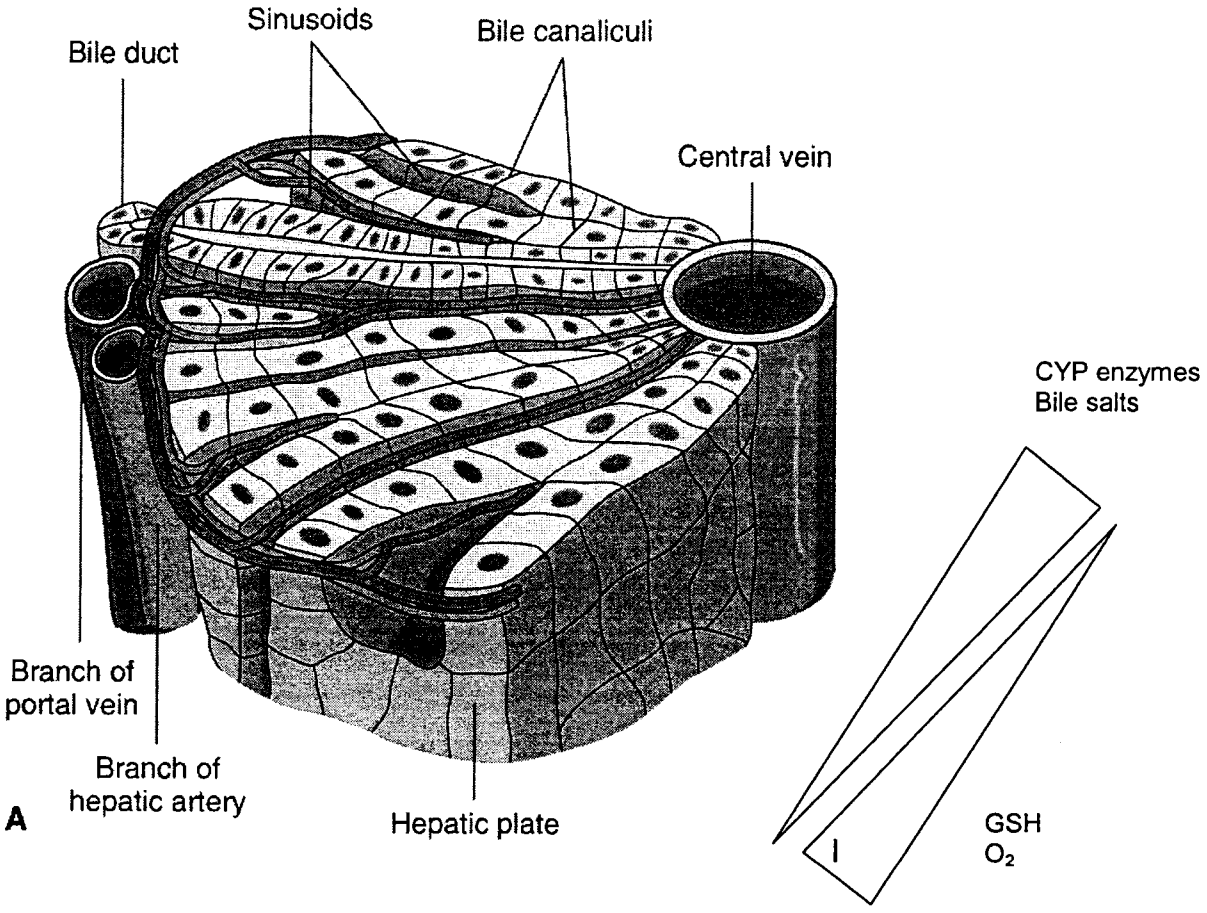
membranes aid the entry of non-lipophilic toxins [25]. Thus the liver, due to its function of removing and metabolizing toxins, is one of the most vulnerable organs to toxicity [25].

The liver is divided into lobules, which are further divided into centrolobular, midzonal and periportal regions (Figure 1.6). In the centrolobular area are the central veins. At the corners of the lobules are branches of the hepatic artery, portal veins and bile duct [25]. The cells in the perilobular area receive the most oxygen and have higher bile salt and GSH concentrations [25]. The centrolobular area has the highest number of P450 enzymes and less GSH and oxygen [25].

Hepatic injury resulting from a toxin is dependent on the nature of the toxicant [25]. Common reactions include hepatic lipidosis, hepatocyte death, bile duct damage, sinusoidal disorders, fibrosis and neoplasia [25]. The response depends on whether an insult is acute or chronic, the concentration of the toxic insult, and population of affected cells [25]. For instance, hepatic lipidosis is a reversible change characterized by an increase in the lipid content of hepatocytes and is commonly seen with acute toxicity. With a more severe or prolonged injury, hepatocytes can also show diffuse, random or focal apoptosis or necrosis. Focal necrosis is often centrolobular in location. Fibrosis and biliary proliferation are seen with chronic toxicity [25]. Hepatic neoplasia can occur following exposure to certain toxins such as aflatoxin [25,26].

Figure 1.6: The liver lobule

The liver is divided into lobules. At the corner of each lobule is a portal triad containing a hepatic artery, portal vein and bile duct. At the centre of each lobule is the central vein. The CYP enzymes are most concentrated close to the central vein. The oxygen and GSH concentrations are highest adjacent to the portal triad. (Modified from J Cullen, Chapter 8, Biliary System and Exocrine Pancreas In: Pathologic Basis of Veterinary Disease. McGavin MD, Zachari JF Editors. Elsevier, 2007, pg 393-461)



The location of the hepatic injury is dependent on the toxin and site of reactive metabolite production [25]. For example, the highly reactive trichloromethyl free radical ($\cdot CCl_3$) is produced from carbon tetrachloride (CCl_4) by CYP enzyme catalyzed oxidation [25]. Thus CCl_4 toxicity is associated with centrolobular necrosis because the centrolobular regions of the liver lobules have a high concentration of CYP enzymes and low concentration of GSH [25]. The oxygen-rich perilobular area is affected by iron overload because that is the location of hepatic uptake of iron and there is more oxygen to contribute to the oxidizing affects [25]. Bile ducts are affected by metabolites that are concentrated in the bile. Ito cells, which store vitamin A, are affected by vitamin A toxicity [25]. Thus hepatotoxins are associated with characteristic pathological changes that depend largely on where the toxin is taken up or metabolized to a reactive intermediate.

1.6 Hematotoxicity

Hematotoxicity refers to adverse effects of toxicants on blood-forming organs such as the bone marrow, and on the constituents of blood, including platelets, neutrophils, monocytes, lymphocytes and erythrocytes [64]. In this thesis, I will confine my discussions to the erythrocytes.

Mature erythrocytes have no nuclei or organelles. The main protein constituent of the erythrocyte cytosol is hemoglobin [65]. Its primary purpose is the transportation of oxygen from the lungs to the tissues of the body [65].

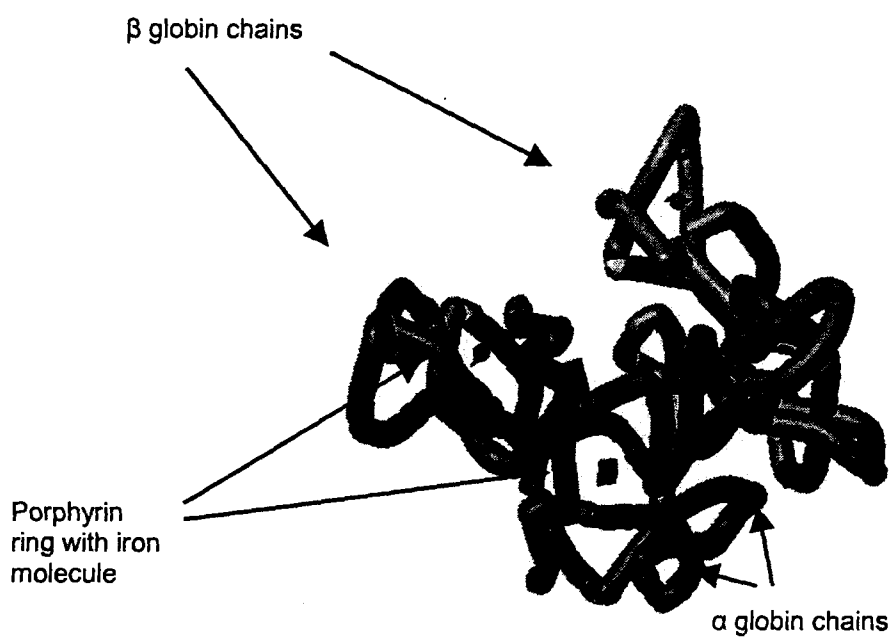
Hemoglobin is a heterotetramer of 2 pairs of different polypeptide globin chains (α and non-α) in a helical pattern that form tight hydrophobic pockets for four heme groups, composed of a porphyrin ring and an iron molecule [65] (Figure 1.7).

Oxygen transportation occurs by attachment and dissociation of oxygen to the iron (Fe) molecules. The binding and releasing of oxygen to Fe occurs by structural changes of the hemoglobin through realignment of the helices [65]. The helical structure changes are controlled by external influences such as pH, temperature, and binding of the chemical bisphosphoglycerate (2,3 BPG). Oxygen binding is also affected by whether the Fe is in the ferric or ferrous form. Oxyhemoglobin contains Fe in the ferrous form. Oxygen cannot bind to hemoglobin containing Fe in the ferric form [65].

There are 1-3 million new red blood cells produced per second in the bone marrow in humans [65]. Red cell proliferation and differentiation occurs following stimulation of early erythrocyte precursors by growth factors such as erythropoietin [65,66]. The maturation and differentiation involves the progressive synthesis of hemoglobin and loss of nucleoli, cytoplasmic golgi apparatus, mitochondria and ribosomes [67]. There is also a reduction in cell volume and expulsion of the pyknotic nucleus [67]. Immature red blood cells that first enter circulation are called reticulocytes or polychromatophils. Reticulocytes contain precipitated mitochondria, RNA and some organelles that are lost within 48 hours in most species [67]. Mature red blood cells contain no mitochondria and

Figure 1.7: Structure of hemoglobin

Hemoglobin is composed of 2 α and 2 non-α (usually β) globin chains. Each chain forms a three dimensional pocket containing a heme group composed of a porphyrin ring and a molecule of iron. (Courtesy of Dr. Alphonso Lopez)



therefore cannot perform oxidative metabolism [67]. Instead, erythrocytes rely on anaerobic glycolysis to metabolize glucose to lactate. This pathway is called the Embden-Meyerhof pathway and produces a net gain of 2 moles of adenosine tri-phosphate (ATP) [67]. Approximately 10% of the glucose metabolized in the Embden-Meyerhof pathway is shunted to the pentose phosphate pathway for NADPH production[68].

Red blood cells are highly vulnerable to toxicity. This is because the marked proliferation rate, lack of organelles and limited ability to make energy limit the ability of red blood cells to respond to toxins [64]. Hematotoxicity can be associated with decreased erythrocyte function, or decreased numbers of red blood cells (anemia) secondary to decreased bone marrow production or increased destruction [64].

Erythrocyte production is decreased by agents that affect mitosis. For example doxorubicin forms a complex with DNA and the DNA repair enzyme topoisomerase that results in double stranded DNA breaks [69]. Sulfasalazine can cause decreased production of red blood cells through competition with folate which is required for making nucleic acids [70]. Lead toxicity causes anemia by inhibition of delta aminolevulinic acid dehydrogenase which interferes with the production of the porphyrin ring of hemoglobin [71].

A common type of drug-associated hematotoxicity is anemia due to immune-mediated hemolytic destruction. This occurs by three mechanisms [72]. In hapten-mediated destruction, a small drug acts as a hapten after binding to

the red blood cell [72]. A common example is penicillin[73,74]. Most individuals treated with penicillin develop IgM antibodies to the erythrocyte benzylpenicilloyl determinants but these do not cause hemolysis [73,74]. Low numbers of penicillin-treated patients develop IgG antibodies to proteins on drug-coated red blood cells. The IgG leads to phagocytosis of erythrocytes by splenic macrophages [73,74]. The second type of a drug-associated immune-mediated reaction is a ternary complex mechanism [72]. In this type of a reaction, a neoantigen is created by a drug binding to a specific site on a blood cell, with the combination recognized by the immune system[72]. Destruction is usually via complement [72]. An example of a drug acting via this mechanism is temafloxacin [75]. The third mechanism of drug-induced hemolytic anemia is via an autoantibody reaction in which antibodies are produced against erythrocytes after the drug is no longer present [72]. The classic example of a drug acting in this fashion is α-methyl dopa which may bind to red cell membranes of early precursor red blood cells causing an autoantibody to form against the mature red blood cell [76,77].

Oxidation can affect both the erythrocyte lifespan and function [64]. Erythrocytes have protective anti-oxidant systems, including NADH methemoglobin reductase, GSH, catalase and superoxide dismutase [64]. If the anti-oxidant systems are overwhelmed, oxidation can occur in 3 areas in the red blood cell: lipids in the red cell membrane, proteins in the globin chains or iron in the hemoglobin. Oxidation causes erythrocyte changes such as eccentrocyte

formation, Heinz bodies and methemoglobin production [64]. Eccentrocytes, formerly known as blister cells, are red blood cells with oxidative damage to red cell skeletal proteins followed by external forces that push the membrane together resulting in the eccentrically displaced hemoglobin [64].

Heinz bodies are homogeneous round inclusions in erythrocytes that occur following the development of disulphide bonds in the hemoglobin sulfhydryl groups [64]. This results in precipitation of the hemoglobin which then adheres to the inner cell membrane [64]. Peroxidation of unsaturated fatty acids and polymerization of phospholipids and protein may also occur, causing an increased permeability to cations and tendency of the erythrocytes to swell [64]. The erythrocytes become less deformable and are prone to damage when passing through the micro-circulation, and are subsequently removed by macrophages [64].

Methemoglobin is hemoglobin with iron in the oxidized ferric (Fe^{3+}) form [78]. The tight hydrophobic pocket formed by the globin chains around the heme groups usually maintains a non-polar environment in which it is difficult to oxidize ferrous (Fe^{2+}) iron to Fe^{3+} despite the proximity of iron to oxygen [78]. However, approximately 0.5-3.0 % of Fe^{2+} in red blood cells is oxidized to Fe^{3+} daily [78]. This may be associated with an oxidizing agent or a nucleophile such as Cl^- replacing the bound oxygen, which allows the oxygen to discharge as superoxide, leaving Fe in the ferric form (Fe^{3+}) [68,78].

Ferric iron is unable to bind to oxygen. The shape and stability of the

other hemes in an erythrocyte are also affected by a heme group containing ferric iron [78]. The other hemes are unable to dissociate from oxygen [78].

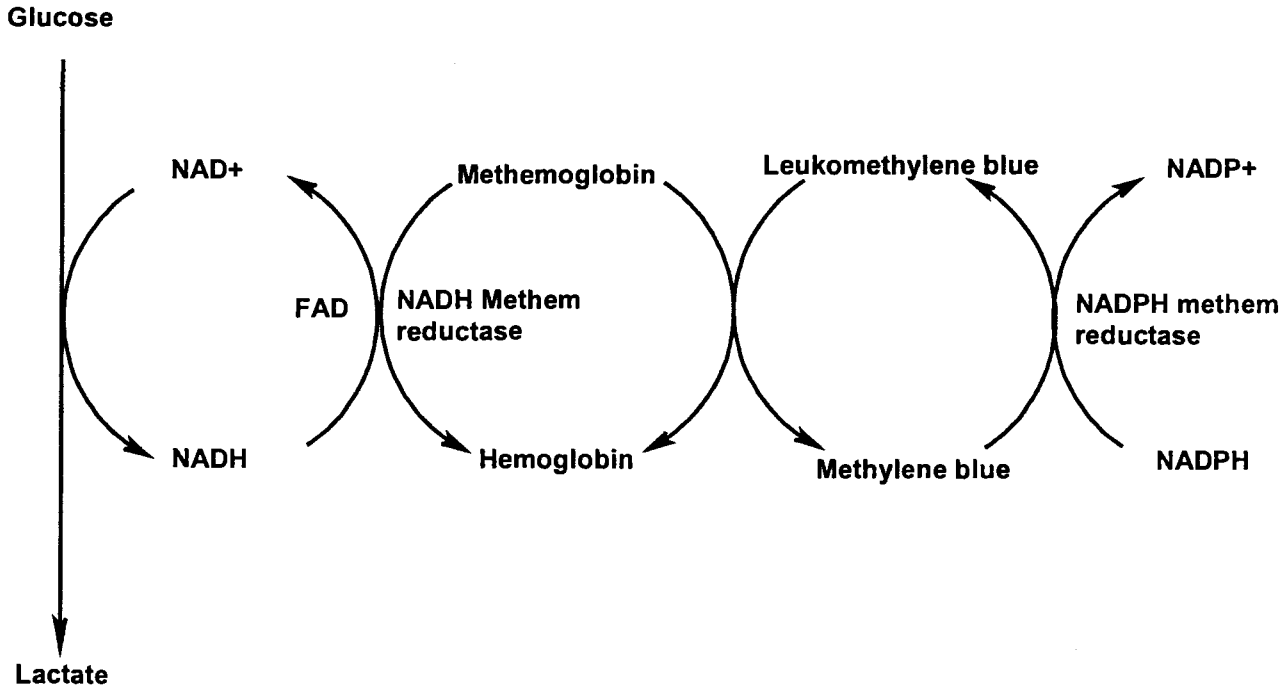
At any one time, the methemoglobin percentage of total hemoglobin in most animals is <1.0% [68]. This is because erythrocytes have adapted to the inadvertent oxidation of iron by developing efficient reduction. The primary method of methemoglobin reduction (>95%) is via methemoglobin reductase (also named cytochrome b_5 reductase (Cb_5R) or NADH diaphorase) [78]. This enzyme is NADH-dependent and requires flavin dinucleotide (FAD) as a cofactor [78]. There is also a minor contribution by NADPH-dependent methemoglobin reductase (<5%) toward the total methemoglobin reduction activity as well as non-enzymatic reduction by glutathione (GSH) or ascorbic acid (Figure 1.8).

Methemoglobin concentrations of 10-25% usually produce brown or blue discoloration of mucous membranes and skin [79]. At percentages of 35-40% methemoglobin, clinical signs such as fatigue, headaches, tachycardia and slight exercise intolerance are seen in people [79]. At 60% methemoglobinemia, marked lethargy and stupor occur, and concentrations >70% are usually fatal [79].

Causes of methemoglobinemia can be categorized as exogenous or endogenous. Exogenous oxidants that can induce methemoglobin formation include acetaminophen in dogs and cats, benzocaine, lidocaine and phenazopyridine in cats, nitrates or nitrites in ruminants, and methylene blue in cats [80-83]. Endogenous etiologies include various genetic mutations of the

Figure 1.8: Reduction of methemoglobin

The majority of methemoglobin (>90%) is reduced by NADH-dependent methemoglobin reductase with the cofactor flavin adenine dinucleotide (FAD). The activity of NADPH methemoglobin reductase is negligible except during times of toxicity. The NADPH methemoglobin reductase requires an electron carrier such as methylene blue. An electron is transferred from NADPH to the electron carrier which then transfers the electron to methemoglobin.



different cofactors or enzymes involved in methemoglobin reduction [78]. These include cytochrome b_5 reductase deficiency, G6PD deficiency, FAD deficiency, or abnormal hemoglobin structure such as hemoglobin M in humans [78].

Cytochrome b_5 reductase deficiency, also known as NADH-dependent methemoglobin reductase deficiency, is the most frequently reported enzyme deficiency causing methemoglobinemia reported in dogs and cats [79,84,85]. It has an autosomal recessive inheritance in humans, but the mode of inheritance in dogs and cats has not been investigated [79,86]. Affected animals and humans do not show any clinical signs other than constant cyanosis [68,78]. Most affected dogs are not diagnosed until maturity or at the time of surgery when the blood is discovered to be dark in colour [79,86]. Red blood cell numbers are increased due to a secondary erythrocytosis [79,86]. Severity of clinical signs in dogs with this deficiency appears to relate to acuteness of onset rather than degree of methemoglobinemia [79,86]. Diagnosis requires documenting the increased methemoglobin percentage, ruling out access to an oxidant and measurement of cytochrome b_5 reductase activity [79,86]. The standard treatment for humans with NADH dependent methemoglobin reductase deficiency is methylene blue, with a response usually seen within 1-2 hours of therapy [78].

Flavin adenine dinucleotide deficiency has been reported in one horse. The 20-year-old Spanish mustang mare had a history of persistent methemoglobinemia, eccentrocytosis and pyknocytosis that were not due to oxidants [87]. She had no clinical signs other than cyanosis [87].

Hemoglobin M is reported in humans but has never been reported in animals [65]. Heme groups are held in pockets between the E and F α helices of each of the four globin chains [65]. The iron forms four bonds with the porphyrin ring and one covalent bond with the imidazole nitrogen of the proximal histidine residue on the F α globin helix [65]. Hemoglobin M has a tyrosine substituted for either this proximal histidine or a distal histidine on the opposite side of the ring adjacent to the iron [65]. Unlike histidine, tyrosine forms a complex with Fe^{3+} that cannot be reduced back to the ferrous state following oxidation [65]. This disease is inherited in humans as an autosomal dominant disorder [65].

Glucose-6-phosphate-dehydrogenase deficiency is the most common enzyme deficiency in humans with >200 million people affected world-wide [88]. This deficiency is associated with increased resistance to malaria and therefore is quite common in Africa and the Middle East [88]. This enzyme is required for the production of NADPH, which is required for active GSH and catalase [88]. Glucose-6-phosphate-dehydrogenase deficiency results in a decreased ability to withstand oxidation. The G6PD gene is located on the X chromosome, so homozygous males are more likely to be clinically affected than heterozygote females [88]. In females, one of the X chromosomes in each cell is randomly inactivated during embryonic development (lyonization). Therefore, the disease in females varies in severity from mild to severe depending on the effect of the random lyonization of X chromosomes. In humans with this deficiency, clinical signs of hemolytic anemia and methemoglobinemia are usually only seen

following exposure to oxidants [88]. A G6DP deficiency has been reported in a six-month-old male American Saddlebred horse that had no clinical signs associated with its deficiency [89]. Coincidental findings of eccentrocytes and pyknocytes during a referral examination for dermatitis led to the diagnosis of G6PD deficiency [89].

1.7 Acetaminophen

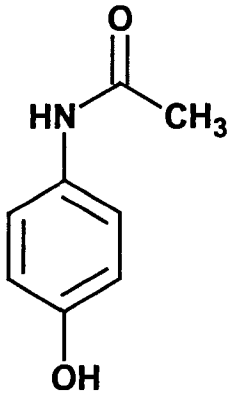
Acetaminophen and paracetamol are generic names for the chemical *N*-acetyl-*para*-aminophenol (APAP) (Figure 1.9). Acetaminophen was developed in the late 1800's following the mistaken release of acetanilide to two doctors investigating the use of napthalene as an antiparasitic agent [90]. Acetanilide (Figure 1.9), a biproduct of the dye industry never previously given to humans, had no antiparasitic effect but was noticeably antipyretic. Within two years, Bayer was selling the closely related drug, phenacetin (Figure 1.9). Although acetaminophen was developed shortly after phenacetin, common use didn't begin until the 1950's when phenacetin was removed from the market due to excess abuse-related nephropathies and hemolytic anemias [91,92]. The McNeil Laboratories began marketing acetaminophen as a single product, Tylenol®, in the mid 1950's. Acetaminophen is now one of the most commonly used analgesics and antipyretics worldwide and is present in over 200 different medications including over-the-counter and prescription drugs.

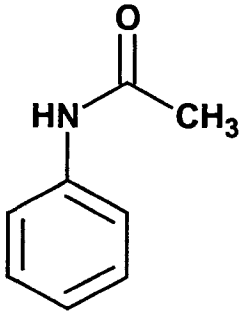
Acetaminophen is an aminophenol derivative with an acetyl group at the

Figure 1.9: Chemical structures of acetaminophen, acetanilide and phenacetin.

Acetaminophen

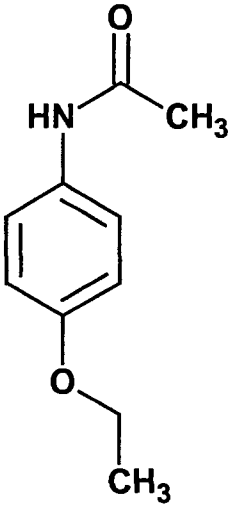
***N*-acetyl-*para*-aminophenol**





Acetanilide

***N*-phenylacetamide**



Phenacetin

***N*-(4-ethoxyphenyl)acetamide**

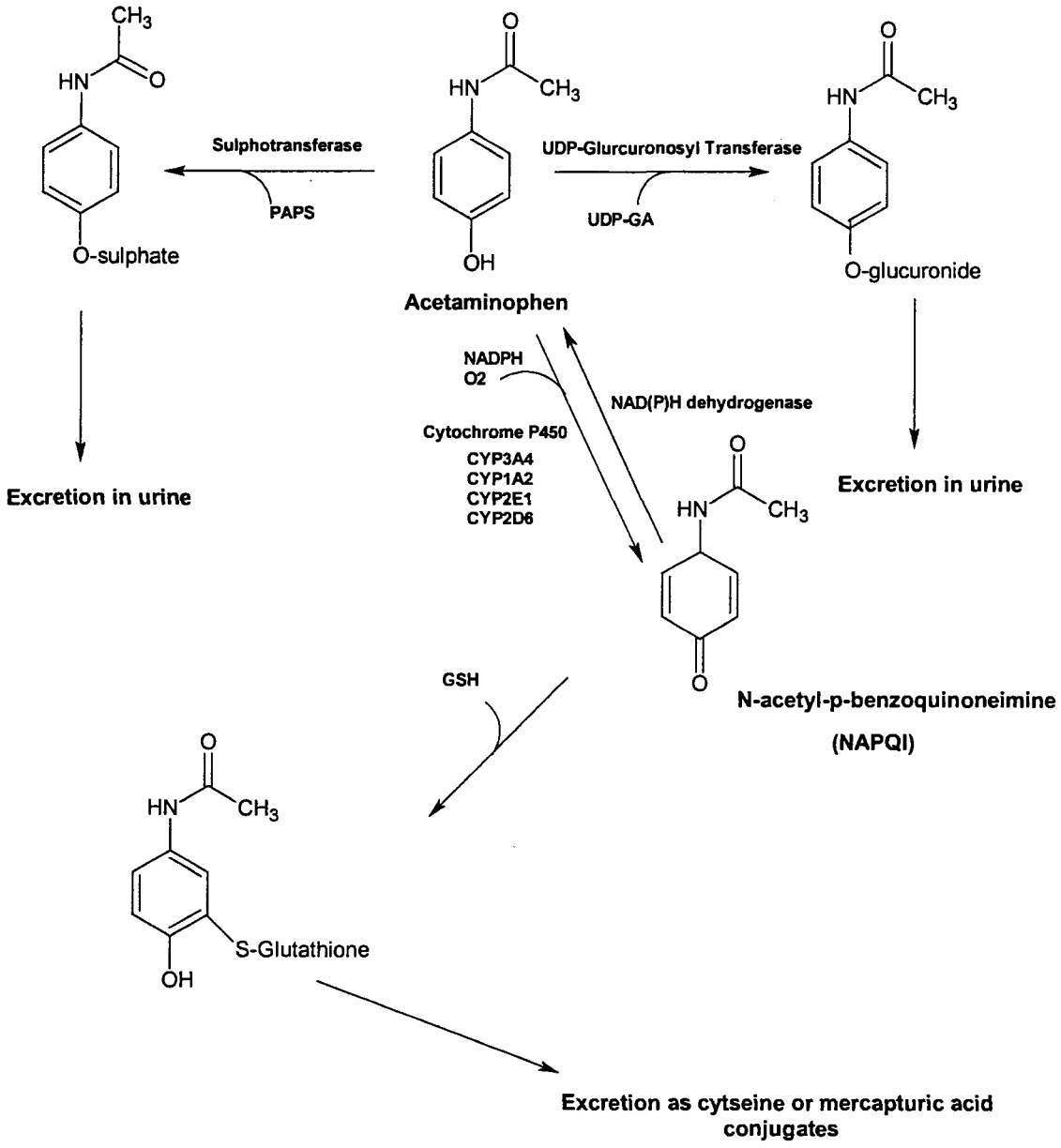
N position. Until recently, the mechanism of action of APAP was unknown [93]. It now appears that the analgesic effect of APAP is mediated through cannabinoid receptors in the central nervous system [94-96].

In people, 85-90% of APAP is metabolized in the liver and excreted in the urine as conjugates within 24 hours of ingestion [97]. In all species evaluated to date, the major biotransformation pathways are sulfation, glucuronidation and oxidation [97-100]. Following oxidation, NAPQI can be reduced back to APAP by NAD(P)H dehydrogenase or conjugated with glutathione and excreted as cysteine or mercapturic acid conjugates (Figure 1.10) [97]. The percentages of the varying metabolite pathways vary with the species [97-100]. In humans, approximately 20-30 % of APAP is sulfated, 45-55 % is glucuronidated and 15-25% is oxidized by P450 enzymes to the reactive metabolite NAPQI [97]. In rats, 10-20% of the urinary metabolites are cysteine or mercapturic acid conjugates while in dogs they account for 4-5 %, mice 15% and in cats 5-15% of the urinary metboilites [98,101,102].

1.8 Acetaminophen toxicity

Acetaminophen toxicity is a type A dose dependent adverse drug reaction [97,98]. Although APAP is typically considered safe at therapeutic doses, toxicity due to overdosage in humans is common [2-4]. Acetaminophen is the drug most frequently associated with self-poisoning in Britain and the United States [2-4]. Sulfate and glucuronosyl transferases become saturated with increasing

Figure 1.10: The major biotransformation pathways of acetaminophen



dosages of APAP. This leads to increased oxidation and NAPQI production [97]. The NAPQI continues to bind to GSH and be excreted as an innocuous product until GSH is depleted to <20% of its normal concentration [6]. The reactive intermediate NAPQI subsequently binds covalently to cysteine groups on hepatocellular proteins, eventually leading to hepatocellular functional abnormalities and cell death [5,60]. This hepatocellular death is characterized by centrolobular necrosis due to the higher concentration of P450 enzymes and lower concentration of GSH in the centrolobular regions of the liver [25]. Clinical signs of abdominal pain, vomiting and icterus begin at 36-48 hours post ingestion in humans [1]. Sensitivity and toxicity vary with species, but most non-human species including rats, rabbits, mice and hamsters also develop hepatic centrolobular necrosis [6]. Species sensitivity varies with the amount of oxidation and the quantity of NAPQI [6,99].

Dogs and cats are unique in that they primarily experience hematotoxicity associated with APAP toxicosis, characterized by methemoglobinemia and hemolytic anemia [10,13,103]. Much of the research concerning APAP toxicity in cats has involved retrospective or prospective studies describing the clinical effects and toxic dose of APAP [10,104]. Aronson in 1996 compiled data from 17 cases of feline APAP toxicity, providing an excellent summary of the clinical signs, toxic dose, therapy and outcome [10]. Finco in 1975 described two clinical and two experimental cases of feline APAP toxicosis [104]. Hematotoxicity from APAP administration is not as well recognised in dogs as it is in cats [13].

However, Savides et al 1984 described hematotoxicity in both dogs and cats at low, medium and high doses of APAP [98], and researchers have had to repeatedly overcome the development of methemoglobinemia while trying to develop an animal model for fulminant hepatic toxicity by using APAP in dogs [14,15,105]. Based on these studies and numerous published clinical cases, the toxic dosage of APAP in dogs is 150-200 mg/kg and in cats is 75 mg/kg [10,98]. Affected animals typically show profound depression, cyanosis, vomiting, tachypnea, facial or forelimb edema and brown coloured blood within 4-6 hours of intoxication due to the rapid development of methemoglobinemia [10,13,103]. If the animal survives, a hemolytic anemia can occur at approximately 48 hours [10,13,103]. Dogs can show hepatic centrolobular necrosis if they survive the initial hematotoxicity [14,15]. Cats have never been reported to develop centrilobular necrosis but may show hepatocellular degeneration with subsequent increases in serum activities of hepatic enzymes such as alanine transferase (ALT), likely due to hypoxia [98,106]. Keratoconjunctivitis sicca has also been rarely reported in dogs and cats with APAP toxicity [107].

Savides et al 1984 determined the metabolic pathways for APAP at low, medium and high doses in dogs and cats [98]. They demonstrated that the half-life of APAP increases with higher APAP doses in both species, but the increase is most pronounced in cats. The feline half-life of APAP increased from a half-life of 0.6 hours at 20 mg/kg APAP to a half-life of 4.8 hours at 120 mg/kg APAP [98]. This publication also reported an increase in oxidation of APAP in cats with

increasing APAP doses [98]. The percentage of APAP-cysteine and mercapturic acid metabolites in cats increased from 5% at 20 mg/kg to 10% at 120 mg/kg [98]. Savides (1984) and Welch (1966) have both demonstrated that cats glucuronidate <5% of APAP during biotransformation [98,108]. The minimal APAP glucuronidation activity present in cats is now known to be due to a feline pseudogene for UGT1A6 [109].

The APAP-induced hematotoxicity in cats has been attributed to a combination of 1) decreased glucuronidation of APAP leading to greater NAPQI formation, and 2) a higher number of reactive sulfhydryl groups in feline hemoglobin making feline erythrocytes a susceptible target to oxidative damage [16,98,108,109].

Gunn rats also have a marked decrease in glucuronidation due to a molecular defect. These mutant Wistar rats show an increased sensitivity to APAP-induced centrolobular necrosis but hematotoxicity is not observed [110]. Therefore, the decreased glucuronidation in cats may contribute to the susceptibility to APAP toxicity, but is unlikely to be the underlying reason for the hematotoxicity in cats and it cannot explain the hematotoxicity in dogs. Dogs glucuronidate 75% percent of APAP, yet develop hematotoxicity [98,111].

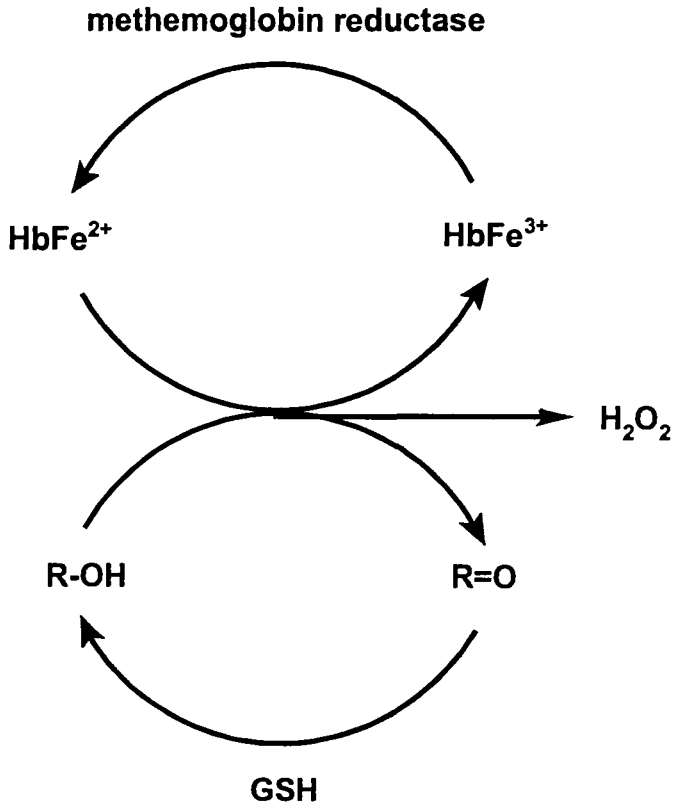
The toxic metabolite responsible for the APAP-induced hematotoxicity in dogs and cats has been presumed to be NAPQI. However, NAPQI does not have the appropriate pharmacokinetic or chemical characteristics to induce methemoglobinemia. It is produced in hepatocytes through oxidation of APAP

by CYP enzymes. Erythrocytes do not contain the CYP enzymes necessary for its production. At therapeutic doses of APAP, NAPQI binds covalently to GSH and is then excreted from the hepatocytes in an inactive form. The NAPQI-GSH complex is excreted in the urine as a cysteine or mercapturic acid conjugate [97]. At toxic doses the supply of GSH is exhausted. The NAPQI then binds covalently to other hepatocellular proteins [112]. The protein-bound NAPQI does not leak into the blood until the hepatocyte becomes necrotic, more than 12 hours post intoxication [60,113]. Clinical methemoglobinemia is evident in dogs and cats by 2-4 hours post-intoxication [10,13,16].

Many chemicals that induce methemoglobinemia, including dapsone hydroxylamine, phenylhydroxamine and sulfamethoxazole hydroxylamine, do so by a co-oxidation reaction with oxyhemoglobin, [19,114]. During co-oxidation reactions, an electron is removed from both the chemical and Fe^{2+} of oxyhemoglobin and is donated to oxygen, creating a reactive oxygen species such as hydrogen peroxide (Figure 1.11) [115,116]. The loss of an electron from oxyhemoglobin results in methemoglobin production. [115,116]. The methemoglobin is then reduced to oxyhemoglobin by methemoglobin reductase and the oxidized compound is reduced to the original metabolite through reduction by GSH [115,116]. This redox cycling of the chemical permits an on-going co-oxidation reaction with oxyhemoglobin to form more methemoglobin [115,116]. If the chemical is not removed from cycling by alternative biotransformation pathways, the ability of the cell to reduce the methemoglobin

Figure 1.11: Co-oxidation of oxyhemoglobin to methemoglobin

Some drugs can be co-oxidized with oxyhemoglobin. An electron is lost from both the drug and Fe^{2+} and donated to oxygen to form a reactive oxygen species such as hydrogen peroxide while oxidizing the drug and oxyhemoglobin.



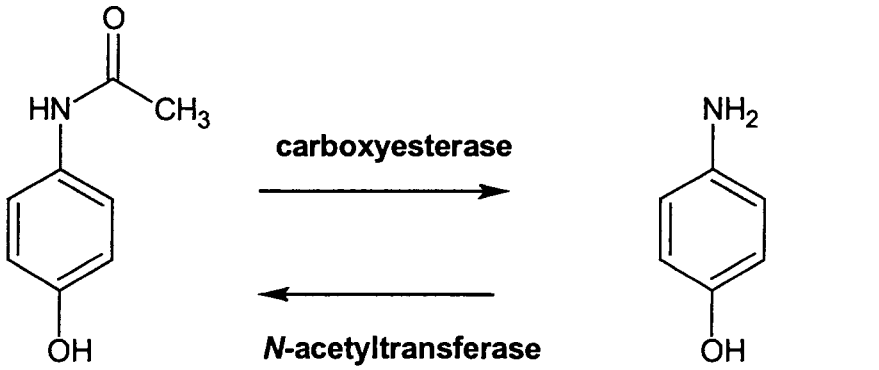
formed can be overwhelmed, leading to overt methemoglobinemia [115,116]. The reactive intermediate NAPQI does not have the chemical characteristics necessary to redox cycle with oxyhemoglobin. Covalent binding of NAPQI with GSH or another protein results in a stable conjugate [97]. There is, however, an APAP metabolite, PAP, that is known to co-oxidize with oxyhemoglobin [116].

1.9 *para*-Aminophenol

A minor APAP metabolic pathway is the deacetylation of APAP by microsomal carboxyesterases in the liver and kidney to the active metabolite PAP (Figure 1.12) [117-119]. This metabolite has been known to induce methemoglobinemia for many years [120]. *Para*-aminophenol contributes to the methemoglobinemia associated with aniline dye toxicity in humans [19,121]. Methemoglobin is produced via a co-oxidation reaction of PAP and oxyhemoglobin. The reaction produces a reactive oxygen species, methemoglobin, and oxidized PAP (benzoquinoneimine (BQI)) [115,116] (Figure 1.13). The BQI is reduced to PAP by GSH and the PAP continues to react with oxyhemoglobin until it is removed by biotransformation [116]. In rats and mice, PAP is primarily detoxified by *N*-acetylation to the parent compound APAP or through binding with GSH [122-124]. In rats, the *N*-acetylation and GSH binding is so efficient that the half-life of aminophenols is <5 minutes [19]. However, dogs and cats are deficient in *N*-acetyltransferase; this could predispose them to prolonged redox cycling

Figure 1.12: Deacetylation and *N*-acetylation of *para*-aminophenol

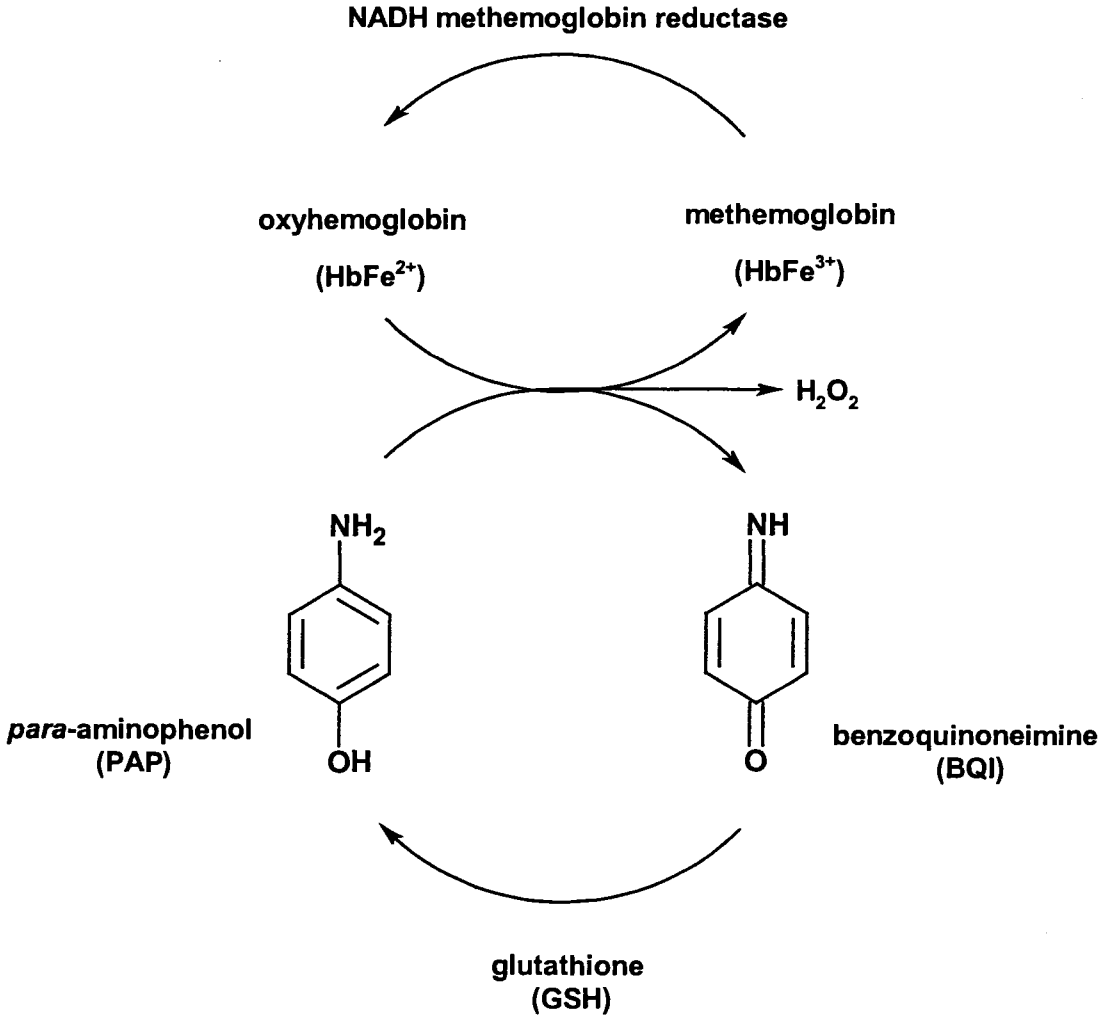
Acetaminophen is deacetylated by hepatic microsomal carboxyesterases to *para*-aminophenol. The majority of PAP is quickly reacetylated by *N*-acetyltransferase to the parent compound, APAP in rats and mice [122-124].



Acetaminophen **para-aminophenol**

Figure 1.13: Co-oxidation of *para*-aminophenoi and oxyhemoglobin

The co-oxidation of PAP and oxyhemoglobin produces methemoglobin, reactive oxygen species such as hydrogen peroxide and benzoquinoneimine (BQI).



of PAP and oxyhemoglobin and result in greater production of methemoglobin [20,21].

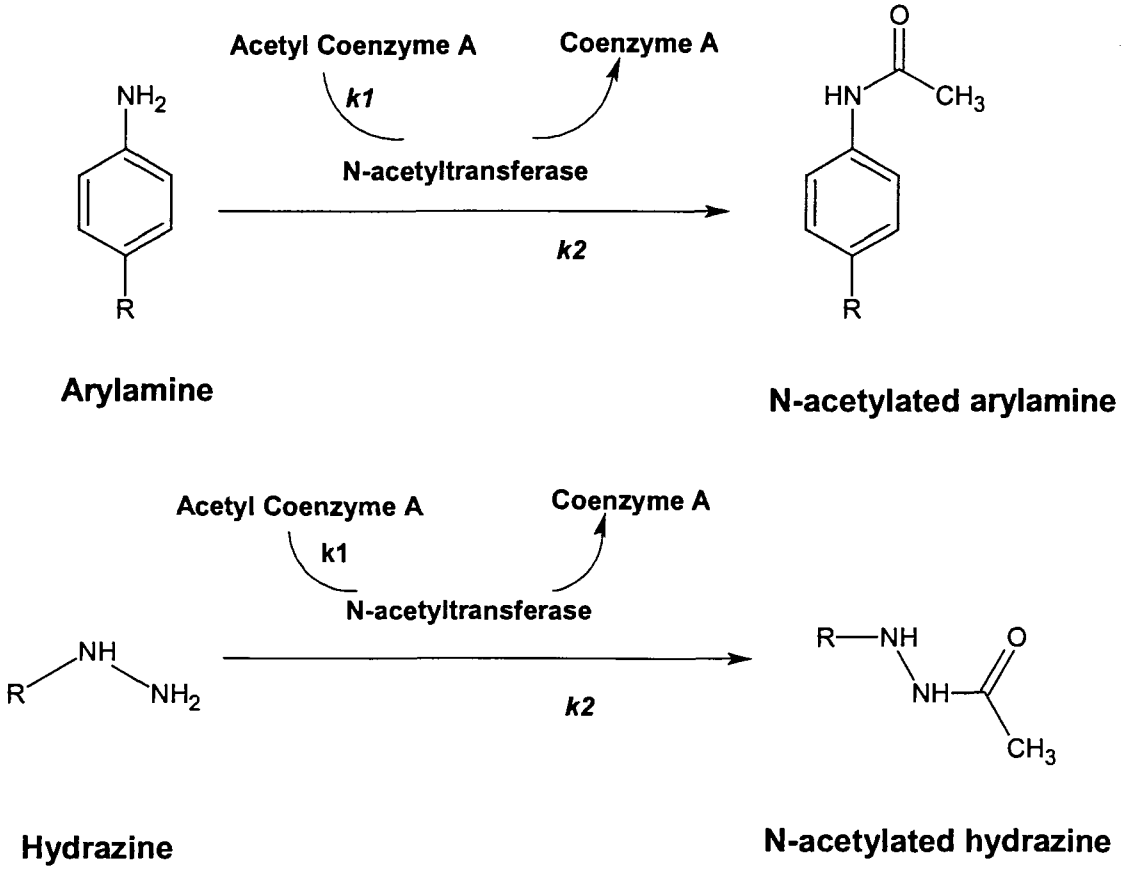
1.10 *N*-acetyltransferase

N-acetyltransferases (NAT; EC2.3.1.5) are cytosolic phase II biotransformation enzymes important in the metabolism of drugs and carcinogens [125]. The endogenous role of NAT enzymes appears to involve folate metabolism, because the folate metabolite *N*-acetylated *p*-aminobenzoylglutamate has been identified in urine [126]. Human fetal tissues contain NAT1 and this enzyme may be involved in neural tube development [127]. The NAT enzymes catalyze the acetylation of the nitrogen in primary amines of arylamines and hydrazines via a "bi bi ping pong" mechanism using an acetyl group from the donor acetyl coenzyme A (AcCoA) [128] (Figure 1.13). Bi bi ping pong enzymatic reactions are reactions that involve two substrates and two products. In *N*-acetylation, the first substrate is the acetyl group from acetyl coenzyme A that is transferred to the NAT enzyme (product one). Only once it is bound to an acetyl group can NAT bind with the chemical substrate (substrate two). The NAT then transfers the acetyl group to the substrate forming the second product [129].

Most species have two NAT isoenzymes, NAT1 and NAT2 [130]. The *NAT1* and *2* genes are both located on chromosome 8 in humans and it is speculated that *NAT2* may have originally been a duplication of *NAT1* [131].

Figure 1.14: *N*-acetylation of arylamines and hydrazines

N-acetylation occurs via a bi bi ping pong mechanism. An acetyl group is transferred to the NAT enzyme from acetyl coenzyme A (AcCoA). Only after AcCoA has been released, can NAT transfer the acetyl group an arylamine or hydrazine [129].



Although they show considerable homology, NAT1 and NAT2 differ in their preferred substrate and distribution in the body [132,133]. Human NAT1 is located in most tissues throughout the body while NAT2 is mainly in the liver and intestine [132,133]. A small amount of NAT2 is usually found in tissues containing NAT1 [132,133].

Human NAT1 catalyzes the acetylation of smaller molecules such as *p*-aminosalicylic acid (PAS) and *p*-aminobenzoic acid (PABA) [132]. Larger molecules, including many drugs such as sulfamethazine and procainamide, are acetylated by NAT2 [132]. The binding site on NAT enzymes is a catalytic triad containing cysteine [134,135]. The amino acids arginine (Arg) and phenylalanine (Phe) are important in the substrate specificity [136]. In particular, Phe contributes through steric control of recognition [136]. The phenyl ring of Phe may limit the entry of larger substrates to the catalytic triad in NAT1 [136]. There is also a flexible loop associated with the triad which may account for the overlap in substrate specificity [137]. Mutations in this area could also contribute to NAT1 polymorphisms.

Human NAT2 is polymorphic with individuals divided into slow, intermediate and fast acetylators [132]. The rate of acetylation can predispose to ADR or neoplasia. For example, individuals who are slow acetylators are more prone to sulfonamide-induced immune-mediated adverse drug reactions if they have additional predisposing factors such as a low ascorbate or GSH concentration [46,138]. Slow acetylators are also more predisposed to arylamine-

associated bladder cancer, while fast acetylators are more likely to develop arylamine-associated colon cancer [139]. Polymorphisms of NAT1 are uncommon and do not typically result in phenotypic variation in acetylation, however an increased incidence of a NAT1 rapid polymorphism has been demonstrated in some cancers [140-143].

The underlying mechanism of the *N*-acetylation polymorphism varies both between and within species. Slow acetylators in New Zealand White rabbits have a gene deletion of NAT2, [144] while human slow acetylators have the gene and the mRNA but quantitatively less NAT2 enzyme due to either less translation or decreased protein stability [35,145,146].

The enzymes NAT1 and NAT2 are differentiated by the amino acid sequence and substrate specificity [125]. The terminology between species can be confusing. In 1998, it was decided by the *N*-acetyltransferase nomenclature committee to maintain previous nomenclature [133]. Therefore, contrary to what happens in humans, NAT1 in most rodents including hamsters, mice and rats is polymorphic and has a substrate preference and distribution similar to that of human NAT2 while the rodent NAT2 is monomorphic with a substrate preference and distribution similar to that of human NAT1 [147,148]. Rabbit NAT1 and 2 have a similar substrate preference and distribution to human NAT1 and 2 [149]. Mice and rats also have a *NAT3* [150,151]. The NAT3 enzyme in rats has good activity but NAT3 in mice is poorly functional [150,151]. Humans have a pseudogene, *NATP,* that contains several deletions and does not produce an

enzyme. [152].

Canids and *Suncus murinus*, an Asian insectivore, are the only known mammals with no NAT genes [20,153]. Cats only have NAT1 [21]. Common ancestors of the dog and cat may have lacked NAT2, and subsequent to dividing into two separate species, NAT1 was also lost from dogs[20].

Although the NAT enzymes are well conserved across species, they are not required for survival. Lacking the enzymes or having only one enzyme appears to have no effect on dogs and cats other than an altered biotransformation of some drugs and carcinogens [154,155]. Dogs, because they lack both NAT enzymes, are essentially the ultimate slow acetylators.

1.11 Cimetidine

Several studies have investigated the prevention of and therapy for APAP hematotoxicity. Cats fed a diet high in the oxidant propylene glycol develop greater methemoglobin with APAP toxicity [156] while cats fed a diet rich in the antioxidant bioflavonoid, or treated with the anti-oxidant S-adenosylmethionine (SAMe) show no difference in methemoglobinemia but do demonstrate a protective effect against erythrocyte oxidation changes such as Heinz bodies [157,158]. Savides found intravenous *N*-acetylcysteine and intravenous sodium sulphate to be equally effective [98]. *N*-acetylcysteine has been shown to decrease the degree of both methemoglobinemia and hemolysis in cats [106,159,160]. The use of methylene blue, which is a common treatment for

methemoglobinemia in humans, has been used successfully in dogs in the fulminant hepatonecrosis animal model studies [14,15]. However methylene blue has not been found to be efficacious in cats, except when used in combination with *N*-acetylcysteine in females [160]. Thus the the primary treatment for APAP toxicosis in humans, dog and cats is *N*-acetylcysteine [160,161]. *N*-acetylcysteine increases available GSH by providing the GSH precursor cysteine and by providing sulfhydryl groups [160]. Cimetidine has also been proposed as an additional therapy for human, cat and dog APAP toxicosis [16,162,163]. The use of cimetidine for APAP toxicity is based on initial *in vivo* and *in vitro* studies in mice, rats and humans which indicated that cimetidine decreases NAPQI production by inhibition of P450 enzymes [162,163]. Subsequent studies in humans have shown no significant effect of cimetidine on NAPQI production and thus cimetidine is no longer recommended as adjunct therapy for human APAP toxicity [161,164-166]. However, cimetidine continues to be recommended for feline and canine APAP toxicity in some veterinary journal articles [16].

Svensson and Tomilo have demonstrated that cimetidine inhibits *N*-acetylation of PABA and procainamide in rats [167]. This is of potential concern in cats. If cimetidine also inhibits feline *N*-acetylation, then cimetidine may inhibit PAP detoxification both in the liver where PAP is produced and in erythrocytes where NAT1 may be instrumental in the removal of PAP from redox cycling during the co-oxidation process [168,169]. This could prolong APAP-induced methemoglobinemia in cats and would offer no protective effect if NAPQI is not

responsible for the methemoglobinemia and hepatotoxicity is not a major problem in cats [10]. Inhibition of *N*-acetylation by cimetidine is of no consequence in canine therapy as dogs have no NAT enzymes [16].

1.12 Summary

Acetaminophen hepatotoxicity and hematotoxicity are predictable, dose dependent, type A adverse drug reactions. In many species, including humans and many laboratory animals, APAP toxicity is associated with hepatotoxicity. Dogs and cats are unique in that they develop hematotoxicity characterized by hemolytic anemia and methemoglobinemia. It has often been assumed that the limited glucuronidation in cats leads to increased NAPQI production by oxidation, and that the high numbers of exposed hemoglobin sulfhydryl groups in cats results in a different target organ. However, other species, such as the Gunn rat, lack glucuronide transferase enzymes but do not show APAP-induced hematotoxicity. Instead, the Gunn rat is more sensitive to APAP-induced hepatoxicity than other rats. The dog, which has excellent glucuronidation conjugation activity and fewer sulfhydryl groups than the cat, still develops hematotoxicity after APAP exposure. In addition, NAPQI is not present in the blood to any large extent until after there has been significant hepatocellular degeneration, which does not occur until 12 hours post intoxication. Feline and canine methemoglobinemia peaks by 4-6 hours post APAP intoxication, which appears too early for NAPQI to be acting in the circulation. As well, NAPQI does

not have the chemical characteristics required to induce methemoglobinemia by either co-oxidation or removal of an electron from oxyhemoglobin. Circulating NAPQI in the blood is in an inactive form as it is still bound to hepatocellular proteins and is thus unable to bind to other proteins.

A minor biotransformation pathway of APAP is deacetylation by carboxyesterases. This produces the reactive metabolite PAP. This metabolite is known to induce methemoglobinemia by a co-oxidation reaction with oxyhemoglobin. Detoxification of PAP occurs primarily by *N*-acetylation via NAT enzymes to the parent compound APAP in rats and mice. Dogs have no NAT enzymes and cats have only NAT1. Therefore, dogs and cats may be exposed to larger amounts of PAP for a prolonged period of time following toxic doses of APAP, resulting in methemoglobinemia and hemolytic anemia.

1.13 Hypotheses

Based on this reasoning, the central hypothesis guiding this thesis is:

Dogs and cats experience hemolytic anemia and methemoglobinemia after exposure to toxic doses of acetaminophen because APAP is deacetylated to PAP. Dogs and cats are unable to or deficient at reacetylating PAP back to the parent compound APAP due to a deficiency of N-acetyltransferase.

In order to test this hypothesis, the following specific aims were addressed:

1. To determine if APAP is deacetylated to PAP and if PAP is *N*-acetylated to APAP *in vitro* by dog, cat, rat and mouse liver microsomes and cytosols respectively (Chapter 2).

2. To determine the methemoglobin forming potential of acetaminophen and its metabolites and role of *N*-acetyltransferase in species differences in sensitivity (Chapter 2).

3. To determine if *NAT1/NAT2* double knockout mice develop APAP-induced hematotoxicity (Chapter 3).

4. To compare PAP-induction of methemoglobin in *NAT1/NAT2* knockout and wildtype C57BL/6 mice (Chapter 3).

Cimetidine has been proposed as a therapeutic option in APAP toxicity in dogs and cats. However, evidence suggests that cimetidine may inhibit *N*-acetyltransferases. If the main hypothesis is correct, then inhibition of *N*-acetyltransferases could aggravate hematotoxicity in cats. Therefore we also tested the hypothesis that:

Cimetidine inhibits feline N-acetylation of PAP.

In order to test this hypothesis, the following specific aim was addressed:

1. To determine if cimetidine has an inhibitory effect on feline *N*-acetylation of PAP *in vitro* (Chapter 4).

1.14 References

1. Prescott LF, Roscoe P, Wright N, Brown SS. Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet 1971;1:519-22.

2. Watson WA, Litovitz TL, Klein-Schwartz W, et al. 2003 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2004;22:335-404.

3. Camidge DR, Wood RJ, Bateman DN. The epidemiology of self-poisoning in the UK. Br J Clin Pharmacol 2003;56:613-9.

4. Nourjah P, Ahmad SR, Karwoski C, Willy M. Estimates of acetaminophen (Paracetomal)-associated overdoses in the United States. Pharmacoepidemiol Drug Saf 2006;15:398-405.

5. Landin JS, Cohen SD, Khairallah EA. Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol 1996;141:299-307.

6. Davis DC, Potter WZ, Jollow DJ, Mitchell JR. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci 1974;14:2099-109.

7. Hornfeldt CS, Murphy MJ. American Association of Poison Control Centers report on poisonings of animals, 1993-1994. J Am Vet Med Assoc 1998;212:358-61.

8. Jones RD, Baynes RE, Nimitz CT. Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989-1990). J Am Vet Med Assoc 1992;201:475-7.

9. Villar D, Buck WB, Gonzalez JM. Ibuprofen, aspirin and acetaminophen toxicosis and treatment in dogs and cats. Vet Hum Toxicol 1998;40:156-62.

10. Aronson LR, Drobatz K. Acetaminophen toxicosis in 17 cats. J Vet Emerg Crit Care 1996;6:65-9.

11. Savides MC, Oehme FW. Acetaminophen and its toxicity. J Appl Toxicol 1983;3:96-111.

12. Nash SL, Savides MC, Oehme FW, Johnson DE. The effect of acetaminophen on methemoglobin and blood glutathione parameters in

the cat. Toxicol 1984;31:329-34.

13. MacNaughton SM. Acetaminophen toxicosis in a Dalmatian. Can Vet J 2003;44:142-4.

14. Kelly JH, Koussayer T, He DE, et al. An improved model of acetaminophen-induced fulminant hepatic failure in dogs. Hepatol 1992;15:329-35.

15. Gazzard BG, Hughes RD, Mellon PJ, Portmann B, Williams R. A dog model of fulminant hepatic failure produced by paracetamol administration. Br J Exp Pathol 1975;56:408-11.

16. Taylor NS, Dhupa N. Acetaminophen Toxicity in Dogs and Cats. Compend Contin Educ Pract Vet 2003;12:160-9.

17. Bernheim F, Berheim M, Michel HO. The action of p-aminophenol on certain tissue oxidations. J Pharmacol Exp Ther 1937;61:311-20.

18. Smith RP, Alkaitis AA, Shafer PR. Chemically induced methemoglobinemias in the mouse. Biochem Pharmacol 1967;16:317-28.

19. Harrison JH, Jr., Jollow DJ. Contribution of aniline metabolites to aniline-induced methemoglobinemia. Mol Pharmacol 1987;32:423-31.

20. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol 1997;54:73-80.

21. Trepanier LA, Cribb AE, Spielberg SP, Ray K. Deficiency of cytosolic arylamine N-acetylation in the domestic cat and wild felids caused by the presence of a single NAT1-like gene. Pharmacogenetics 1998;8:169-79.

22. Page S.W., Maddison J.E. Principles of clinical pharmacology. In: Maddison J, Page S, Church D, editors. Small Animal Clinical Pharmacology. Philadelphia: W.B.Saunders; 2002. p.1-26.

23. Dyke TM, Maddison JE, Page SW. Clinical pharmacokinetics. In: Maddison JE, Page SW, Church D, editors. Small Animal Clinical Pharmacology. Philadelphia: W.B. Saunders; 2002. p.27-39.

24. Wilkinson GR. Pharmacokinetics The dynamics of drug absorption, distribution, and elimination. In: Harman JG, Limbird LE, Gilman AG, editors. Goodman and GIlman's The Pharmacological Basis of

Therapeutics. Tenth ed. New York: McGraw-Hill; 2001. p.3-29.

25. Treinen-Moslen M. Toxic responses of the liver. In: Klaassen CD, editor. Casarett & Doull's Toxicology, The basic science of poisons. Sixth ed. New York: McGraw-Hill; 2001. p.471-90.

26. Crawford J. Liver and biliary tract. In: Kumar V, Abbas AK, Fausto N, editors. Pathologic Basis of Disease. Seventh ed. Philadelphia: Elsevier Saunders; 2005. p.878-937.

27. Parkinson A. Biotransformation of xenobiotics. In: Kalssen CD, editor. Casarett & Doull's Toxicology, The Basic Science of Poisons. New York: McGraw-Hill; 2001. p.133-224.

28. Maddison JE, Page SW. Adverse drug reactions. In: Maddison JE, Page SW, Church D, editors. Small Animal Clinical Pharmacology. Philadelphia: W.B/ Saunders; 2002. p.41-52.

29. Court MH, Greenblatt DJ. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. Biochem Pharmacol 1997;53:1041-7.

30. Iyanagi T. Molecular basis of multiple UDP-glucuronosyltransferase isoenzyme deficiencies in the hyperbilirubinemic rat (Gunn rat). J Biol Chem 1991;266:24048-52.

31. Klaassen CD, Liu L, Dunn RT. Regulation of sulfotransferase mRNA expression in male and female rats of various ages. Chem Biol Interact 1998;109:299-313.

32. Boothe DM. Anticonvulsants and other neurologic therapies in small animals. In: Boothe DM, editor. Small Animal Clinical Pharmacology and Therapeutics. Philadelphia: W.B.Saunders; 2001. p.431-56.

33. Boothe DM. Control of Pain in Small Animals: Opiod Agonists and Antagonists and Other Locally and Centrally Acting Analgesics. In: Boothe DM, editor. Small Animal Clinical Pharmacology and Therpeutics. Philadelphia: W.B. Saunders; 2001. p.405-24.

34. Robinson EP, Sams RA, Muir WW. Barbiturate anesthesia in greyhound and mixed-breed dogs: comparative cardiopulmonary effects, anesthetic effects, and recovery rates. Am J Vet Res 1986;47:2105-12.

35. Blum M, Demierre A, Grant DM, Heim M, Meyer UA. Molecular mechanism of slow acetylation of drugs and carcinogens in humans. Proc

Natl Acad Sci U S A 1991;88:5237-41.

36. Meyer UA, Gut J. Genomics and the prediction of xenobiotic toxicity. Toxicol 2002;181-182:463-6.

37. Meyer UA. Pharmacogenetics and adverse drug reactions. Lancet 2000;356:1667-71.

38. Meyer UA, Zanger UM. Molecular mechanisms of genetic polymorphisms of drug metabolism. Annu Rev Pharmacol Toxicol 1997;37:269-96.

39. Meyer UA, Zanger UM, Skoda RC, Grant D, Blum M. Genetic polymorphisms of drug metabolism. Prog Liver Dis 1990;9:307-23.

40. Mealey KL, Bentjen SA, Gay JM, Cantor GH. Ivermectin sensitivity in collies is associated with a deletion mutation of the mdr1 gene. Pharmacogenetics 2001;11:727-33.

41. Nelson OL, Carsten E, Bentjen SA, Mealey KL. Ivermectin toxicity in an Australian Shepherd dog with the MDR1 mutation associated with ivermectin sensitivity in Collies. J Vet Intern Med 2003;17:354-6.

42. Paulson SK, Engel L, Reitz B, et al. Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. Drug Metab Dispos 1999;27:1133-42.

43. Court MH, Hay-Kraus BL, Hill DW, Kind AJ, Greenblatt DJ. Propofol hydroxylation by dog liver microsomes: assay development and dog breed differences. Drug Metab Dispos 1999;27:1293-9.

44. Yasar U, Tybring G, Hidestrand M, et al. Role of CYP2C9 polymorphism in losartan oxidation. Drug Metab Dispos 2001;29:1051-6.

45. Lee CR, Pieper JA, Hinderliter AL, Blaisdell JA, Goldstein JA. Losartan and E3174 pharmacokinetics in cytochrome P450 2C9*1/*1, *1/*2, and *1/*3 individuals. Pharmacotherapy 2003;23:720-5.

46. Rieder MJ, Shear NH, Kanee A, Tang BK, Spielberg SP. Prominence of slow acetylator phenotype among patients with sulfonamide hypersensitivity reactions. Clin Pharmacol Ther 1991;49:13-7.

47. Magon AM, Leipzig RM, Zannoni VG, Brewer GJ. Interactions of glucose-6-phosphate dehydrogenase deficiency with drug acetylation and hydroxylation reactions. J Lab Clin Med 1981;97:764-70.

48. Waxman DJ. P450 gene induction by structurally diverse xenochemicals:

central role of nuclear receptors CAR, PXR, and PPAR. Arch Biochem Biophys 1999;369:11-23.

49. Whitlock JP, Jr. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 1999;39:103-25.

50. Ciaccio PJ, Duignan DB, Halpert JR. Selective inactivation by chloramphenicol of the major phenobarbital-inducible isozyme of dog liver cytochrome P-450. Drug Metab Dispos 1987;15:852-6.

51. Levine M, Bellward GD. Effect of cimetidine on hepatic cytochrome P450: evidence for formation of a metabolite-intermediate complex. Drug Metab Dispos 1995;23:1407-11.

52. World Health Organization. International drug monitoring: The role of the hospital. Technical Report Series No. 425. 1966. Geneva, Switzerland, World Health Organization.
Ref Type: Pamphlet

53. Pirmohamed M, James S, Meakin S, et al. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. BMJ 2004;329:15-9.

54. Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients - A meta-analysis of prospective studies. JAMA 1998;279:1200-5.

55. Gregus Z, Klaassen CD. Mechanisms of toxicity. In: Klaassen CD, editor. Casarett & Doull's Toxicology The Basic Science of Poisons. Sixth ed. New York: McGraw-Hill; 2001. p.35-81.

56. Pirmohamed M, Park BK. Adverse drug reactions: back to the future. Br J Clin Pharmacol 2003;55:486-92.

57. Cribb AE. Adverse Drug Reactions. In: Peterson ME, Talcott PA, editors. Small Animal toxicology. Philadelphia: W.B. Saunders Company; 2001. p.134-50.

58. Hawkey CJ. Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterol 2000;119:521-35.

59. Potter DW, Hinson JA. Mechanisms of acetaminophen oxidation to N-acetyl-P-benzoquinone imine by horseradish peroxidase and cytochrome P-450. J Biol Chem 1987;262:966-73.

60. Pumford NR, Roberts DW, Benson RW, Hinson JA. Immunochemical

quantitation of 3-(cystein-S-yl)acetaminophen protein adducts in subcellular liver fractions following a hepatotoxic dose of acetaminophen. Biochem Pharmacol 1990;40:573-9.

61. Gibson JD, Pumford NR, Samokyszyn VM, Hinson JA. Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. Chem Res Toxicol 1996;9:580-5.

62. Trepanier LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. J Vet Pharmacol Ther 2004;27:129-38.

63. Salama A, Mueller-Eckhardt C. On the mechanisms of sensitization and attachment of antibodies to RBC in drug-induced immune hemolytic anemia. Blood 1987;69:1006-10.

64. Bloom JC, Brandt JT. Toxic responses of the blood. In: Klassen CD, editor. Casarett & Doull's Toxicology The Basic Science of Poisons. Sixth ed. New York: McGraw-Hill; 2001. p.389-417.

65. Ranney HM, Sharma V. Structure and function of hemoglobin. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, editors. Williams Hematology. Sixth ed. New York: McGraw-Hill; 2001. p.345-53.

66. Car BD. Erythropoiesis and erythrokinetics. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm's Veterinary Hematology. Fifth ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p.105-9.

67. Fernadez FR, Grindem CB. Reticulocyte response. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm's Veterinary Hematology. Philadelphia: Lippincott Williams & Wilkins; 2000. p.110-6.

68. Harvey JW. Erythrocyte Metabolism. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm's Veterinary Hematology. Fifth ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p.125-8.

69. Zunino F, Capranico G. DNA topoisomerase II as the primary target of anti-tumor anthracyclines. Anticancer Drug Des 1990;5:307-17.

70. Rio B, Froquet R, Parent-Massin D. In vitro effect of lead acetate on human erythropoietic progenitors. Cell Biol Toxicol 2001;17:41-50.

71. Lindenbaum J. Drugs and vitamin B12 and folate metabolism. Curr Concepts Nutr 1983;12:73-87.

72. Packman C. Drug-related Immune Hemolytic Anemia. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, editors. Williams

Hematology. Sixth ed. New York: McGraw-Hill; 2001. p.657-63.

73. Kerr RO, Cardamone J, Dalmasso AP, Kaplan ME. Two mechanisms of erythrocyte destruction in penicillin-induced hemolytic anemia. N Engl J Med 1972;287:1322-5.

74. Petz LD, Fudenberg HH. Coombs-positive hemolytic anemia caused by penicillin administration. N Engl J Med 1966;274:171-8.

75. Blum MD, Graham DJ, McCloskey CA. Temafloxacin syndrome: review of 95 cases. Clin Infect Dis 1994;18:946-50.

76. Kelton JG. Impaired reticuloendothelial function in patients treated with methyldopa. N Engl J Med 1985;313:596-600.

77. Territo MC, Peters RW, Tanaka KR. Autoimmune hemolytic anemia due to levodopa therapy. JAMA 1973;226:1347-8.

78. Beutler E. Methemoglobinemia and other causes of cyanosis. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, editors. Williams Hematology. Sixth ed. New York: McGraw-Hill; 2001. p.581-610.

79. Fine DM, Eyster GE, Anderson LK, Smitley A. Cyanosis and congenital methemoglobinemia in a puppy. J Am Anim Hosp Assoc 1999;35:33-5.

80. Schechter RD, Schalm OW, Kaneko JJ. Heinz body hemolytic anemia associated with the use of urinary antiseptics containing methylene blue in the cat. J Am Vet Med Assoc 1973;162:37-44.

81. Bruning-Fann CS, Kaneene JB. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. Vet Hum Toxicol 1993;35:237-53.

82. Wilkie DA, Kirby R. Methemoglobinemia associated with dermal application of benzocaine cream in a cat. J Am Vet Med Assoc 1988;192:85-6.

83. Lagutchik MS, Mundie TG, Martin DG. Methemolgoibinemia induced by a benzocaine-based topically administered anesthetic in eight sheep. J Am Vet Med Assoc 1992;201:1407-10.

84. Atkins CE, Kaneko JJ, Congdon LL. Methemoglobin reductase deficiency and methemogobinemia in a dog. J Am Anim Hosp Assoc 1981;17:829-32.

85. Harvey JW, Dahl M, High ME. Methemoglobin reductase deficiency in a

cat. J Am Vet Med Assoc 1994;205:1290-1.

86. Harvey JW, Ling GV, Kaneko JJ. Methemoglobin reductase deficiency in a dog. J Am Vet Med Assoc 1974;164:1030-3.

87. Harvey JW, Stockham SL, Scott MA, et al. Methemoglobinemia and eccentrocytosis in equine erythrocyte flavin adenine dinucleotide deficiency. Vet Pathol 2003;40:632-42.

88. Beutler E. Glucose-6-Phosphate-Dehydrogenase Deficiency. N Eng J Med 1991;324:169-74.

89. Stockholm SL, Harvey JW, Kinden A. Equine glucose-6-phosphate dehydrogenase deficiency. Vet Pathol 1994;31:518-27.

90. McNeill Consumer Healthcare Company Worldwide Consumer Pharmaceutical Intranet Site, History of Tyelenol. 2002. Nancy West Communications.
Ref Type: Pamphlet

91. Davidson RJ. Phenacetin-induced haemolytic anaemia. J Clin Pathol 1971;24:537-41.

92. Tan GH, Robbibo M.D., Hopper JJr. Is phenacetin a nephrotoxin? A report on twenty-three users of the drug. Calif Med 1964;101:73-7.

93. Graham GG, Scott KF. Mechanism of action of paracetamol. Am J Ther 2005;12:46-55.

94. Bertolini A, Ferrari A, Ottani A, et al. Paracetamol: new vistas of an old drug. CNS Drug Rev 2006;12:250-75.

95. Ottani A, Leone S, Sandrini M, Ferrari A, Bertolini A. The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. Eur J Pharmacol 2006;531:280-1.

96. Hogestatt ED, Jonsson BA, Ermund A, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem 2005;280:31405-12.

97. Davis M, Simmons CJ, Harrison NG, Williams R. Paracetamol overdose in man: relationship between pattern of urinary metabolites and severity of liver damage. Q J Med 1976;45:181-91.

98. Savides MC, Oehme FW, Nash SL, Leipold HW. The toxicity and

biotransformation of single doses of acetaminophen in dogs and cats. Toxicol Appl Pharmacol 1984;74:26-34.

99. Gregus Z, Madhu C, Klaassen CD. Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. J Pharmacol Exp Ther 1988;244:91-9.

100. Moldeus P. Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. Biochem Pharmacol 1978;27:2859-63.

101. Hjelle JJ, Klaassen CD. Glucuronidation and biliary excretion of acetaminophen in rats. J Pharmacol Exp Ther 1984;228:407-13.

102. Fischer LJ, Green MD, Harman AW. Levels of acetaminophen and its metabolites in mouse tissues after a toxic dose. J Pharmacol Exp Ther 1981;219:281-6.

103. Ilkiw JE, Ratcliffe RC. Paracetamol toxicity in a cat. Aust Vet J 1987;64:245-7.

104. Finco DC, Duncan JR, Schall WD, Prasse KW. Acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1975;166:469-72.

105. Francavilla A, Makowka L, Polimeno L, et al. A dog model for acetaminophen-induced fulminant hepatic failure. Gastroenterol 1989;96:470-8.

106. St Omer VV, McKnight ED, III. Acetylcysteine for treatment of acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1980;176:911-3.

107. Mariani L, Baratelli M. [Paracetamol: overdose and hepatic lesions]. ClinTher 1981;98:117-52.

108. Welch RM, Conney AH, Burns JJ. The metabolism of acetophenetidin and N-acetyl-p-aminophenol in the cat. Biochem Pharmacol 1966;15:521-31.

109. Court MH, Greenblatt DJ. Molecular genetic basis for deficient acetaminophen glucuronidation by cats: UGT1A6 is a pseudogene, and evidence for reduced diversity of expressed hepatic UGT1A isoforms. Pharmacogenetics 2000;10:355-69.

110. de Morais SM, Wells PG. Deficiency in bilirubin UDP-glucuronyl transferase as a genetic determinant of acetaminophen toxicity. J

Pharmacol Exp Ther 1988;247:323-31.

111. Snow NS. Some observations on the reactive sulphydryl groups in haemoglobin. Biochem J 1962;84:360-4.

112. Gillette JR, Nelson SD, Mulder GJ, et al. Formation of chemically reactive metabolites of phenacetin and acetaminophen. Adv Exp Med Biol 1981;136 Pt B:931-50.

113. Muldrew KL, James LP, Coop L, et al. Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of acetaminophen using high-performance liquid chromatography with electrochemical detection. Drug Metab Dispos 2002;30:446-51.

114. Reilly TP, Woster PM, Svensson CK. Methemoglobin formation by hydroxylamine metabolites of sulfamethoxazole and dapsone: implications for differences in adverse drug reactions. J Pharmacol Exp Ther 1999;288:951-9.

115. Kiese M. The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol Rev 1966;18:1091-161.

116. Kiese M., Pekis M. The reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

117. Emeigh Hart SG, Wyand DS, Khairallah EA, Cohen SD. Acetaminophen nephrotoxicity in the CD-1 mouse. II. Protection by probenecid and AT-125 without diminution of renal covalent binding. Toxicol Appl Pharmacol 1996;136:161-9.

118. Newton JF, Kuo CH, Gemborys MW, Mudge GH, Hook JB. Nephrotoxicity of p-aminophenol, a metabolite of acetaminophen, in the fischer 344 rat. Toxicol Appl Pharmacol 1982;65:336-44.

119. Carpenter HM, Mudge GH. Acetaminophen nephrotoxicity: studies on renal acetylation and deacetylation. J Pharmacol Exp Ther 1981;218:161-7.

120. Berheim F, Berheim MLC., Michel HO. The action of p-aminophenol on certain tissue oxidations. J Pharmacol Exp Ther 1937;61:311-20.

121. Harrison JH, Jr., Jollow DJ. Role of aniline metabolites in aniline-induced

hemolytic anemia. J Pharmacol Exp Ther 1986;238:1045-54.

122. Song H, Chen TS. p-Aminophenol-induced liver toxicity: tentative evidence of a role for acetaminophen. J Biochem Mol Toxicol 2001;15:34-40.

123. Dressler WE, Appelqvist T. Plasma/blood pharmacokinetics and metabolism after dermal exposure to para-aminophenol or para-phenylenediamine. Food Chem Toxicol 2006;44:371-9.

124. Klos C, Koob M, Kramer C, Dekant W. p-aminophenol nephrotoxicity: biosynthesis of toxic glutathione conjugates. Toxicol Appl Pharmacol 1992;115:98-106.

125. Grant DM. Molecular genetics of the N-acetyltransferases. Pharmacogenetics 1993;3:45-50.

126. Minchin RF. Acetylation of p-aminobenzoylglutamate, a folic acid catabolite, by recombinant human arylamine N-acetyltransferase and U937 cells. Biochem J 1995;307 (Pt 1):1-3.

127. Smelt VA, Upton A, Adjaye J, et al. Expression of arylamine N-acetyltransferases in pre-term placentas and in human pre-implantation embryos. Hum Mol Genet 2000;9:1101-7.

128. Weber WW, Cohen SN. N-acetylation of drugs: isolation and properties of an N-acetyltransferase from rabbit liver. Mol Pharmacol 1967;3:266-73.

129. Palmer T. Kinetics of multi-substrate enzyme-catalysed reactions. In: Palmer T, editor. Understanding Enzymes. Fourth ed. Hemel Hampstead: Prentice Hall/Ellis Horwood; 1991. p.155-74.

130. Grant DM, Lottspeich F, Meyer UA. Evidence for two closely related isozymes of arylamine N-acetyltransferase in human liver. FEBS Lett 1989;244:203-7.

131. Hickman D, Risch A, Buckle V, et al. Chromosomal localization of human genes for arylamine N-acetyltransferase. Biochem J 1994;297 (Pt 3):441-5.

132. Grant DM, Blum M, Beer M, Meyer UA. Monomorphic and polymorphic human arylamine N-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. Mol Pharmacol 1991;39:184-91.

133. Hein DW, Grant DM, Sim E. Update on consensus arylamine N-

acetyltransferase gene nomenclature. Pharmacogenetics 2000;10:291-2.

134. Dupret JM, Grant DM. Site-directed mutagenesis of recombinant human arylamine N-acetyltransferase expressed in Escherichia coli. Evidence for direct involvement of Cys68 in the catalytic mechanism of polymorphic human NAT2. J Biol Chem 1992;267:7381-5.

135. Mushtaq A, Payton M, Sim E. The COOH terminus of arylamine N-acetyltransferase from Salmonella typhimurium controls enzymic activity. J Biol Chem 2002;277:12175-81.

136. Goodfellow GH, Dupret JM, Grant DM. Identification of amino acids imparting acceptor substrate selectivity to human arylamine acetyltransferases NAT1 and NAT2. Biochem J 2000;348 Pt 1:159-66.

137. Rodrigues-Lima F, Delomenie C, Goodfellow GH, Grant DM, Dupret JM. Homology modelling and structural analysis of human arylamine N-acetyltransferase NAT1: evidence for the conservation of a cysteine protease catalytic domain and an active-site loop. Biochem J 2001;356:327-34.

138. Lavergne SN, Kurian JR, Bajad SU, et al. Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. Toxicol 2006;222:25-36.

139. Hein DW, McQueen CA, Grant DM, et al. Pharmacogenetics of the arylamine N-acetyltransferases: a symposium in honor of Wendell W. Weber. Drug Metab Dispos 2000;28:1425-32.

140. Butcher NJ, Ilett KF, Minchin RF. Functional polymorphism of the human arylamine N-acetyltransferase type 1 gene caused by C190T and G560A mutations. Pharmacogenetics 1998;8:67-72.

141. Risch A, Wallace DM, Bathers S, Sim E. Slow N-acetylation genotype is a susceptibility factor in occupational and smoking related bladder cancer. Hum Mol Genet 1995;4:231-6.

142. Geylan YS, Dizbay S, Guray T. Arylamine N-acetyltransferase activities in human breast cancer tissues. Neoplasma 2001;48:108-11.

143. Hughes NC, Janezic SA, McQueen KL, et al. Identification and characterization of variant alleles of human acetyltransferase NAT1 with defective function using p-aminosalicylate as an in-vivo and in-vitro probe. Pharmacogenetics 1998;8:55-66.

144. Blum M, Grant DM, Demierre A, Meyer UA. N-acetylation pharmacogenetics: a gene deletion causes absence of arylamine N-acetyltransferase in liver of slow acetylator rabbits. Proc Natl Acad Sci U S A 1989;86:9554-7.

145. Leff MA, Fretland AJ, Doll MA, Hein DW. Novel human N-acetyltransferase 2 alleles that differ in mechanism for slow acetylator phenotype. J Biol Chem 1999;274:34519-22.

146. Grant DM, Morike K, Eichelbaum M, Meyer UA. Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine N-acetyltransferase in human liver. J Clin Invest 1990;85:968-72.

147. Levy GN, Martell KJ, DeLeon JH, Weber WW. Metabolic, molecular genetic and toxicological aspects of the acetylation polymorphism in inbred mice. Pharmacogenetics 1992;2:197-206.

148. Kawamura A, Graham J, Mushtaq A, et al. Eukaryotic arylamine N-acetyltransferase. Investigation of substrate specificity by high-throughput screening. Biochem Pharmacol 2005;69:347-59.

149. Hein DW, Hirata M, Glowinski IB, Weber WW. Biochemical evidence for the coexistence of monomorphic and polymorphic N-acetyltransferase activities on a common protein in rabbit liver. J Pharmacol Exp Ther 1982;220:1-7.

150. Estrada-Rodgers L, Levy GN, Weber WW. Substrate selectivity of mouse N-acetyltransferases 1, 2, and 3 expressed in COS-1 cells. Drug Metab Dispos 1998;26:502-5.

151. Walraven JM, Doll MA, Hein DW. Identification and characterization of functional rat arylamine N-acetyltransferase 3: comparisons with rat arylamine N-acetyltransferases 1 and 2. J Pharmacol Exp Ther 2006;319:369-75.

152. Blum M, Grant DM, McBride W, Heim M, Meyer UA. Human arylamine N-acetyltransferase genes: isolation, chromosomal localization, and functional expression. DNA Cell Biol 1990;9:193-203.

153. Nakura H, Itoh S, Kusano H, et al. Evidence for the lack of hepatic N-acetyltransferase in suncus (Suncus murinus). Biochem Pharmacol 1995;50:1165-70.

154. Cribb AE, Spielberg SP. An in vitro investigation of predisposition to

sulphonamide idiosyncratic toxicity in dogs. Vet Res Commun 1990;14:241-52.

155. Cribb AE. Idiosyncratic reactions to sulfonamides in dogs. J Am Vet Med Assoc 1989;195:1612-4.

156. Weiss DJ, McClay CB, Christopher MM, Murphy M, Perman V. Effects of propylene glycol-containing diets on acetaminophen-induced methemoglobinemia in cats. J Am Vet Med Assoc 1990;196:1816-9.

157. Allison RW, Lassen ED, Burkhard MJ, Lappin MR. Effect of a bioflavonoid dietary supplement on acetaminophen-induced oxidative injury to feline erythrocytes. J Am Vet Med Assoc 2000;217:1157-61.

158. Webb CB, Twedt DC, Fettman MJ, Mason G. S-adenosylmethionine (SAMe) in a feline acetaminophen model of oxidative injury. J Feline Med Surg 2003;5:69-75.

159. Gaunt SD, Baker DC, Green RA. Clinicopathologic evaluation N-acetylcysteine therapy in acetaminophen toxicosis in the cat. Am J Vet Res 1981;42:1982-4.

160. Rumbeiha WK, Lin YS, Oehme FW. Comparison of N-acetylcysteine and methylene blue, alone or in combination, for treatment of acetaminophen toxicosis in cats. Am J Vet Res 1995;56:1529-33.

161. Dart RC, Erdman AR, Olson KR, et al. Acetaminophen poisoning: an evidence-based consensus guideline for out-of-hospital management. Clin Toxicol (Phila) 2006;44:1-18.

162. Mitchell MC, Schenker S, Speeg KV, Jr. Selective inhibition of acetaminophen oxidation and toxicity by cimetidine and other histamine H2-receptor antagonists in vivo and in vitro in the rat and in man. J Clin Invest 1984;73:383-91.

163. Al Mustafa ZH, Al Ali AK, Qaw FS, Abdul-Cader Z. Cimetidine enhances the hepatoprotective action of N-acetylcysteine in mice treated with toxic doses of paracetamol. Toxicol 1997;121:223-8.

164. Hazai E, Vereczkey L, Monostory K. Reduction of toxic metabolite formation of acetaminophen. Biochem Biophys Res Commun 2002;291:1089-94.

165. Slattery JT, McRorie TI, Reynolds R, et al. Lack of effect of cimetidine on acetaminophen disposition in humans. Clin Pharmacol Ther 1989;46:591-

7.

166. Zed PJ, Krenzelok EP. Treatment of acetaminophen overdose. Am J Health Syst Pharm 1999;56:1081-91.

167. Svensson CK, Tomilo M. Effect of H2-receptor antagonists on rat liver cytosolic acetyl CoA:arylamine N-acetyltransferase activity. Drug Metab Dispos 1992;20:74-8.

168. Risch A, Smelt V, Lane D, et al. Arylamine N-acetyltransferase in erythrocytes of cystic fibrosis patients. Pharmacol Toxicol 1996;78:235-40.

169. Ward A, Hickman D, Gordon JW, Sim E. Arylamine N-acetyltransferase in human red blood cells. Biochem Pharmacol 1992;44:1099-104.

2. METHEMOGLOBIN-FORMING POTENTIAL OF ACETAMINOPHEN AND ITS METABOLITES AND THE ROLE OF *N*-ACETYLTRANSFERASE IN SPECIES DIFFERENCES IN SENSITIVITY

2.1 Introduction

Acetaminophen (APAP) is a commonly used human analgesic and antipyretic. Although considered safe at therapeutic doses, APAP is the most frequent drug overdose reported to human poison control centers in the United States and Britain [1-3]. Veterinary poison control centers also report numerous cases of APAP toxicity in dogs and cats [4]. In humans and most laboratory species, APAP toxicity is associated with hepatic centrilobular necrosis with clinical signs such as abdominal pain and icterus developing by 48 hours post ingestion [5,6]. Dogs and cats are unique in that they develop methemoglobinemia and hemolytic anemia resulting in the clinical signs of profound depression, cyanosis, vomiting, tachypnea, facial or forelimb edema and brown coloured blood within 4-6 hours of intoxication [7-11]. Dogs can also develop hepatic centrilobular necrosis if they survive the initial hematotoxicity [12,13]. Cats have never been shown to develop centrilobular necrosis but may show hepatocellular degeneration with associated elevated liver enzymes, likely due to hypoxia [14].

Acetaminophen is primarily metabolized by sulfation and glucuronidation in the liver followed by excretion of the conjugates in the urine in most species [6,15-17]. A small percentage of APAP is oxidized by cytochrome P450 (CYP) enzymes to the oxidative metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [16]. At therapeutic doses of APAP, NAPQI binds to glutathione (GSH) and is then

excreted in the urine as cysteine and mercapturic acid metabolites [5]. At toxic doses, the sulfate and glucuronosyl transferases become saturated and increased NAPQI production occurs [5]. This can lead to exhaustion of available GSH [18]. If GSH is depleted to < 20% of its usual concentration, NAPQI binds covalently to cysteine groups on hepatocellular proteins in the cell membrane, mitochondria or cytosol, leading to hepatocellular functional abnormalities and cell death [18,19]. The difference in species sensitivity to APAP toxicity is due to variation in NAPQI production [5,20,21]. Species that are highly sensitive to APAP-induced hepatotoxicity produce more NAPQI and subsequently show greater covalent binding to hepatocellular proteins [5,20,21].

The metabolite responsible for the APAP-induced hematotoxicity in dogs and cats has never been demonstrated. In the veterinary literature, the reaction is often attributed to NAPQI [22,23]. It is postulated that cats may produce more NAPQI than other species as they have a limited ability to glucuronidate APAP. This is due to the presence of a feline pseudogene for uridine-diphosphate (UDG)-glucuronosyl transferase 1A6 (UGT1A6), the glucuronosyl transferase responsible for glucuronidation of APAP in other species [24]. The feline gene for UGT1A6 has several mutations including two premature stop codons. Because of this, there is no transcribed UGT1A6 in cats [25]. The metabolism of APAP in cats is primarily by conjugation with sulfates (92%), with minimal glucuronidation (1.3%) and oxidation by CYP enzymes (4.7%) [15]. The different target organ in cats is presumed to be due to high numbers of exposed sulfhydryl groups on feline hemoglobin predisposing feline hemoglobin to oxidation and subsequent

Heinz body formation [23,26]. Cats have eight sulfhydryl groups per hemoglobin molecule as opposed to four in dogs and two in humans [27,28].

Dogs are the only other species known to develop APAP-induced hematotoxicity. Unlike cats, glucuronidation is the predominant pathway (76%) in canine APAP metabolism [15]. In addition, canine hemoglobin has fewer reactive sulfhydryl groups per molecule than feline hemoglobin and does not show the same exquisite sensitivity to oxidation as does feline hemoglobin [27].

There are flaws in the hypothesis that NAPQI is the reactive metabolite responsible for APAP-induced hematotoxicity. First, NAPQI is not likely produced in erythrocytes as they lack the necessary CYP enzymes for a two electron oxidation. Second, reactive NAPQI is not released into the blood from hepatocytes. Most NAPQI covalently bound to hepatic proteins remains within the necrotic hepatocytes but small amounts of inactive NAPQI, can leak into the plasma, either covalently bound to either GSH or hepatocyte proteins [18,29,30]. Minute quantities of cysteine-bound NAPQI can be detected in the blood immediately after APAP overdose, but most NAPQI does not enter the blood until significant hepatic degeneration or necrosis occurs 6-12 hours post intoxication[18,29,30]. Clinical signs of methemoglobinemia in dogs and cats can occur within 2-4 hours of intoxication, much earlier than the leakage of inactive NAPQI [7,23].

Third, NAPQI does not have the chemical characteristics necessary to redox cycle with oxyhemoglobin effectively. Many chemicals known to induce methemoglobinemia do so by a co-oxidation reaction with oxyhemoglobin

(Figures 1.11 and 1.13). Examples include dapsone hydroxylamine, phenylhydroxamine and sulfamethoxazole hydroxylamine [31,32]. Co-oxidation reactions involve the removal of an electron from both the chemical and the ferrous iron (Fe^{2+}) in the oxyhemoglobin. The electrons are donated to oxygen, creating a reactive oxygen species such as hydrogen peroxide, and oxidizing both the oxyhemoglobin (to methemoglobin) and the chemical to a nitroso compound [33,34]. The methemoglobin is subsequently reduced to oxyhemoglobin by NADH methemoglobin reductase, and the oxidized compound is reduced to the original metabolite through reduction by GSH [33,34]. This redox cycling of the chemical permits an on-going co-oxidation reaction with oxyhemoglobin to form more methemoglobin. If the chemical is not removed from cycling by alternative biotransformation pathways, the ability of the cell to reduce the methemoglobin formed can be overwhelmed, leading to overt methemoglobinemia. Covalent binding of NAPQI with GSH results in a stable conjugate, effectively preventing it from redox cycling. The APAP metabolite that induces methemoglobin must be able to either undergo co-oxidation with oxyhemoglobin or to donate an electron directly to oxyhemoglobin.

A metabolite of APAP that can redox cycle with methemoglobin is *para*-aminophenol (PAP). This is a minor APAP metabolite produced by deacetylation of APAP by hepatic microsomal carboxyesterases [35]. *para*-Aminophenol has been demonstrated in cats, rats and mice [36-38]. *para*-Aminophenol is also a metabolite of chemicals such as aniline dyes and has long been known to induce methemoglobinemia through a co-oxidation reaction with hemoglobin [39,40].

In rats and mice, >70% of PAP is removed by biliary excretion of GSH and *N*-acetyl conjugates [37,38]. *N*-acetylation of drugs is catalysed by two closely related enzymes: *N*-acetyltransferase 1 and *N*-acetyltransferase 2 (NAT1 and NAT2; EC2.3.1.5) [41]. Although NAT1 and NAT2 show considerable homology, these phase II biotransformation enzymes differ in their preferred substrates and distribution in the body [42]. Human NAT1 is located in most tissues including red blood cells while NAT2 is found mainly in the liver and small intestine [42-44]. Human NAT1 tends to catalyze acetylation of smaller molecules such as *para*-aminobenzoic acid (PABA), while larger molecules such as procainamide and some sulfonamide antimicrobials are *N*-acetylated by NAT2 [42]. Unlike other species, cats only have NAT1 and dogs have no NAT enzymes [45,46]. The NAT1 activity in cats is considerably less than in other species [45]. Therefore, dogs and cats are deficient in their ability to *N*-acetylate arylamine-containing compounds. Cats and dogs may subsequently be exposed to more PAP during APAP toxicity than other species.

In summary, APAP-induced hematotoxicity in dogs and cats has not been adequately explained. It has been postulated that the limited glucuronidation in cats results in higher NAPQI production, which then targets erythrocytes because of the high sensitivity of feline hemoglobin to oxidative damage. However, this reasoning does not apply to dogs because canine glucuronidation of APAP is excellent and canine erythrocytes are not highly sensitive to oxidants. In addition, active NAPQI is not released from hepatocytes. In rats and mice, PAP is removed from the redox cycling largely by *N*-acetylation. Dogs and cats are

completely or partially deficient in NAT enzymes, respectively. Therefore, they may not remove PAP from redox cycling as efficiently as other species, thus prolonging co-oxidation and methemoglobin formation.

We therefore hypothesized that PAP, not APAP or NAPQI, would induce methemoglobinemia *in vitro*. Moreover, we hypothesized that there would be species differences in susceptibility to the methemoglobin forming potential of PAP due to the deficient *N*-acetylation in dogs and cats.

The following studies compare the *in vitro* deacetylation of APAP and *N*-acetylation of PAP in dogs, cats, rats and mice. The studies also examine the difference in the methemoglobin forming potential of APAP, NAPQI and PAP *in vitro* in dog, cat, mice and rat erythrocytes, and the difference in species sensitivity to PAP-induced *in vitro* methemoglobin formation.

2.2 Materials

The following compounds were obtained from Sigma-Aldrich Inc (St. Louis, MO): *N*-acetyl-*p*-benzoquinoneimine (NAPQI), potassium ferricyanide, potassium cyanide, K_2HPO_4, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) free acid, $NaH_2PO_4.H_2O$, anhydrous dimethyl sulfoxide (Me_2SO, DMSO), para-aminobenzoic acid (PABA), cimetidine, leupeptin, phenylmethylsulfonyl fluoride (PMSF), 2,6-di-tert-butyl-4-methylphenol (BHT), acetyl DL-carnitine hydrochloride, carnitine acetyltransferase, acetyl coenzyme A (AcCoA), triethylamine (TEA), DL-dithiothreitol (DTT), potassium chloride, acetaminophen, butyric anhydride and ethylenediaminetetraacetic acid (EDTA).

Anhydrous dextrose, NaCl, KCl, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, perchloric acid (PCA), acetonitrile and glacial acetic acid were obtained from Fischer Scientific (Fairhaven, NJ). KH_2PO_4 was obtained from BDH Inc, Toronto, Canada.

Triethanolamine-HCl, KCl, bovine serum albumin (BSA), 4-acetamidobenzoic acid (Nac-PABA) and 4-aminophenol (PAP) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI).

The Bio-Rad DC Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA).

2.3 Animals

All procedures were done in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the University of Prince Edward Island Animal Care Committee. Written informed consent was obtained from all owners prior to blood sampling healthy pet cats by venipuncture of the jugular vein and dogs by venipuncture of the cephalic vein. Healthy male Sprague-Dawley rats aged 10-12 weeks and healthy male and female wildtype (C57BL/6) mice aged 6-7 weeks were obtained from Charles River, St. Hyacinthe, QC and acclimatized for two or more weeks. C57BL/6 double knockout mice for NAT1 and NAT2 were donated by Dr. Denis Grant of the University of Toronto and maintained in a breeding colony at the University of Prince Edward Island.

Rats were sedated with 5 mg/kg xylazine (20 mg/ml Rompun® , Bayer, Toronto, ON) and 70 mg/kg ketamine (100 mg/ml Vetalar ®, Bioniche Animal

Health Canada, Belleville, ON) intraperitoneally (IP) for venipuncture of the ventral tail artery. Euthanasia of rats for liver collection was by inhalation of CO_2 and exsanguination by cardiac puncture. Euthanasia of mice was done by IP injection of 80 mg/kg pentobarbital followed by cardiac puncture and exsanguination for blood collection followed by removal of livers.

Livers were obtained from mature healthy cats and dogs deemed inappropriate for adoption by the Prince Edward Island Humane Society. Cats were sedated intramuscularly with 15 mg/kg ketamine (100 mg/ml Vetalar®, Bioniche Animal Health Canada, Belleville, ON) and 0.7 mg/kg xylazine (20 mg/ml Rompun®, Bayer Animal Health, Toronto, ON). Dogs were sedated with 12 mg/kg ketamine and 0.8 mg/kg xylazine. The sedated cats and dogs were then euthanized by an overdose of intravenous 200 mg/ml embutramide, 50 mg/ml mebozonium and 5 mg/ml tetracaine hydrochloride (T61-Euthanasia Solution, Whitby, ON).

2.4 Liver fractionation

Immediately following euthanasia, livers were removed and washed and perfused with cold phosphate buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, pH 7.4), snap frozen in liquid nitrogen and stored at -80°C until use [47]. Livers were later homogenized on ice by a tissue homogenizer (Tissue Tearor, Model 398, Biospec Products Inc, Dremels, WI) in 4 volumes of TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) with 10 µM leupeptin, 100 µM PMSF, and 18 µM BHT [47].

Initial differential centrifugation was at 9,000 g, for 20 minutes 4°C (Allegra X-15R Centrifuge, Beckman Coulter) [47]. The supernatant was centrifuged at 115,000 g for 1 hour at 4°C (L8-60M Ultracentrifuge, Beckman Coulter). The cytosol was separated from the pellet containing the microsomes and aliquots of cytosol frozen at -80°C until use [47]. Pellets (microsomes) were re-suspended in 0.25 M sucrose and 5 mM HEPES at a pH of 7.4 and aliquots were immediately frozen at -80°C. Protein concentrations were determined by BioRad DC Protein Assay, a modified Bradford technique [48].

2.5 *N*-acetyltransferase assay

The assay for *N*-acetyltransferase was performed as previously described [49-51]. Cytosol was diluted appropriately to achieve final protein concentrations ensuring linear metabolism with consumption of less than 10% of substrate over the incubation period (approximately 1.0 mg protein/ml in cats and dogs, 0.3 mg protein/ml in rats and 0.15 mg protein/ml in mice). Rat and mice cytosol was diluted with TEDK containing 1 mg/ml BSA and cat cytosol was diluted with TEDK alone. All analyses were carried out at an AcCoA concentration of 100 µM in the presence of a regenerating system (5 mM acetyl DL-carnitine and 0.22 U/ml carnitine acetyltransferase in a NAT assay buffer (225 mM triethanolamine-HCl, 4.5 mM EDTA, 4.5 mM DTT) to maintain a constant AcCoA concentration [47]. Cytosol, AcCoA and the regenerating system were pre-incubated at 37°C for 5 minutes. Reactions were initiated with the addition of a substrate, PABA or PAP. Incubations were carried out at 37°C for 10 minutes in rats and mice and 15

minutes in cats, and terminated by the addition of 1/10 volume of 15% perchloric acid, placement on ice and vortexing for 5 minutes at 16,000 g. All reactions were carried out in duplicate.

N-acetylated products were quantified as previously described for measurement of NacPABA and modified for APAP.[42] *N*-acetylated PABA and APAP were measured with UV detection at 270 nm. In all cases, 50 µl of the supernatant was injected into the HPLC without further preparation. The mobile phase for NacPABA consisted of water:acetonitrile:glacial acetic acid:triethylamine at a ratio of 89:11:1:.05 (v/v). The mobile phase for analysis of APAP was water:acetonitrile:glacial acetic acid:triethylamine at a ratio of 96:4:1:0.05. Both mobile phases were used isocratically at 1.25 ml/min.

Enzyme velocity measurements were made using 9-10 acceptor substrate concentrations ranging from 2.5-500 µM.

The retention times of APAP and NacPABA were 6.3 and 6.7 minutes respectively. The APAP intraday coefficient of variation (CV) was <1.77% for concentrations of 0.156-5 µM APAP based on five repeated measurements of 0.156-5 µM APAP within one day. The interday variation was 6.29% for 2.5 µM APAP and 14.02% for 0.156 µM APAP based on the measurement of 2.5 µM and 0.156 µM APAP aliquots stored at -80°C before thawing and measuring on four separate days. The lower limit of quantification was 0.156 µM APAP. The interday variation was 7.09% for 2.5 µM NacPABA and 16.89% for 0.156 µM NacPABA based on the measurement of 2.5 µM and 0.156 µM NacPABA aliquots stored at -80°C before thawing and measuring on four separate days.

The lower limit of quantification was 0.156 µM for NacPABA.

2.6 Deacetylation assay

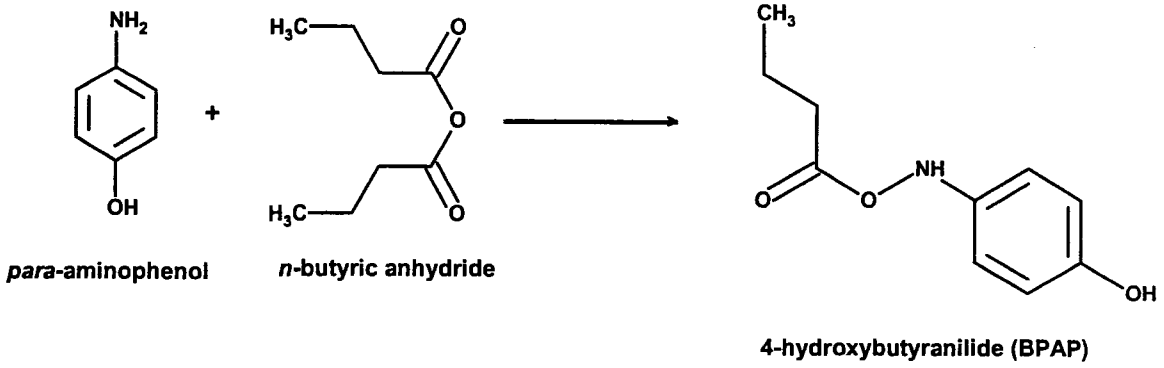
Microsomes of rats, wildtype mice, dogs and cats were diluted appropriately with TEDK to final protein concentrations ensuring linear conditions with less than 10% consumption of substrate (1 mg/ml). The diluted microsomes were pre-incubated for 5 minutes at 37°C. Reactions were initiated by addition of equal volumes of pre-incubated APAP in TEDK and 0.05% DMSO to achieve final concentrations of 1000 and 5000 µM APAP. Mixtures were incubated at 37°C for 30 minutes.

Reactions were stopped by the addition of 3 µl of *n*-butyric anhydride. Samples were then centrifuged for 5 minutes at 16,000 g (Spectrafuge 16 M, Labnet International Inc.). The supernatant containing the stable derivative 4-hydroxybutyranilide (BPAP) was removed from the protein pellet [17] (Figure 2.1). The BPAP was measured the same day by injection of 50 µl of supernatant into the HPLC using a Beckman Ultrasphere ODS C-18 column (150 X 4.6 mm, 5 µm particle size) and a Shimadzu SCL-10A solvent delivery system (Shimadzu Scientific, Columbia, MD). The isocratic mobile phase for BPAP consisted of water:acetonitrile:glacial acetic acid:triethylamine at a ratio of 91:9.0:1.0:0.05 (v/v) at 1.25 ml/min. Detection was at 245 nm.

The retention time of BPAP was 10.2 minutes. The intraday coefficient of variation (CV) was <7% for 0.1-10 µM BPAP, based on five repeated measurements of 0.1-10 µM BPAP within one day. The interday CV was 2.9% for 5 µM and 3.0% for

Figure 2.1: Derivatization of *para*-aminophenol and butyric anhydride

para-Aminophenol is an unstable compound that auto-oxidizes within 30 minutes of formation. Therefore the deacetylation assay was stopped by the addition of butyric anhydride. Butyric anhydride and PAP formed the stable derivative 4-hydroxybutyranilide (BPAP) which was then measured by HPLC.



***para*-aminophenol** ***n*-butyric anhydride**

4-hydroxybutyranilide (BPAP)

0.3 µM BPAP based on the measurement of 5 µM and 0.3 µM BPAP aliquots stored at -80°C, before thawing and measuring on four separate days. The lower limit of quanitification was 0.156 µM.

2.7 Red blood cell preparation.

Two to eight ml of whole blood was collected into three ml EDTA Vacutainers® (Becton Dickinson, Franklin Lakes, NJ) from healthy cats, dogs and rats or 100-400 µl of whole blood was collected into 400 µl EDTA Vacutainers® (Becton Dickinson, Franklin Lakes, NJ) from mice. Red blood cells were separated from plasma by centrifugation for 10 minutes at 1800 g and 4°C for the methemoglobin measurements and at 1000 g and 4°C for the hemolysis studies [52]. Erythrocytes were washed in PBS, pH 7.4 following centrifugation 1-2 times. The final red cell pellets were resuspended in HEPES-buffered medium (15 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid, pH 7.4, 125 M sodium chloride, 6 mM potassium chloride, 1.2 mM magnesium sulphate, 1 mM sodium phosphate, 1 mM calcium chloride and 10 mM glucose) to yield packed cell volumes of 35-50% [52]. Lysed samples of red blood cells were prepared by suspending centrifuged and washed erythrocytes in distilled water at a 1:1 ratio volume/volume and vortexing for 5 seconds at room temperature.

2.8 *In vitro* exposure of red blood cells to chemicals

Dog, cat and rat erythrocytes were exposed to APAP, NAPQI and PAP. To isolate the effect of NAT on species sensitivity to APAP-induced

methemoglobinemia, erythrocytes of *NAT1/NAT2* double knockout C57BL/6 mice were incubated with 500 µM PAP *in vitro* and compared to wildtype C57BL/6 mice.

Stock solutions of APAP, NAPQI and PAP were prepared in 100% DMSO just prior to use and diluted appropriately with HEPES buffered medium to achieve two times the desired final concentration in 0.5% DMSO. Control solutions consisted of 0.5% DMSO in HEPES. Equal volumes of resuspended red blood cells and chemicals or controls were mixed to produce the desired final chemical concentrations and erythrocytes in 0.25% DMSO. Duplicate samples were incubated in a shaking hot water bath at 37°C for 5 or 60 minutes.

2.9 Methemoglobin measurement

Methemoglobin concentrations were determined as previously described with modifications to allow use of a 96 well plate spectrophotometer (Spectra Max 340, Molecular Devices, Sunnyvale, CA) [52]. In brief, 50 µl of sample was added to 1950 µl of distilled water and gently swirled. An aliquot of 2000 µl of potassium phosphate buffer was added and the resulting sample was vortexed to cause lysis. One hundred and fifty µl of vortexed sample was pipetted into each well of 96 well plates. Each duplicate was sampled 6 times to give a final reported value based on the average of two duplicates done in triplicate. Ten µl of 0.60 M $K_3Fe(CN)_6$ was added to one half of the samples and incubated for 2.5 minutes at 20°C. The absorbency was determined at 630 nm. Ten µl of 0.76 M KCN was then added to all wells and the samples incubated for 5.5 minutes before

repeating the measurement of the absorbency at 630 nm to allow subtraction of the background.

2.10 Hemoglobin determination

Samples of washed, resuspended erythrocytes were incubated with APAP, NAPQI or PAP for one hour at 37°C. Post-incubation, the samples were centrifuged at 1000 g for 10 minutes at 4°C. Hemoglobin measurement of the supernatant was performed as previously described with minor modifications to allow use of a 96 well plate spectrophotometer [52]. In brief, 200 µl of plasma was mixed with Drabkins solution (10 mM $NaHCO_3$, 0.61 mM $K_3Fe(CN)_6$, 0.77 mM KCN) and incubated for 5 minutes. The absorbency at 540 nm was measured. The calculation was as follows:

$$\text{Hemolysis at time x} = \frac{\text{value}_{xa} - \text{baseline}_b}{100\%_c - \text{baseline}_b}$$

a) Absorbence of post-incubation sample at time x

b) Absorbence of control (red cells+ 0.025% DMSO)

c) Absorbence of 100% lysed red cells prior to incubation

2.11 Statistical Analysis

Eadie hofstee transformations of *N*-acetylation were performed to demonstrate whether the *N*-acetylation was by single or multiple enzymes in rats, mice and cats. The apparent Michealis-Menton parameters were calculated by curvilinear regression analysis of hyperbola produced by the velocity as a function

of substrate concentration in rats, mice and cats. Time and protein linearity for deacetylation were determined by linear regression. Comparison of species deacetylation was by a one-way analysis of variance (ANOVA).

The NAPQI, and APAP induction of methemoglobin were not analyzed statistically as no clinically significant methemoglobin was produced. Arcsin transformation of the data for PAP induction of methemoglobin was done prior to analysis. The PAP induced methemoglobin was analyzed by repeated measure one-way ANOVA and Dunnett tests. A species comparison of methemoglobin induction by 100, 250 and 500 μM PAP in dogs, cats and rats was done by one-way ANOVAs and Bonferoni tests at each concentration and corrected for the multiple dose points by lowering the level of significance to <0.017 (0.05/3). The generation of methemoglobin in wildtype and knockout mice following incubation with 500 μM PAP were compared using a two tailed t-test.

The generation of methemoglobin in lysed and intact erythrocytes was analyzed with paired t tests following arcsin transformation of the data. The multiple time points were corrected for by lowering the level of significance to <0.017.

All statistical calculations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA. Unless otherwise stated, data are expressed as means ± SEM and significance is taken to be $P<0.05$.

2.12 Results

2.12.1 *N*-acetylation of PABA

N-acetylation of PABA was used as a positive control to confirm the methodology and previously reported species differences in *N*-acetylation activity. The mean *N*-acetylation activity of 100 μM PABA in each species is summarized in Table 2.1.

2.12.2 *N*-acetylation of PAP

The acetylation of PAP was determined in all species. Rat *N*-acetylation of 100 μM PAP was linear (r^2=0.99) with protein concentrations over a range of 0.05 to 1.8 mg/ml cytosolic protein following 15 minutes of incubation (Figure 2.2). Rat acetylation of 100 μM PAP was linear with time (r^2=0.96) over 0-30 minutes. Eadie-Hofstee transformation of the data was curvilinear indicating more than one enzyme involved in rat *N*-acetylation (Figure 2.3). The overall apparent K_m was 10.3 ± 2.9 μM PAP and the overall apparent rat V_{max} was 0.63 ± 0.03 nmol/mg prot/min in rats (Figure 2.4).

Feline acetylation of 100 μM PAP was linear (r^2=0.99) with protein concentrations over a range of 0.07-1.1 mg/ml cytosolic protein at 4-5 concentrations (n=3) (Figure 2.5). Feline *N*-acetylation of 100 μM PAP was linear with time (r^2=0.99) over 0 to 30 minutes. Eadie-Hofstee transformation of the data was linear indicating there was one enzyme responsible for *N*-acetylation in cats (Figure 2.6). The apparent K_m was 6.4 ± 1.6 μM PAP and the apparent V_{max} was 0.09 ± 0.004 nmol/mg prot/min in cats (Figure 2.6).

Wildtype mouse acetylation of 100 μM PAP was linear (r^2=0.99) with protein

Table 2.1: Velocity of *N*-acetylation of PABA in the hepatocyte cytosol of rats, cats, mice, dogs and knockout mice.

Cytosol was incubated with 100 µM PABA in the presence of 100 µM AcCoA and an AcCoA regenerating system at 37°C for 10 minutes in mice and rats, and 15 minutes in cats and dogs.

Animals	**Final protein concentration** (mg prot/ml)	**Velocity** (nmol/mg prot/min)
Rats (n=4)	1.30	0.436 ± 0.027
Cats (n=4)	1 .0	0.058 ± 0.006
Wildtype mice (C57BL/A6) (n=6)	1.15	1.234 ± 0.205
Dogs (n=4)	1.0	Below detectable limits
NAT1/NAT2 knockout mice (C57BL/A6) (n=6)	1.30	Below detectable limits

Figure 2.2: Protein and time linearity of *N*-acetylation of 100 µM PAP in rats

(Top) Protein linearity of PAP *N*-acetylation at 5 concentrations of cytosol protein over a range of 0.05-1.8 mg/ml, (n=3). (Bottom) Time linearity of rat *N*-acetylation of PAP at 6 times points over the range of 0-30 minutes at a cytosol protein concentration of 0.3g (n=4).

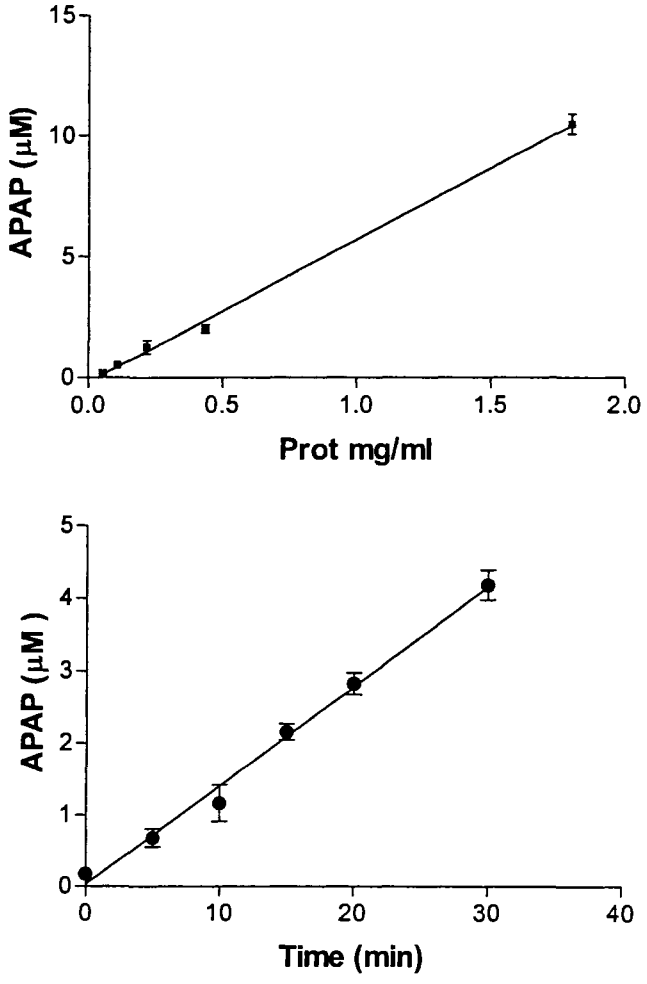


Figure 2.3: Eadie-Hofstee transformations of PAP *N*-acetylation in 4 individual rats

The PAP *N*-acetylation in four rats using 8-10 acceptor substrate concentrations ranging from 2.5-500 μM at a constant AcCoA concentration of 100 μM was plotted as Eadie-Hofstee transformations. The curvilinear pattern indicates that more than one enzyme *N*-acetylates PAP in rats. Velocity (V). Velocity/substrate concentration (V/S).

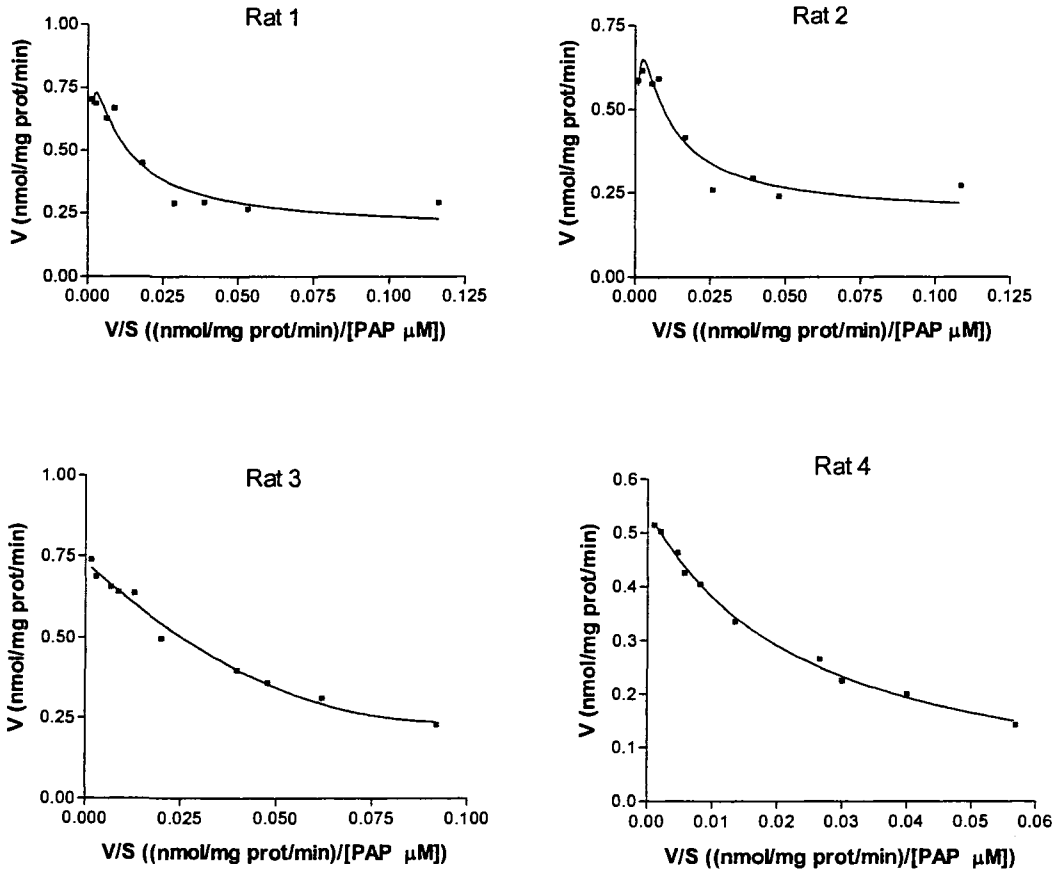


Figure 2.4: Determination of PAP *N*-acetylation enzyme kinetics in rats

In rats, the overall apparent K_m was 10.3 ± 2.0 µM and apparent V_{max} was 0.63 ± 0.03 nmol/mg protein/minute at a constant AcCoA concentration of 100 µM (n=4).

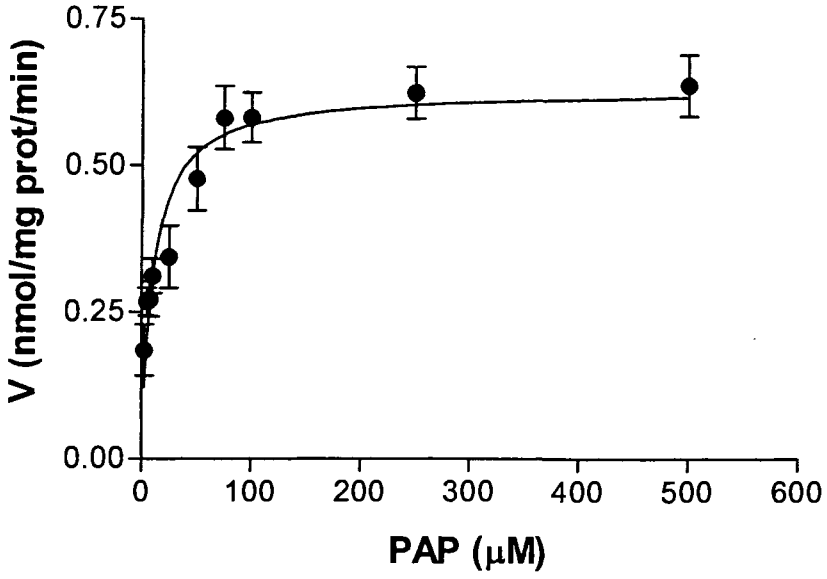


Figure 2.5: Protein and time linearity of *N*-acetylation of 100 µM PAP in cats

(Top) Protein linearity of feline *N*-acetylation of 100 µM PAP at 4-5 concentrations ranging from 0.07-1.1 mg prot/ml in 3 cats. (Bottom) Time linearity of feline *N*-acetylation of 100 µM PAP at 6 times points ranging from 0-30 minutes (n=3).

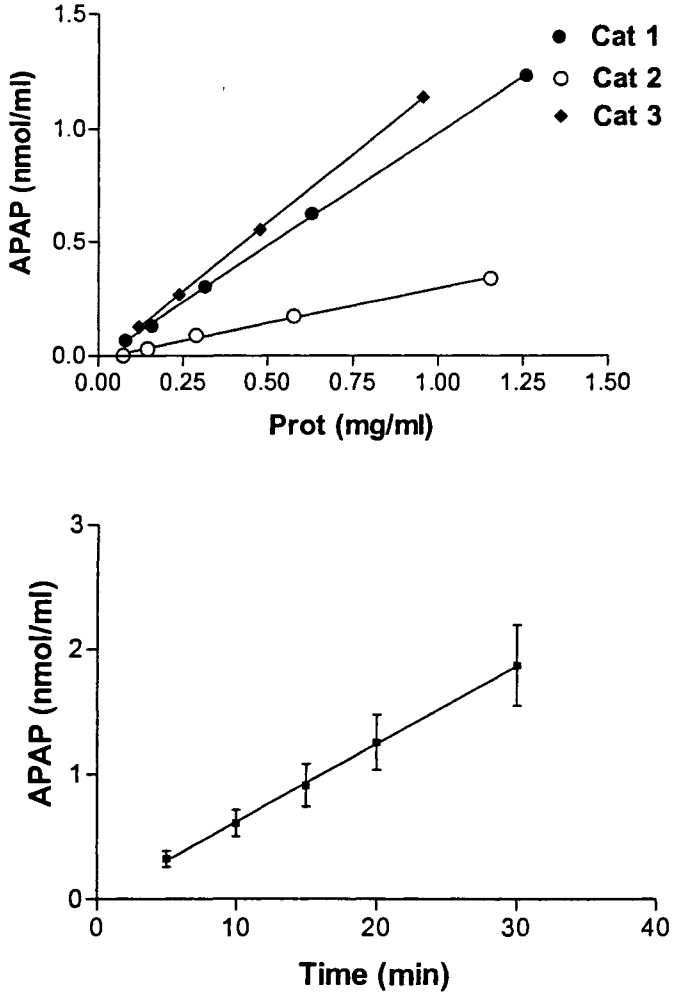


Figure 2.6: Eadie-Hofstee transformations of PAP *N*-acetylation in six individual cats

Feline PAP *N*-acetylation using 9-10 acceptor substrate concentrations ranging from 2.5-500 µM at a constant AcCoA concentration of 100 µM.

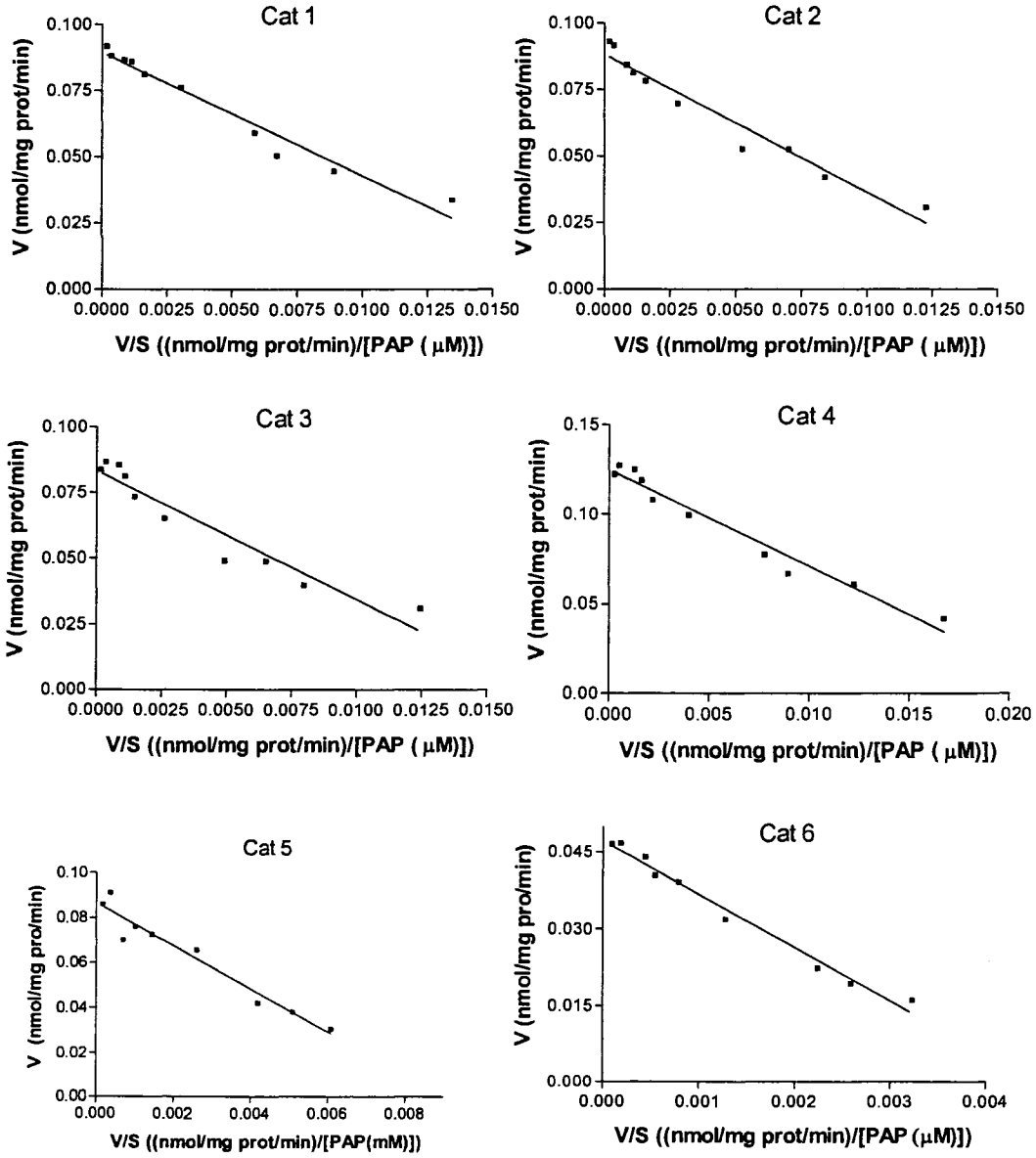
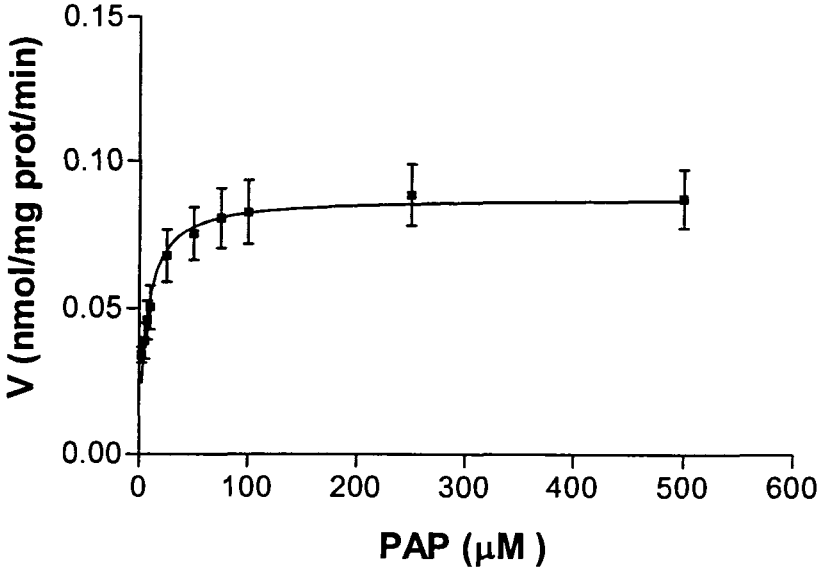


Figure 2.7: Determination of PAP *N*-acetylation enzyme kinetics in cats.

The K_m of PAP *N*-acetylation was 6.4 ± 1.6 µM and the V_{max} was 0.09 ± 0.004 nmol/mg protein/minute at a constant AcCoA concentration of 100 µM in cats (n=6).



concentration over a range of 0.08-1.08 mg/ml cytosol protein following 15 minutes of incubation (Figure 2.8). Eadie-Hofstee transformation of the data was curvilinear indicating more than one enzyme involved in the *N*-acetylation (Figure 2.9). The overall apparent mouse K_m was 23.2 ± 6.0 μM PAP and overall apparent V_{max} was 1.42 ± 0.10 nmol/mg prot/min (n=3) (Figure 2.10). There was no *N*-acetylation of APAP in dogs and knockout mice (n=4).

2.12.3 Deacetylation

Deacetylation of APAP was carried out under linear conditions. All species exhibited deacetylation activity. There was no significant difference in the deacetylation between species (P=0.12), (Figure 2.11).

2.12.4 *In vitro* methemoglobin

There was no increase of methemoglobin following exposure of red blood cells of all species to a final concentration of 0.25% DMSO in HEPES. The mean methemoglobin concentrations of 0.25% DMSO in HEPES were 2.4 ± 0.6%, 1.2 ± 0.4% and 1.5 ± 0.5% in rats, dogs, and cats respectively (n=5-6). A time course for PAP-induced methemoglobin formation was performed in dogs (Figure 2.12) and cats. The feline and canine time courses had a curvilinear pattern over a 60 minute period. Five minutes was chosen to represent the initial rate of methemoglobin formation because we were unable to accurately do a shorter time. Sixty minutes was chosen to represent the plateau concentration. The compounds APAP, NAPQI and PAP were analyzed for methemoglobin

Figure 2.8: Wildtype (C57BL/6) mouse *N*-acetylation protein linearity

N-acetylation of 100 µM PAP at 5 concentrations of cytosol protein over a range of 0.05-1.08 mg/ml (n=3).

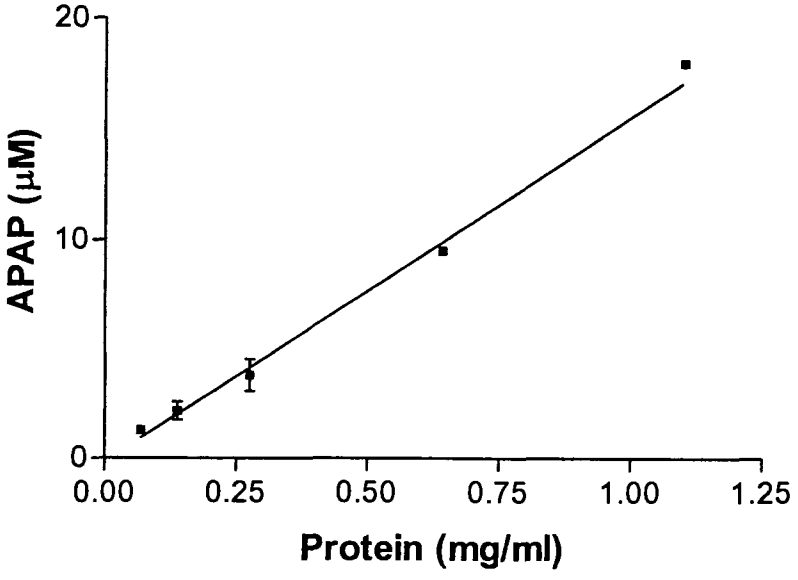


Figure 2.9: Eadie-Hofstee transformations of *N*-acetylation of PAP in 3 individual wildtype (C57BL/6) mice

N-acetylation of PAP using 9-10 acceptor substrate concentrations ranging from 2.5-500 µM at a constant AcCoA concentration of 100 µM.

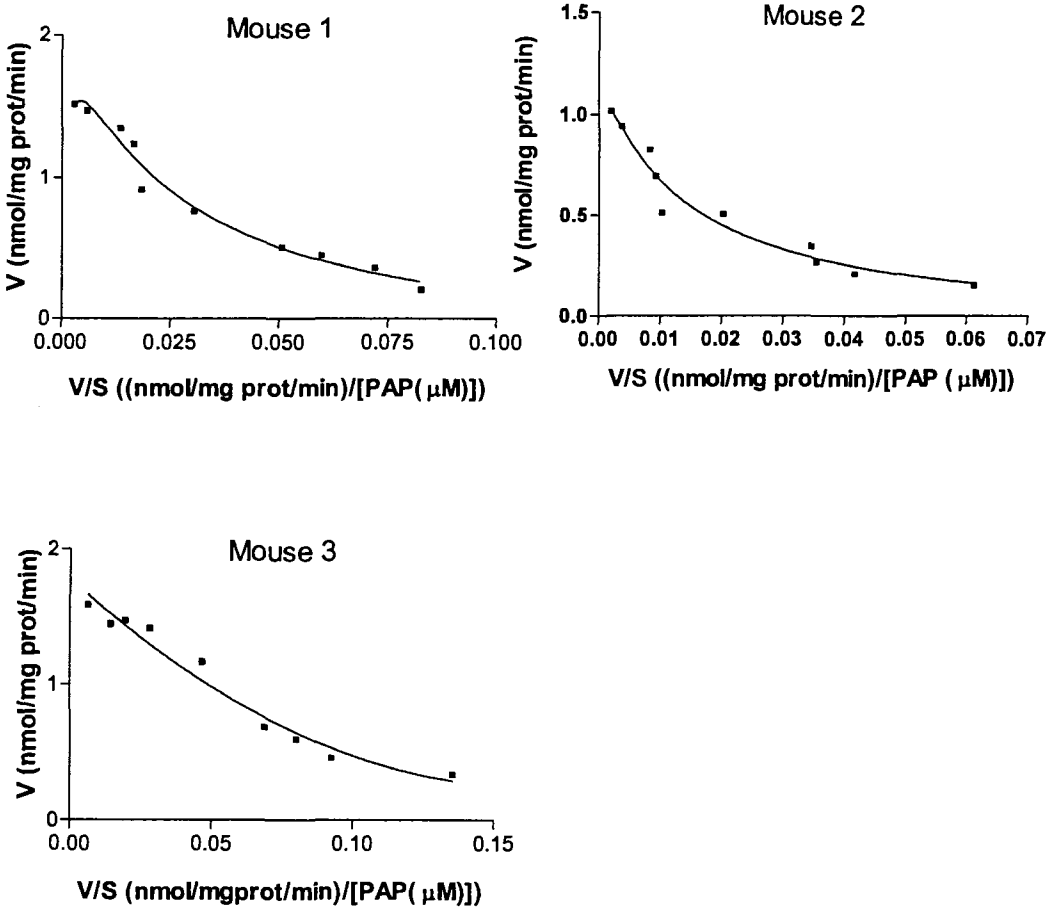


Figure 2.10: Wildtype C57BL/6 mouse enzyme kinetics

Determination of PAP *N*-acetylation enzyme kinetics in mice. In wildtype mice, the overall apparent K_m was 23.2 ± 6.0 µM and apparent V_{max} was 1.42 ± 0.10 nmol/mg protein/minute at a constant AcCoA concentration of 100 µM (n=4).

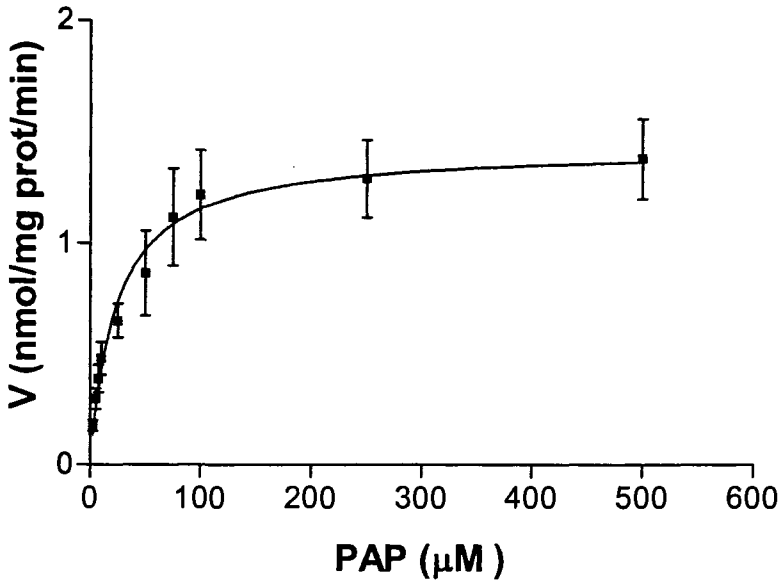


Figure 2.11: Species comparison of deacetylation of 5,000 µM APAP *in vitro*

The velocity of APAP deacetylation to PAP by microsomal decarboxyesterases was determined by HPLC measurement of the stable derivative 4-hydroxybutyranilide (BPAP) following the addition of butyric anhydride to stop the enzyme reaction (n=4 in all species).

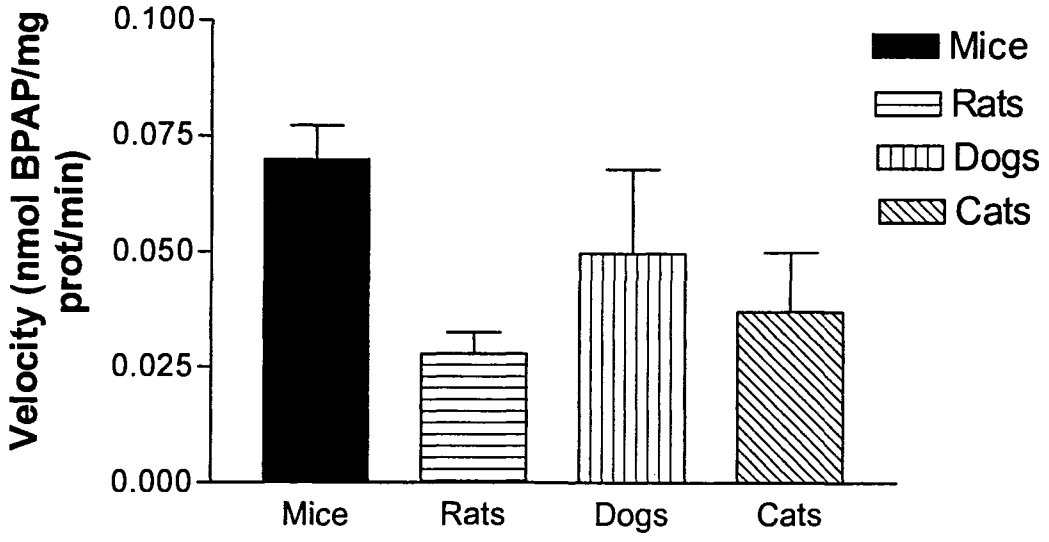
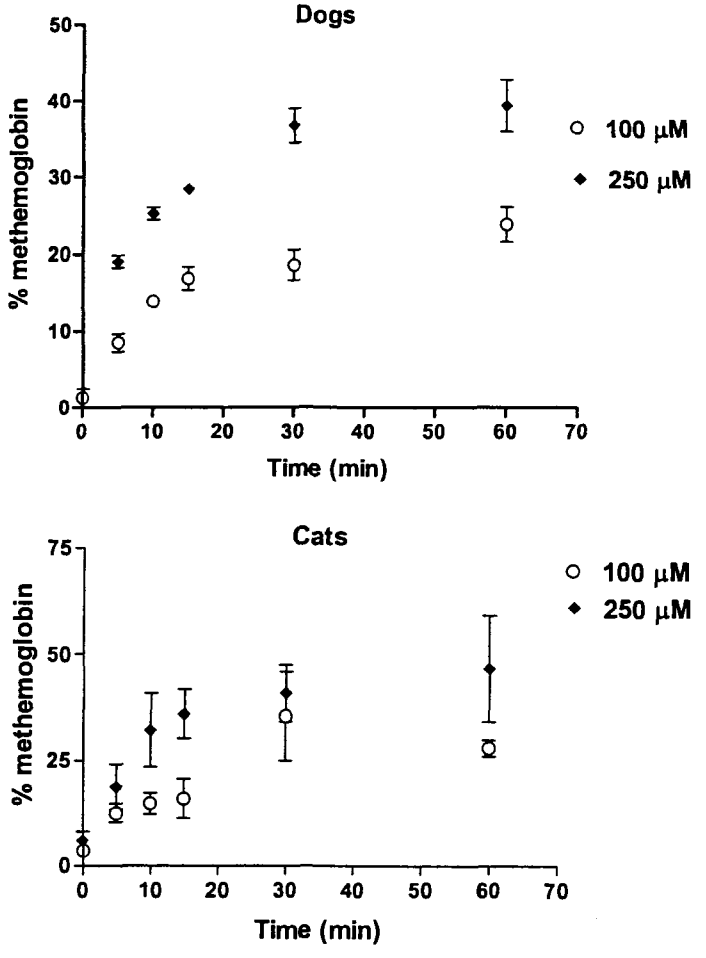


Figure 2.12: Time course of PAP-induction of methemoglobin formation

(Top) Time course of methemoglobin induction by 100 and 250 μM PAP in dogs at 6 time points ranging from 0 to 60 minutes (n=4). (Bottom) Time course of methemoglobin induction by 100 and 250 μM PAP in cats at 6 time points ranging from 0 to 60 minutes (n=4).



induction. There was no biologically significant methemoglobin formation following incubation with 1000 or 5000 μM APAP for 5 or 60 minutes in feline, rat or canine erythrocytes. (Figure 2.13). There was also no biologically significant induction of methemoglobin formation in rat, feline or canine erythrocytes by 100, 250 or 500 μM NAPQI following 5 0r 60 minutes of incubation (Figure 2.14).

There was significant generation of methemoglobin in rat erythrocytes *in vitro* following incubation of erythrocytes with 500 μM PAP ($P<0.05$), cat erythrocytes at a concentration of 25-500 μM ($P<0.01$) and in canine erythrocytes at 25 μM PAP ($P<0.05$) and at 50-500 μM PAP ($P<0.01$) following 5 minutes of incubation (Figure 2.15). There was significant methemoglobin formation at concentrations of 12.5-500 μM PAP in canine and feline erythrocytes and in rat erythrocytes at 100-500 μM PAP following 60 minutes of incubation ($P<0.01$) *in vitro*. Therefore, PAP was the only chemical tested that induced clinically significant methemoglobin formation in dogs, cats and rats (Figure 2.16).

Methemoglobin induction by 500 μM PAP was compared at 60 minutes in dogs, cats, rats, wildtype mice (C57Bl/A6) and *NAT1, NAT2* double knockout mice (Figure 2.17). There was no significant difference between methemoglobin generation in dog and cat erythrocytes at 5 or 60 minutes ($P>0.05$). There was a significantly higher PAP induced methemoglobin formation in canine and feline erythrocytes compared to rat, wildtype and knockout mice ($P<0.001$). Knockout mice erythrocytes had significantly more methemoglobin induction than wildtype mice ($P=0.03$).

Methemoglobin formation was compared in lysed and intact erythrocytes

Figure 2.13: Methemoglobin induction by APAP

APAP did not induce biologically significant methemoglobin formation in rat, feline, or canine erythrocytes *in vitro* following 5 minutes (Top) or 60 minutes (Bottom) of incubation (n=6).

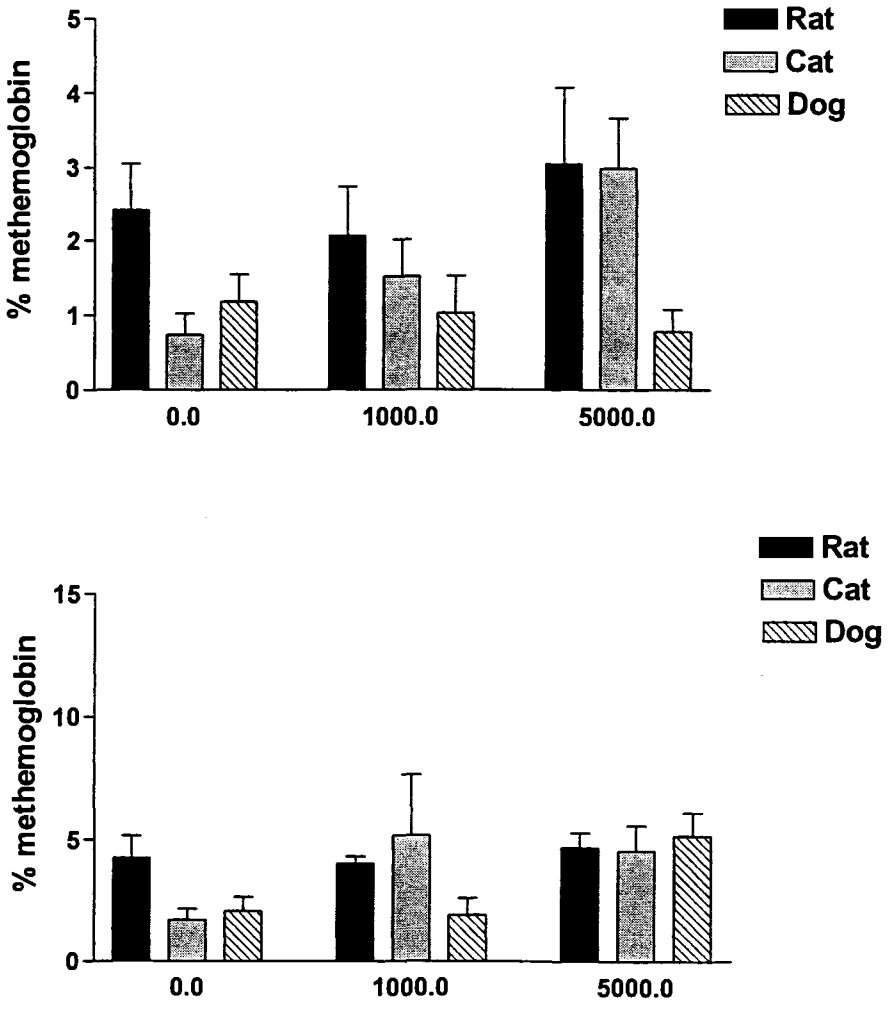


Figure 2.14: Methemoglobin induction by NAPQI

NAPQI did not induce biologically significant methemoglobin formation in rat, feline, or canine erythrocytes *in vitro* following 5 minutes (Top) or 60 minutes (Bottom) of incubation (n=6).

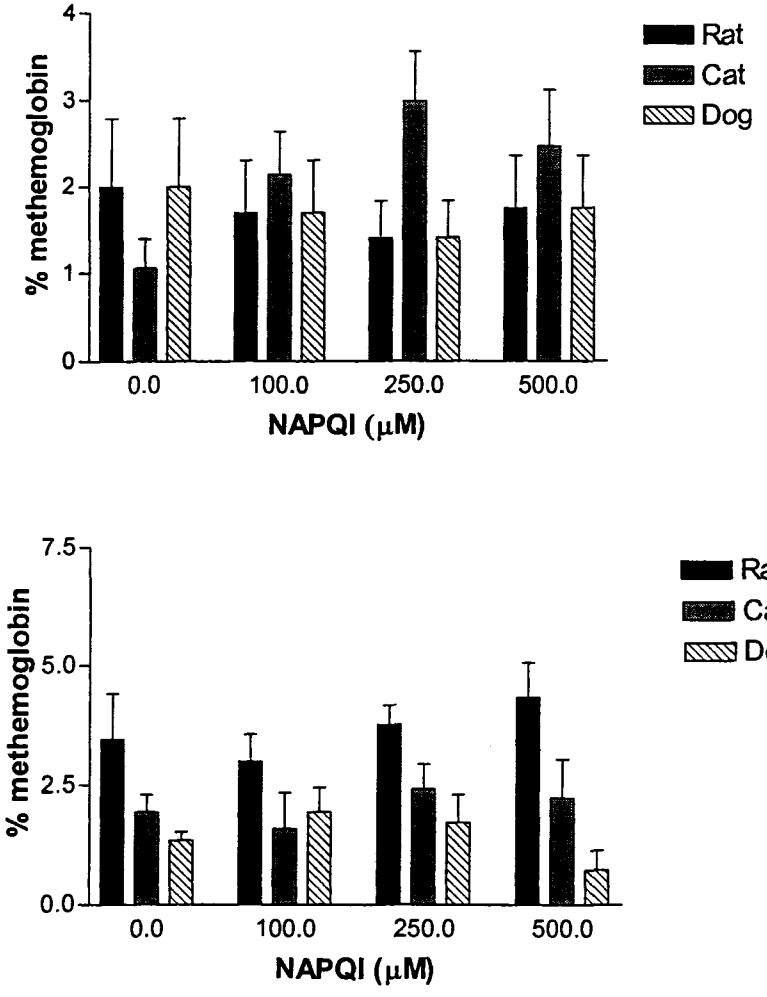


Figure 2.15: Dose-dependent methemoglobin induction by PAP

(Top) PAP dose-dependent methemoglobin induction at 5 minutes *in vitro* in canine, feline and rat erythrocytes (n=6). (Bottom) PAP dose-dependent methemoglobin induction at 60 minutes *in vitro* in canine, feline and rat erythrocytes (n=6). (*P<0.05, **P<0.01), describe significant methemoglobin induction compared to control of 0.25% DMSO in HEPES for that species. "A" indicates methemoglobin induction is significantly greater than rat methemoglobin induction.

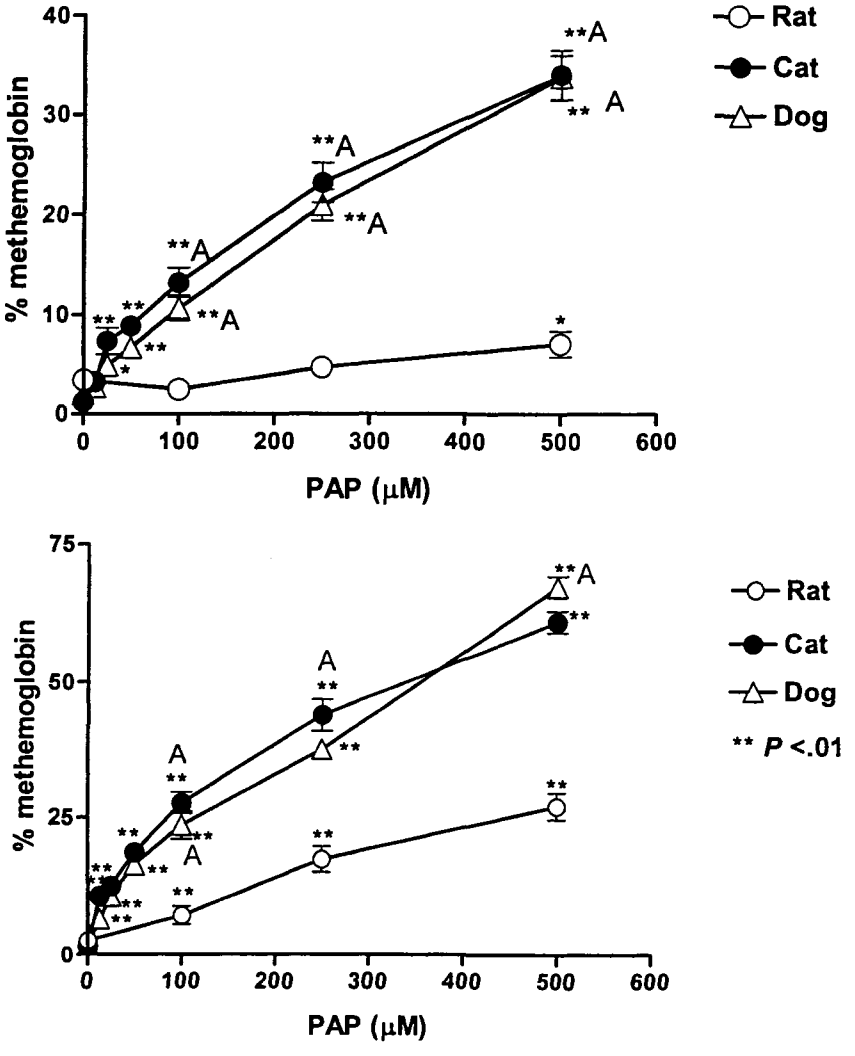


Figure 2.16: APAP, NAPQI and PAP methemoglobin induction

Methemoglobin formation following 0, 5 and 60 minutes exposure to APAP, NAPQI and PAP in dog (top), cat (middle) and rat (bottom) erythrocytes *in vitro*. (n=6). (*P<0.05,**P<0.01)

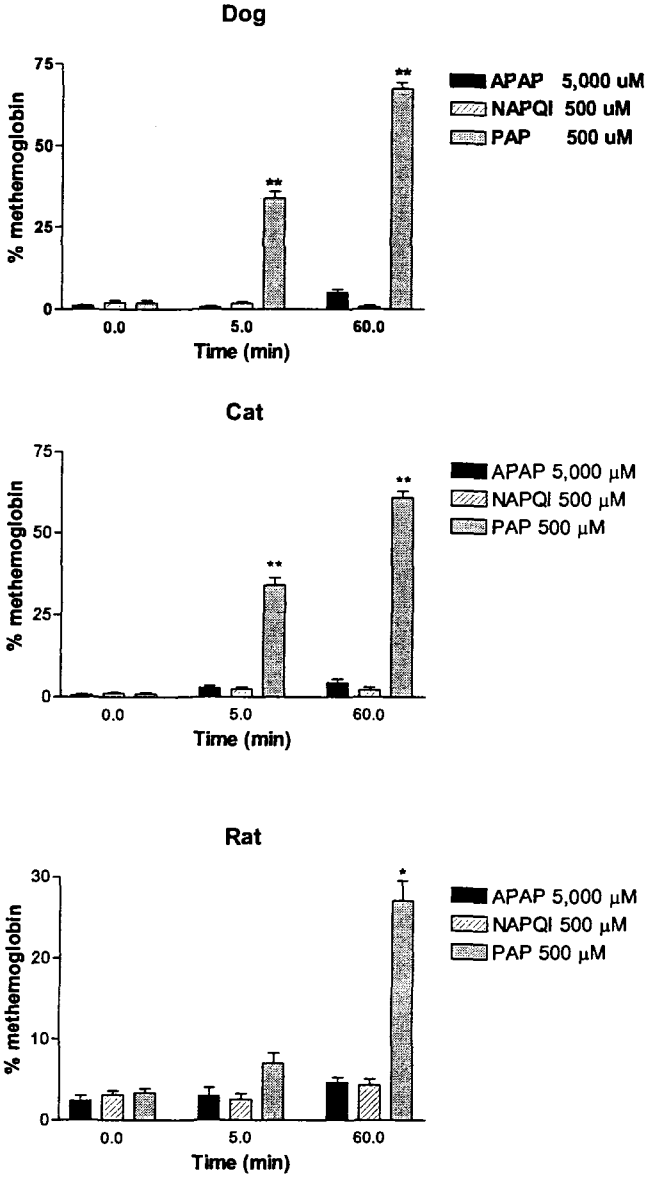
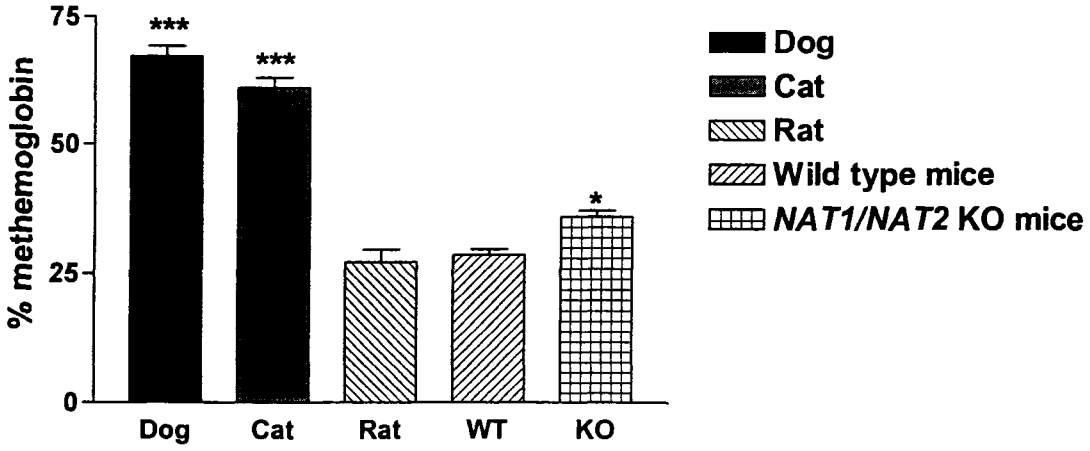


Figure 2.17: Comparison of *in vitro* induction of methemoglobin formation in dogs, cats, rats, wildtype C57BL/6 and *NAT1/NAT2* knockout mice by 500 μM PAP at 60 minutes.

The methemoglobin formation in dog and cat erythrocytes was significantly greater than rat, wildtype (WT) and NAT1/NAT2 knockout mouse (KO) erythrocytes (***P<0.001). Methemoglobin generation in KO erythrocytes was significantly greater than in WT erythrocytes (*P<0.05) (n=6 in dogs and cats, n=4 in wildtype and knockout mice).



exposed to 250 μM PAP (Figure 2.18). In rats and C57BL/6 wildtype mice, there was significantly more methemoglobin induction in lysed cells than in intact red blood cells following 30 minutes exposure (P=0.013 and P=0.015 respectively). There was also more methemoglobin generation in intact rat red blood cells following 1 hour of incubation (P=0.019), however, it was not statistically significant following lowering of the level of significance. In dogs and cats there was more methemoglobin induction in intact cells than lysed cells at 30 (P=0.029 and 0.0007 respectively) and at 60 minutes (P=0.057 and P=0.023). There was no significant difference in methemoglobin production in lysed versus intact erythrocytes in knockout mice (P=1.0).

No biologically significant hemolysis was caused *in vitro* by APAP, PAP or NAPQI in dogs or cats over six hours of incubation (Figure 2.19). Hemolysis was not measured in rats or mice given that there was no hemolysis in the main species of interest.

Figure 2.18: Methemoglobin generation in intact and lysed erythrocytes following incubation with 250 μM PAP.

(*P<0.05, **P<0.01, ***P<0.001).

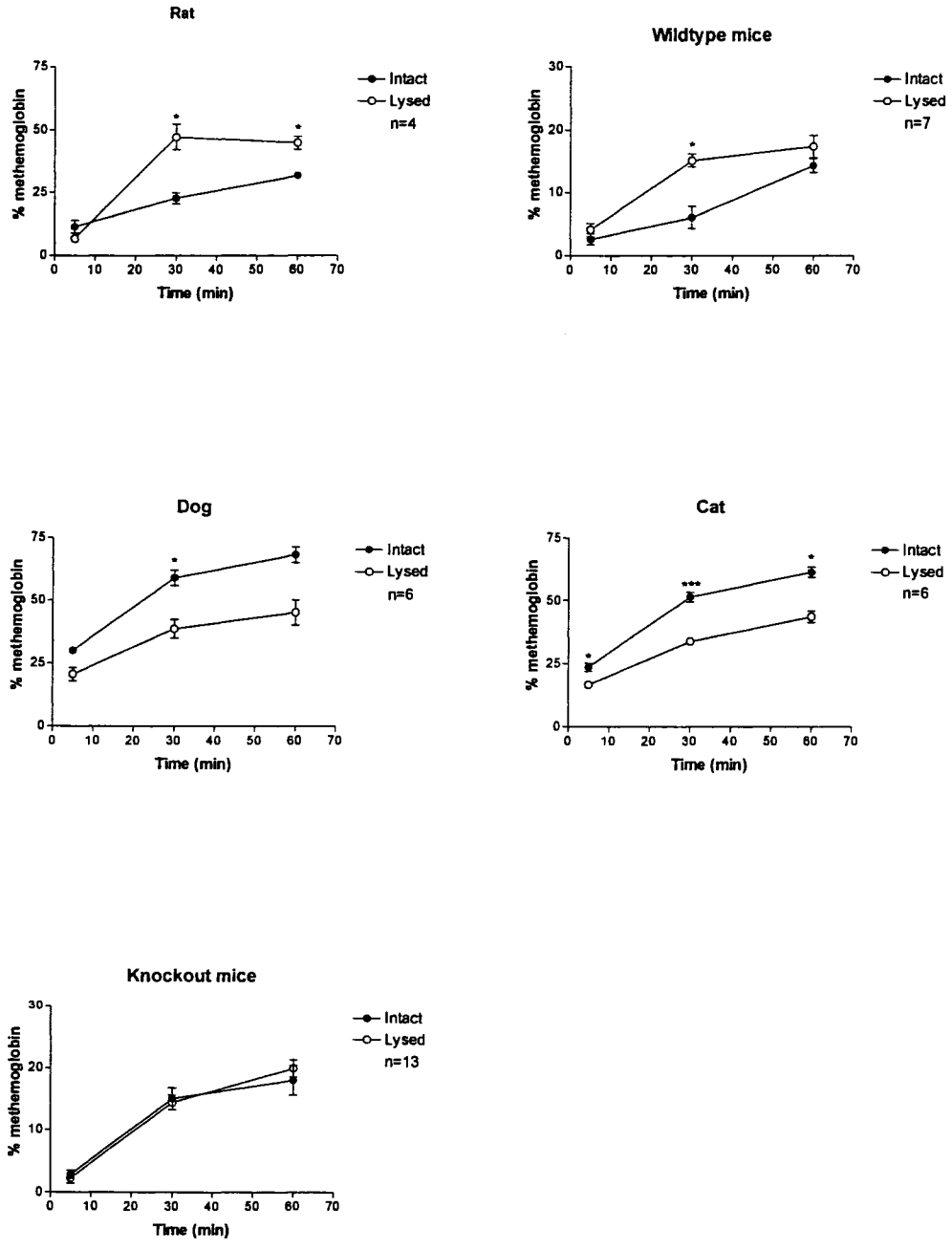
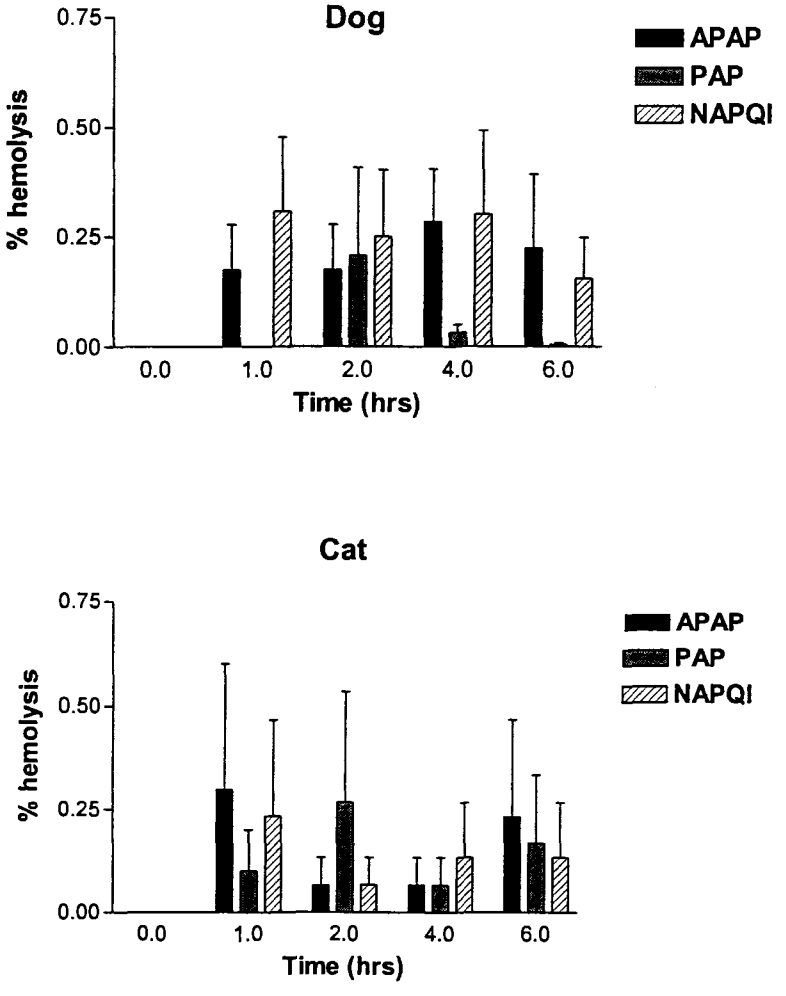


Figure 2.19: APAP, PAP and NAPQI induction of hemolysis in dogs and cats

No biologically significant hemolysis occurred in canine (top) or feline (bottom) erythrocytes following 6 hours exposure to APAP, PAP or NAPQI (n=4).



2.12 Discussion

Dogs and cats are the only reported species in which acetaminophen toxicity is associated with hemolytic anemia and methemoglobinemia. The reaction has often been attributed to NAPQI, the toxic metabolite responsible for APAP-induced hepatotoxicity in humans [23,26]. The minor APAP metabolite PAP has been overlooked, despite previously being associated with aniline dye-induced methemoglobinemia in humans [32].

para-Aminophenol induces methemoglobin formation by co-oxidation with oxyhemoglobin [32,53,54]. During co-oxidation, an electron is lost from both the PAP hydroxyl group and the ferrous iron in the oxyhemoglobin, creating oxidized PAP (benzoquinoneimine (BQI)) and methemoglobin (Figure 1.13). The electrons are donated to O_2 resulting in the production of a reactive oxygen species such as hydrogen peroxide or superoxide. The PAP is recycled within the erythrocyte through reduction of BQI to PAP by GSH. The PAP reacts repeatedly with oxyhemoglobin until it is removed by an alternative biotransformation pathway. Greater than 70% of PAP is removed by *N*-acetylation in rats and mice, but *N*-acetylation is poor in cats and non-existent in dogs. This is because dogs lack NAT enzymes and cats only have NAT1 [45,46]. Therefore, the elimination of PAP may be slower in dogs and cats which could result in prolonged redox cycling of PAP and greater methemoglobin production. The aim of our experiments was to determine if there is an *in vitro* species difference in deacetylation and *N*-acetylation of APAP and PAP. We also wished to determine the methemoglobin forming potential of APAP, NAPQI and PAP. Finally, we wished to determine

whether there is a species difference in the PAP-induction of methemoglobin in isolated red blood cells due to the deficient NAT activity in dogs and cats.

Our results demonstrate that dogs, cats, rats and C57BL/6 wildtype mice can de-acetylate APAP to PAP *in vitro*. This suggests that *in vivo*, all four species are capable of producing PAP from APAP. It is important to note that the advantage of using *in vitro* enzymatic reactions is the ability to isolate a particular enzyme and control its access to cofactors and substrates. The drawback is that extrapolation to *in vivo* can be misleading as there may be competition for cofactors or substrates *in vivo* from other biochemical pathways or a variation in the cell enzyme concentration that is not reflected *in vitro*.

The Eadie-Hofstee plots of *N*-acetylation in rats and wildtype mice were curvilinear, indicating more than one contributing enzyme to rat and mouse PAP *N*-acetylation. This is compatible with previously reported rat and mouse *N*-acetylation activity [55-57]. Rats have 3 NAT enzymes: NAT1 and NAT2 as well as the recently discovered functional NAT3 [55]. Mice also have 3 NAT enzymes: NAT1, NAT2 and NAT3 [56,57]. Mouse NAT3 is poorly functional [56,57]. The enzymes NAT1 and NAT2 have different but overlapping substrate preferences. Although NAT2 has a higher affinity for small molecules such as PABA, NAT1 can also catalyze the *N*-acetylation of small molecules to a lesser degree [58]. Dogs showed no *N*-acetylation of PAP in the work presented here, reflecting their lack of NAT enzymes. Feline *N*-acetylation Eadie-Hofstee transformations were linear indicating *N*-acetylation by a single enzyme. The level of activity in feline PABA *N*-acetylation in our studies was similar to that published by Trepanier [45].

The *N*-acetylation activity varied between species (mice>rats>cats>dogs), with rats and mice showing significantly more *N*-acetylation activity than cats. The lower PAP *N*-acetylation in dogs and cats supports the idea that any PAP that is produced from APAP *in vivo* may have a longer half-life in cats and dogs than in rats and mice. This could result in exposure of red blood cells to higher concentrations of PAP during the metabolism of APAP. In addition, any PAP that redox cycles with oxyhemoglobin would not be removed as quickly by biotransformation and thus would continue to redox cycle, continuing to produce methemoglobin.

The hematotoxic concentration of APAP in dogs is ~1000 μM APAP [15]. Acetaminophen did not induce significant methemoglobin at concentrations of 1000 or 5000 μM *in vitro*. This suggests that APAP-induced methemoglobin requires metabolism of APAP to reactive metabolites.

The reactive APAP metabolite responsible for hepatotoxicity in many species is NAPQI. It has also been proposed as the causative agent in dog and cat hematotoxicity. However, the reactive intermediate NAPQI is produced in the liver and does not enter the circulating blood. Instead, an inactive glutathione or hepatic protein conjugated metabolite of NAPQI leaks out of hepatocytes [59,60]. In our studies, NAPQI concentrations that cause *in vitro* hepatocellular toxicity in rats and mice [59,60] did not cause significant methemoglobin formation *in vitro* at 5 or 60 minutes in the species investigated here. This supports that NAPQI is unlikely to be the reactive metabolite responsible for methemoglobinemia in dogs and cats.

Methemoglobin formation associated with PAP has been demonstrated previously [32,61]. The aminophenols are weaker methemoglobin inducers than hydroxylamines [40,62,63]. Similar to our studies, Harrison et al [32] found that the maximum production of methemoglobin in rats was approximately 35% with aminophenols, which was attributed to the short half life of aminophenols in rats

The PAP concentrations were chosen to match NAPQI concentrations for comparative purposes. Actual *in vivo* PAP concentrations in red blood cells are unknown. Previous studies examining the methemoglobin forming potential of PAP have used the production of methemoglobin as their end points and have not determined the concentration of PAP within the erythrocytes [32,34]. In addition, studies often measure the PAP metabolite PAP-GSH but not PAP itself [37,38,64]. As PAP is quickly reacetylated to APAP, these studies likely underestimate the total PAP production.

There was no significant difference in the induction of methemoglobin in canine and feline erythrocytes by 500 µM PAP, but the methemoglobin formation was significantly greater in both feline and canine erythrocytes than in rat and mouse red blood cells. This indicates a difference in species susceptibility to PAP induction of methemoglobin formation. The difference in sensitivity could have several contributing factors. Diffusion of PAP into erythrocytes may differ. There may be inherent differences in oxyhemoglobin reactivity to PAP, rate of PAP detoxification or a difference in reduction of the methemoglobin by NADH methemoglobin reductase.

There was significantly more methemoglobin produced in the knockout

erythrocytes than in wildtype mouse erythrocytes. However, the degree of methemoglobin formation in knockout mouse erythrocytes was significantly lower than the degree of methemoglobin formation in canine and feline erythrocytes. This supports our hypothesis that decreased NAT activity contributes to APAP methemoglobinemia in dogs and cats, but indicates that there must be other contributing factors.

Co-oxidation reactions have been shown to be inversely proportional to the speed at which the reactive compounds are removed from cycling by conjugation reactions [65]. It is important to note that we isolated the effect of NAT on removal of PAP from redox cycling *in vitro. In vivo*, NAT activity would also affect PAP detoxification in hepatocytes and therefore could have an additional effect on the systemic PAP concentration.

We examined methemoglobin induction in intact versus lysed erythrocytes *in vitro*. The lysed cell response isolates the effect on the hemoglobin, removes variations in cell penetration and decreases the activity of detoxification enzymes such as methemoglobin reductase and NAT by dilution. As PAP is able to penetrate red blood cells, the significantly higher methemoglobin formation in lysed cells than in intact cells in wildtype mice and rats was likely due to the decreased methemoglobin reductase and NAT activity in the lysed cells due to dilution [40]. Mice and rats have a greater methemoglobin reductase activity than dogs and cats and therefore the loss of both the reduction by methemoglobin reductase and PAP biotransformation by NAT in the lysed cells could account for the higher methemoglobin production in lysed rat and mouse erythrocytes [66,67].

Contrary to what was seen in mice and rats, there was significantly higher methemoglobin formation in the intact cells from dogs and cats treated with 500 μM PAP. Canine and feline methemoglobin reductase is less active than rat and mouse methemoglobin reductase [68]. Therefore, the loss of methemoglobin reductase activity in dog and cat intact erythrocytes would have less effect on the methemoglobin formation than in intact rat and mouse erythrocytes. The deficient feline and canine NAT activity in intact cells, however would allow prolonged redox cycling of PAP and oxyhemoglobin leading to more methemoglobin formation in intact cells than lysed erythrocytes. The knockout mice did not show a significant difference in methemoglobin formation between intact and lysed erythrocytes. This supports that a deficiency of NAT predisposes to a higher PAP-induction of methemoglobin generation..

Canine and feline APAP hematotoxicity is also associated with hemolysis. Hemolysis can be broadly catagorized into intravascular (erythrocyte lysis within the blood stream) or extravascular (erythrocyte removal by tissue macrophages) [69]. In clinical feline and canine APAP toxicity, there can be mild intravascular hemolysis within the first few hours of intoxication and marked extravascular hemolysis due to oxidative damage characterized by Heinz bodies at 4-6 days [70-72]. In our *in vitro* studies, direct hemolysis did not occur following incubation with any of the chemicals. This is in agreement with previous work that showed that exposure of rats to PAP *in vivo* or *in vitro* does not cause immediate or delayed removal of red blood cells from circulation [40]. Therefore, a metabolite other than PAP or NAPQI is likely responsible for APAP-induced hemolysis. Grossman and

Jollow have shown that *N*-hydroxy dapsone is responsible for the oxidative hemolytic anemia in humans that can occur following therapy with dapsone [63]. Phenylhydroxylamine is another hydroxylated chemical that causes extravascular hemolysis by oxidative damage[40]. Therefore, a hydroxylated metabolite of APAP, potentially even hydroxylated PAP, could be investigated as a metabolite that could potentially induce hemolytic anemia.

In summary, APAP itself does not have the ability to induce methemoglobin formation. Our studies show that acetaminophen must be metabolized to a reactive metabolite for methemoglobinemia to occur in canine and feline APAP toxicity. We have demonstrated that dogs, cats, rats and mice can deacetylate APAP to the reactive metabolite PAP *in vitro*. We found no significant difference in the deacetylation activity between species, indicating that all four species have the potential to produce PAP *in vivo*.

We have also shown that rats and mice have significantly more *N*-acetylation activity than cats, while dogs show no PAP *N*-acetylation. As *N*-acetylation appears to be the predominant biotransformation pathway of PAP, dogs and cats may be exposed to higher concentrations of PAP than rats and mice. This could promote prolonged redox cycling with oxyhemoglobin, resulting in increased methemoglobinemia. This theory was supported by the significantly higher PAP-induction of methemoglobin formation in cats and dogs than in mice and rats. This theory was also supported by the significantly higher methemoglobin induction in *NAT1/NAT2* double knockout mice compared to wildtype mice of the same strain. It was further supported by the variation in the

in vitro response of lysed and intact erythrocytes from wildtype and knockout mice to PAP methemoglobin induction.

There appear to be other contributing factors to the high species sensitivity of dogs and cats to PAP-induction of methemoglobin. The most likely other contributing factor is the enzyme NADH methemoglobin reductase which is the primary reducing system for methemoglobin. This enzyme has been shown elsewhere to be present in lower concentrations in dogs and cats compared to rats and mice [68]. This species variability could have a considerable effect on the final methemoglobin concentration.

Based on these results, further investigation into therapies involving inhibition of APAP deacetylation or augmentation of methemoglobin reduction is warranted for treatment of APAP toxicity in dogs and cats.

2.13 REFERENCES

1. Bialas MC, Evans RJ, Hutchings AD, Alldridge G, Routledge PA. The impact of nationally distributed guidelines on the management of paracetamol poisoning in accident and emergency departments. National Poison Information Service. J Accid Emerg Med 1998;15:13-7.

2. Dargan PI, Jones AL. Management of paracetamol poisoning. Trends Pharmacol Sci 2003;24:154-7.

3. Watson WA, Litovitz TL, Klein-Schwartz W, et al. 2003 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2004;22:335-404.

4. Jones RD, Baynes RE, Nimitz CT. Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989-1990). J Am Vet Med Assoc 1992;201:475-7.

5. Davis DC, Potter WZ, Jollow DJ, Mitchell JR. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci 1974;14:2099-109.

6. Prescott LF, Roscoe P, Wright N, Brown SS. Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet 1971;1:519-22.

7. Aronson LR, Drobatz K. Acetaminophen toxicosis in 17 cats. J Vet Emerg Crit Care 1996;6:65-9.

8. MacNaughton SM. Acetaminophen toxicosis in a Dalmatian. Can Vet J 2003;44:142-4.

9. Schlesinger DP. Methemoglobinemia and anemia in a dog with acetaminophen toxicity. Can Vet J 1995;36:515-7.

10. Ilkiw JE, Ratcliffe RC. Paracetamol toxicity in a cat. Aust Vet J 1987;64:245-7.

11. Nash SL, Savides MC, Oehme FW, Johnson DE. The effect of acetaminophen on methemoglobin and blood glutathione parameters in the cat. Toxicology 1984;31:329-34.

12. Gazzard BG, Hughes RD, Mellon PJ, Portmann B, Williams R. A dog model of fulminant hepatic failure produced by paracetamol administration. Br J Exp Pathol 1975;56:408-11.

13. Kelly JH, Koussayer T, He DE, et al. An improved model of acetaminophen-

induced fulminant hepatic failure in dogs. Hepatology 1992;15:329-35.

14. St Omer VV, McKnight ED, III. Acetylcysteine for treatment of acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1980;176:911-3.

15. Savides MC, Oehme FW, Nash SL, Leipold HW. The toxicity and biotransformation of single doses of acetaminophen in dogs and cats. Toxicol Appl Pharmacol 1984;74:26-34.

16. Davis M, Simmons CJ, Harrison NG, Williams R. Paracetamol overdose in man: relationship between pattern of urinary metabolites and severity of liver damage. Q J Med 1976;45:181-91.

17. Gemborys MW, Mudge GH. Formation and disposition of the minor metabolites of acetaminophen in the hamster. Drug Metab Dispos 1981;9:340-51.

18. Pumford NR, Roberts DW, Benson RW, Hinson JA. Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen protein adducts in subcellular liver fractions following a hepatotoxic dose of acetaminophen. Biochem Pharmacol 1990;40:573-9.

19. Landin JS, Cohen SD, Khairallah EA. Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol 1996;141:299-307.

20. de Morais SM, Wells PG. Deficiency in bilirubin UDP-glucuronyl transferase as a genetic determinant of acetaminophen toxicity. J Pharmacol Exp Ther 1988;247:323-31.

21. Tee LB, Davies DS, Seddon CE, Boobis AR. Species differences in the hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. Biochem Pharmacol 1987;36:1041-52.

22. Allen AL. The diagnosis of acetaminophen toxicosis in a cat. Can Vet J 2003;44:509-10.

23. Taylor NS, Dhupa N. Acetaminophen Toxicity in Dogs and Cats. Compend Contin Educ Pract Vet 2003;12:160-9.

24. Court MH, Greenblatt DJ. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. Biochem Pharmacol 1997;53:1041-7.

25. Court MH, Greenblatt DJ. Molecular genetic basis for deficient acetaminophen glucuronidation by cats: UGT1A6 is a pseudogene, and

evidence for reduced diversity of expressed hepatic UGT1A isoforms. Pharmacogenetics 2000;10:355-69.

26. Desnoyers M. Anemias associated with heinz bodies. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm's Veterinary Hematology. Fifth ed. Philadelphia: Lippincott WIlliams & Wilkins; 2000. p.178-84.

27. Snow NS. Some observations on the reactive sulphydryl groups in haemoglobin. Biochem J 1962;84:360-4.

28. Riggs AF, WOLBACH RA. Sulfhydryl groups and the structure of hemoglobin. J Gen Physiol 1956;39:585-605.

29. Muldrew KL, James LP, Coop L, et al. Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of acetaminophen using high-performance liquid chromatography with electrochemical detection. Drug Metab Dispos 2002;30:446-51.

30. Gillette JR, Nelson SD, Mulder GJ, et al. Formation of chemically reactive metabolites of phenacetin and acetaminophen. Adv Exp Med Biol 1981;136 Pt B:931-50.

31. Reilly TP, Woster PM, Svensson CK. Methemoglobin formation by hydroxylamine metabolites of sulfamethoxazole and dapsone: implications for differences in adverse drug reactions. J Pharmacol Exp Ther 1999;288:951-9.

32. Harrison JH, Jr., Jollow DJ. Contribution of aniline metabolites to aniline-induced methemoglobinemia. Mol Pharmacol 1987;32:423-31.

33. Kiese M. The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol Rev 1966;18:1091-161.

34. Kiese M. The reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

35. Newton JF, Kuo CH, Gemborys MW, Mudge GH, Hook JB. Nephrotoxicity of p-aminophenol, a metabolite of acetaminophen, in the fischer 344 rat. Toxicol Appl Pharmacol 1982;65:336-44.

36. Welsh RM, Conney AH, Burns JJ. The metabolism of acetophenetidin. Biochem Pharmacol 1966;15:521-31.

37. Song H, Chen TS. p-Aminophenol-induced liver toxicity: tentative evidence of

a role for acetaminophen. J Biochem Mol Toxicol 2001;15:34-40.

38. Klos C, Koob M, Kramer C, Dekant W. p-aminophenol nephrotoxicity: biosynthesis of toxic glutathione conjugates. Toxicol Appl Pharmacol 1992;115:98-106.

39. Bernheim F, Berheim M, Michel HO. The action of p-aminophenol on certain tissue oxidations. J Pharmacol Exp Ther 1937;61:311-20.

40. Harrison JH, Jr., Jollow DJ. Role of aniline metabolites in aniline-induced hemolytic anemia. J Pharmacol Exp Ther 1986;238:1045-54.

41. Grant DM, Lottspeich F, Meyer UA. Evidence for two closely related isozymes of arylamine N-acetyltransferase in human liver. FEBS Lett 1989;244:203-7.

42. Grant DM, Blum M, Beer M, Meyer UA. Monomorphic and polymorphic human arylamine N-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. Mol Pharmacol 1991;39:184-91.

43. Hein DW, Kirlin WG, Ferguson RJ, Thompson LK, Ogolla F. Identification and inheritance of inbred hamster N-acetyltransferase isozymes in peripheral blood. J Pharmacol Exp Ther 1986;239:823-8.

44. Ward A, Hickman D, Gordon JW, Sim E. Arylamine N-acetyltransferase in human red blood cells. Biochem Pharmacol 1992;44:1099-104.

45. Trepanier LA, Cribb AE, Spielberg SP, Ray K. Deficiency of cytosolic arylamine N-acetylation in the domestic cat and wild felids caused by the presence of a single NAT1-like gene. Pharmacogenetics 1998;8:169-79.

46. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol 1997;54:73-80.

47. Grant DM, Morike K, Eichelbaum M, Meyer UA. Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine N-acetyltransferase in human liver. J Clin Invest 1990;85:968-72.

48. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

49. Cribb AE, Grant DM, Miller MA, Spielberg SP. Expression of monomorphic

arylamine N-acetyltransferase (NAT1) in human leukocytes. J Pharmacol Exp Ther 1991;259:1241-6.

50. Grant DM, Lottspeich F, Meyer UA. Evidence for two closely related isozymes of arylamine N-acetyltransferase in human liver. FEBS Lett 1989;244:203-7.

51. Andres HH, Klem AJ, Szabo SM, Weber WW. New spectrophotometric and radiochemical assays for acetyl-CoA: arylamine N-acetyltransferase applicable to a variety of arylamines. Anal Biochem 1985;145:367-75.

52. Fairbanks VF, Klee GG. Biochemical Aspects of Hematology. In: Tietz NW, editor. Fundamentals of Clinical Chemistry. 3rd ed. Philadelphia: W.B. Saunders Company; 1987. p.789-824.

53. Berheim F, Berheim MLC., Michel HO. The action of p-aminophenol on certain tissue oxidations. J Pharmacol Exp Ther 1937;61:311-20.

54. Kiese M, Pekis M. THe reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

55. Walraven JM, Doll MA, Hein DW. Identification and characterization of functional rat arylamine N-acetyltransferase 3: comparisons with rat arylamine N-acetyltransferases 1 and 2. J Pharmacol Exp Ther 2006;319:369-75.

56. Fretland AJ, Doll MA, Gray K, Feng Y, Hein DW. Cloning, sequencing, and recombinant expression of NAT1, NAT2, and NAT3 derived from the C3H/HeJ (rapid) and A/HeJ (slow) acetylator inbred mouse: functional characterization of the activation and deactivation of aromatic amine carcinogens. Toxicol Appl Pharmacol 1997;142:360-6.

57. Estrada-Rodgers L, Levy GN, Weber WW. Substrate selectivity of mouse N-acetyltransferases 1, 2, and 3 expressed in COS-1 cells. Drug Metab Dispos 1998;26:502-5.

58. Hein DW, Doll MA, Fretland AJ, et al. Rodent models of the human acetylation polymorphism: comparisons of recombinant acetyltransferases. Mutat Res 1997;376:101-6.

59. McCloskey P, Edwards RJ, Tootle R, et al. Resistance of three immortalized human hepatocyte cell lines to acetaminophen and N-acetyl-p-benzoquinoneimine toxicity. J Hepatol 1999;31:841-51.

60. Harman AW, Kyle ME, Serroni A, Farber JL. The killing of cultured hepatocytes by N-acetyl-p-benzoquinone imine (NAPQI) as a model of the

cytotoxicity of acetaminophen. Biochem Pharmacol 1991;41:1111-7.

61. Kiese M, Pekis M. The reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

62. Jensen CB, Jollow DJ. The role of N-hydroxyphenetidine in phenacetin-induced hemolytic anemia. Toxicol Appl Pharmacol 1991;111:1-12.

63. Grossman SJ, Jollow DJ. Role of dapsone hydroxylamine in dapsone-induced hemolytic anemia. J Pharmacol Exp Ther 1988;244:118-25.

64. Nicholls AW, Farrant RD, Shockcor JP, et al. NMR and HPLC-NMR spectroscopic studies of futile deacetylation in paracetamol metabolites in rat and man. J Pharm Biomed Anal 1997;15:901-10.

65. Kiese M, Rauscher E. The failure of some aromatic amines to produce hemiglobin in vivo in spite of microsomal N-hydroxylation. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1965;251:201-11.

66. Wyman JF, Gray BH, Lee LH, et al. Interspecies variability in propylene glycol dinitrate-induced methemoglobin formation. Toxicol Appl Pharmacol 1985;81:203-12.

67. Srivastava S, Alhomida AS, Siddiqi NJ, Puri SK, Pandey VC. Methemoglobin reductase activity and in vitro sensitivity towards oxidant induced methemoglobinemia in swiss mice and beagle dogs erythrocytes. Mol Cell Biochem 2002;232:81-5.

68. Stolk JM, Smith RP. Species differences in methemoglobin reductase activity. Biochem Pharmacol 1966;15:343-51.

69. Harvey JW, Stockholm SL, Scott MA. Methemoghlobinemia and eccentrocytosis in equine erythrocyte flavin adenine dinucleotide deficiency. Vet Pathol 2003;40:632-42.

70. Rumbeiha WK, Lin YS, Oehme FW. Comparison of N-acetylcysteine and methylene blue, alone or in combination, for treatment of acetaminophen toxicosis in cats. Am J Vet Res 1995;56:1529-33.

71. Harvey JW, French TW, Senior DF. Hematologic abnormalities associated with chronic acetaminophen administration in a dog. J Am Vet Med Assoc 1986;189:1334-5.

72. Finco DC, Duncan JR, Schall WD, Prasse KW. Acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1975;166:469-72.

3. COMPARISON OF THE *NAT1/NAT2* DOUBLE KNOCKOUT MOUSE AND C57BL/6 MOUSE RESPONSE TO HEPATOTOXIC DOSES OF ACETAMINOPHEN AND para-AMINOPHENOL *IN VIVO*

3.1 Introduction

Acetaminophen (APAP) toxicity in dogs and cats causes hematotoxicity, consisting of methemoglobinemia within 2-4 hours and hemolysis at 72 hours [1-3]. In the veterinary literature, this reaction has been attributed to an increased production of *N*-acetyl-*p*-benzoquinoneimine (NAPQI), the metabolite responsible for APAP-associated hepatotoxicity seen in other species, with a different target organ [4,5]. We have shown that both the parent compound APAP and NAPQI do not induce methemoglobin formation *in vitro* (see Chapter 2). Instead, we found that the minor APAP metabolite *para*-aminophenol (PAP) does induce methemoglobin formation. Our studies have demonstrated a high *in vitro* sensitivity to PAP-induction of methemoglobin in canine and feline erythrocytes when compared to rat and mouse erythrocytes.

para -Aminophenol induces methemoglobin by a co-oxidation reaction with oxyhemoglobin [6,7]. *p*-Aminophenol redox cycles with oxyhemoglobin until it is removed by alternative metabolic pathways. In mice and rats, this biotransformation is predominantly *N*-acetylation by *N*-acetyltransferase (NAT) enzymes to APAP (>70%) [8,9]. The majority of the remaining PAP is conjugated with glutathione (GSH) [8,10], or alternatively, is glucuronidated or sulfated [8,9].

Most species have two NAT enzymes. Dogs are one of the few species that have no NAT enzymes and cats have only one enzyme, NAT1 [11,12]. The

resulting deficient *N*-acetylation in dogs and cats may prolong the redox cycling of PAP· and oxyhemoglobin in these species, and thus predispose them to methemoglobin formation following APAP toxicity. In our previous experiments, we compared the *in vitro* methemoglobin induction by PAP in erythrocytes of wildtype C57BL/6 mice and *NAT1/NAT2* knockout C57BL/6 mice (see Chapter 2). Knockout mouse erythrocytes showed significantly higher methemoglobin induction than wildtype mice erythrocytes, supporting the hypothesis that a deficiency of NAT activity contributes to a higher sensitivity to methemoglobin induction by PAP. However, the *in vitro* methemoglobin concentration seen in the knockout mouse red blood cells was significantly less than the methemoglobin concentration in dog and cat erythrocytes, indicating that there are also other contributing factors to the species sensitivity.

The following chapter describes *in vivo* experiments using *NAT1/NAT2* double knockout and wildtype C57BL/6 mice to determine if a deficiency of NAT activity is sufficient to make mice susceptible to APAP hematotoxicity.

3.2 Materials

The following compounds were obtained from Sigma-Aldrich Inc (St. Louis, MO): potassium ferricyanide, potassium cyanide, K_2HPO_4, HEPES free acid, $NaH_2PO_4.H_2O$, leupeptin, phenylmethylsulfonyl fluoride (PMSF), 2,6-di-tert-butyl-4-methylphenol (BHT), acetyl DL-carnitine hydrochloride, carnitine acetyltransferase, acetyl coenzyme A (AcCoA), triethylamine (TEA), DL-dithiothreitol (DTT), potassium chloride, acetaminophen, 4-aminophenol

hydrochloride, triethanolamine-HCl, KCl and ethylenediaminetetraacetic acid (EDTA).

Anhydrous dextrose, NaCl, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, perchloric acid (PCA), acetonitrile, formaldehyde and glacial acetic acid were obtained from Fischer Scientific (Fairhaven, NJ). KH_2PO_4 was obtained from BDH Inc (Toronto, ON). The Bio-Rad DC Protein Assay® was obtained from Bio-Rad Laboratories (Hercules, CA).

3.3 Animals

All procedures were done in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the University of Prince Edward Island Animal Care Committee. Male and female C57BL/6 mice, aged 6-7 weeks, were obtained from Charles River (St. Hyacinthe, QC) and acclimatized for two or more weeks. *NAT1/NAT2* double knockout mice are a mouse model produced by targeting of the genes in mouse embryonic stem cells for the two *N*-acetyltransferase enzymes, NAT1 and NAT2 [13]. Breeding pairs of *NAT1/NAT2* knockout mice were donated by Dr. Denis Grant of the University of Toronto and maintained in a breeding colony at the University of Prince Edward Island. Euthanasia of mice was by an overdose of pentobarbital intraperitoneally (IP) (diluted Euthansol® 340 mg/ml, Schering-Plough Animal Health, Pointe Claire, QC) followed by cardiac puncture and exsanguination.

3.4 Methods

3.4.1 Confirmation of functionality of livers of knockout mice

Livers from four male and female knockout mice bred at the University of Prince Edward Island were prepared and tested for *N*-acetylation activity as per the methods of liver fractionization, *N*-acetyltransferase assay and quantification described in Chapter 2.

3.4.2 *In vivo* APAP trials

Equal numbers of male and female wildtype and knockout mice were divided into groups of 4 in 3 separate APAP *in vivo* experiments. In Experiment #1, 32 knockout and 32 wildtype mice were divided accordingly. Control wildtype and knockout mice were injected with 10 ml/kg saline at 40°C, intraperitoneally (IP). The APAP wildtype and knockout mice were injected with 10 ml/kg of 250 mg/kg APAP in saline at 40°C IP. Control and APAP wildtype and knockout mice were euthanized and sampled at 0, 3, 24, and 48 hours post treatment (n=4 at each time point). Whole blood was collected via cardiac puncture into 400 µl Vacutainers® containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Livers and spleens were removed and placed in 10% formaldehyde.

In Experiment #2, 24 wildtype and 24 knockout mice were divided into groups of 4. Controls were injected with 10 ml/kg saline, and APAP mice were given 250 mg/kg or 400 mg/kg APAP at 40°C, IP at 0 hours and euthanized and sampled as above at 3 and 48 hours post treatment.

In Experiment #3, 24 wildtype and 24 knockout mice were divided into groups of 4 and injected IP with 10 ml/kg saline or 250 mg/kg APAP IP and sacrificed and sampled at 3, 24 or 48 hours post treatment.

3.4.3 *In vivo* PAP trials

Sixteen wildtype and 16 knockout mice consisting of equal numbers of males and females were divided into groups of four in the preliminary PAP time course. The control wildtype and knockout mice were sacrificed and sampled at five minutes post injection of 10 ml/kg saline IP. The remaining mice were sacrificed and sampled at 5, 10 or 20 minutes post injection of 400 mg/kg PAP IP. This preliminary time course demonstrated that saline had no effect on the methemoglobin concentration in C57BL/6 and NAT1/NAT2 knockout mice. Therefore, we compared the response of wildtype and knockout mice to PAP with no control saline groups in subsequent PAP experiments because we were limited in the number of methemoglobin measurements that could be done at our local hospital per day. The first PAP experiment (PAP Experiment #1) was done over two days. Eight wildtype and eight knockout mice, including equal numbers of males and females were sacrificed and sampled at five minutes post injection of 400 mg/kg PAP IP.

The second PAP experiment (PAP Experiment #2) was done to evaluate the effect of fasting PAP induction of methemoglobin. Four wildtype and four knockout mice, including four males and four females, were fasted for 16 hours.

They were then sacrificed and sampled at five minutes post injection of 400 mg/kg PAP IP.

In each experiment, including the time course, 100 to 400 µl of whole blood was collected via cardiac puncture into 400 µl Vacutainers® containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ) and placed in an ice slurry.

3.4.4 Clinical chemistry and hematology

Whole blood was centrifuged in Fisherbrand Micro-hematocrit Capillary Tubes (Fisher Scientific, Pittsburgh, PA) for five minutes to measure the packed cell volume (PCV) (Haemofuge, Heraeus Christ, West Germany). Blood smears were stained with Wright Giemsa (Shandan Instant Hemotoxylin, Fisher Scientific) or New Methylene Blue stains (J.T. Baker, Phillipsburg, NJ) and examined for Heinz bodies and eccentrocytes.

In the APAP *in vivo* trials, erythrocytes were separated from plasma by centrifugation of whole blood for 10 minutes at 1800 g and 4°C (Allegra X-15R Centrifuge, Beckman Coulter). The plasma total bilirubin (Tbili) concentration, aspartate transferase (AST) activity and alanine transferase (ALT) activity were measured by a Hitachi 917 (Roche Diagnostics Canada, Laval, QC). Erythrocytes were resuspended in HEPES for measurement of methemoglobin concentration as per the method in Chapter 2.

In the PAP *in vivo* trials, whole blood was placed in an ice slurry immediately following collection. The methemoglobin concentration in the PAP *in*

vivo trials was measured by co-oximetry (Radiometer Copanhagan ABL-700 (Radiometer Canada, London, ON) at the Queen Elizabeth Hospital, Charlottetown, PE by trained technicians.

3.4.5 Microscopic Examination

Livers and spleens were removed following exsanguination and placed in 10% formaldehyde. Histological sections were prepared and stained with hematoxylin and eosin stains (Hematek® Stain Pack, Bayer HealthCare, Toronto, ON) using standard techniques.

3.5 Statistical analysis

A one-way ANOVA, followed by Bonferroni t-tests was used to compare the PCV, AST activity, ALT activity and Tbili concentration in the APAP experiments. Methemoglobin percentages in the preliminary PAP time course were compared by a Kruskal Wallis test. Methemoglobins in Experiments 1 and 2 were compared by nonparametric Mann-Whitney tests.

All statistical calculations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA). Unless otherwise stated, means are expressed ± SEM in the APAP experiments. Medians and ranges are given for the PAP experiments data. In all cases, significance was taken to be $P<0.05$.

3.6 Results

Livers of four knockout mice were examined for NAT functionality. There was no significant *N*-acetylation of PAP or PABA in cytosol samples from livers of knockout mice bred at our facilities.

Appendix 1 contains data of the APAP experiments. In APAP Experiment #1, there was a significant increase in AST and ALT activities in the knockout mice at 3 hours ($P<0.05$). The lack of statistical significance in the ALT and AST activities at 24 hours in the mice that received APAP was due to marked variability. There was no significant change in the Tbili concentration in mice receiving APAP at 0, 3 or 24 hours. The methemoglobin was not measured due to difficulties with the methodology. There was a significant decrease in the PCV in the knockout mice receiving 250 mg/kg APAP at 48 hours compared to knockout mice receiving saline at 48 hours ($P<0.001$). No changes were seen on the smears of whole blood in control or APAP mice.

In APAP Experiment # 2, there was a significant increase of the ALT activity in the knockout mice at 3 hours post injection of 250 and 400 mg/kg APAP. There was a significant increase of the AST activity in wildtype ($P<0.05$) and knockout ($P<0.05$) mice at 3 hours post injection of 400 mg/kg APAP. There was no significant change in the Tbili, HCT, methemoglobin at any sampling time in experiment #2 ($P>0.05$). No Heinz bodies or eccentrocytes were seen on blood smears at any sampling time.

In APAP Experiment #3, there was no significant change in the Tbili concentration or methemoglobin percentage at 1, 24 or 48 hours post injection of APAP ($P>0.05$). There was a significant increase of ALT and AST activities in

both knockout and wildtype mice at 24 hours post injection of APAP ($P<0.001$ for both enzymes in both groups of mice). There was a significant increase in the PCV at 1 and 24 hours in both wildtype and knockout mice receiving 250 mg/kg APAP compared to the control wildtype and knockout mice ($P<0.05$). No evidence oxidative damage, characterized by Heinz bodies or eccentrocytes, was present on any blood smears.

There were no light microscopic changes in histologic sections of livers or spleens from mice treated with saline (Figure 3.1). Mice treated with 250 or 400mg/kg APAP IP showed no microscopic changes in the spleens. All livers from mice treated with 400 mg/kg APAP developed severe, bridging, centrolobular hepatic necrosis by 48 hours (Figure 3.2). The livers of the mice treated with 250 mg/kg APAP varied. Of the four wildtype and four knockout mice in APAP Experiment #3 treated with 250 mg/kg APAP and sampled at 48 hours post injection, one mouse had mild, single cell necrosis associated with a mild infiltrate of pleomorphic inflammatory cells in the centrolobular region and diffuse vacuolar hepatocellular degeneration. Five mice had moderate centrolobular necrosis and two mice had severe, bridging centrolobular necrosis. The methemoglobin concentrations at 5, 10 and 20 minutes were not significantly different in knockout mice ($P=0.56$) or wildtype mice injected with 400 mg/kg PAP IP. Five minutes was chosen as the exposure time for subsequent *in vivo* PAP Experiments #1-2 based on the trend in the knockout mice towards lower methemoglobin at 10 and 20 minutes (Table 3.1).

Figure 3.1: Histologic section of a knockout mouse liver at 48 hours post injection of saline.
Hematoxylin and eosin. No significant abnormalities. Bar =100 microns.

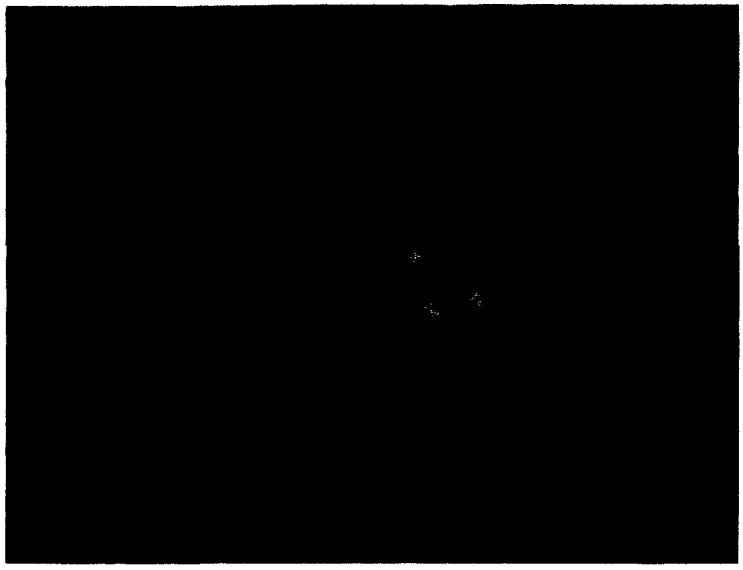


Figure 3.2: Histologic section of a knockout mouse liver at 48 hours post injection of 400 mg/kg APAP.
Hematoxylin and eosin. Marked centrolobular coagulative necrosis with a pleomorphic inflammatory cell infiltrate and evidence of early regeneration, for example, frequent binucleation. Bar = 100 microns.

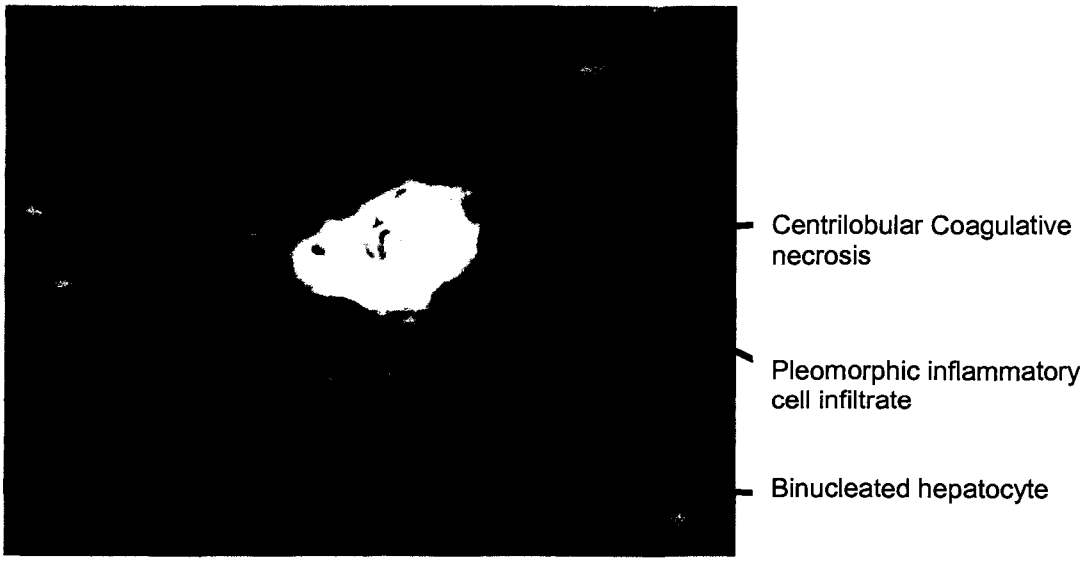


Table 3.1: Preliminary time course for *in vivo* injection of 400 mg/kg PAP

	5 minutes (median % methemoglobin and range)	10 minutes (median % methemoglobin and range)	20 minutes (median % methemoglobin and range)
Knockout mice	14.4 (2.1-76.9)	5.2 (2.9-7.7)	3.9 (2.4-7.7)
Wildtype mice	4.1 (2.2-4.9)	9.2 (0.1-15.4)	5.6 (1.8-16.8)

The methemoglobin concentration of knockout mice treated with PAP in PAP Experiment #1 and 2 had a bimodal distribution (Table 3.2, Figure 3.3). There was a significantly higher (P=0.008) methemoglobin concentration in knockout mice compared to wildtype mice at five minutes post injection of PAP. Female knockout mice had significantly greater methemoglobin than female wildtype mice (P=0.0047). Male knockout mice were not significantly different than male wildtype mice (P=0.69).

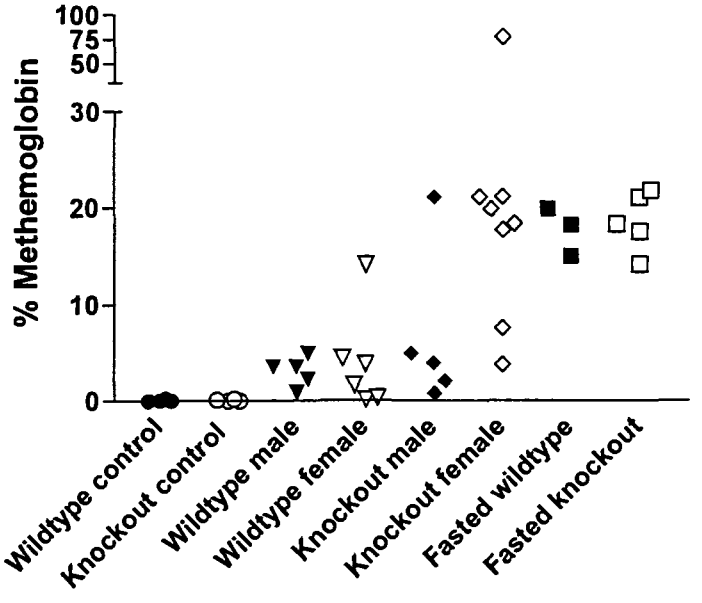
There was no significant difference in the methemoglobin concentration of fasted knockout and wildtype mice at five minutes post injection of PAP (P=0.79)) There was a significantly higher methemoglobin concentration in fasted wildtype mice than non-fasted wildtype mice at five minutes post injection post injection of PAP (P=0.01). There was no significant difference between fasted knockout and non-fasted knockout mice (P=0.49)

The mean PCVs for the wildtype and saline controls were 44.3 ± 0.5% and 45.8 ± 0.9% respectively. The mean PCV's in wildtype and knockout mice injected with PAP were 43.8±0.5 and 42.3 ± 3.0%. There was no significant difference in the PCVs of control and PAP treated mice (P=0.34). Direct blood smears of whole blood at 5 minutes post injection were normal.

Table 3.2: Methemoglobin in wildtype and knockout mice for Experiment #1 and #2.

	(median % methemoglobin and range)	**number of mice**
Control wildtype mice	0.0 (0.0-0.2)	4
Control knockout mice	0.05 (0.0-0.2)	4
Experiment 1		
Total knockout mice	17.1 (0.8-76.9)	13
Male knockout mice	3.9 (0.8-21.1)	5
Female knockout mice	19.2 (3.8-76.9)	8
Total wildtype mice	3.5 (0.1-14.1)	11
Male wildtype mice	3.5 (0.9-4.9)	5
Female wildtype mice	2.7 (0.1-14.1)	6
Experiment 2		
Fasting knockout mice	18.3 (14.1-21.7)	5
Fasting wildtype mice	18.2 (15.0-19.9)	3

Figure 3.3: The percent methemoglobin in wildtype and knockout mice 5 minutes post-injection of 400mg/kg PAP



3.7 Discussion

The purpose of our *in vivo* studies was to determine if a deficiency in *N*-acetylation activity is sufficient to cause APAP-induced hematotoxicity. To isolate the effect of a NAT enzyme deficiency, we compared the response of C57BL/6 wildtype and C57BL/6 *NAT1/NAT2* double knockout mice to hepatotoxic doses of APAP. Mice have 3 NAT enzymes, NAT1, NAT2, and the poorly functional NAT3 [14,15]. Double knockout *NAT1/NAT2* mice retain NAT3 which is shows increased expression [14]. The increased expression of NAT3 is due to a positional artifact of the insertion of the neomycin resistance gene [14]. NAT 3 remains poorly functional [16]. We confirmed that PAP was not acetylated by liver cytosol from knockout mice bred in our facilities.

A hepatotoxic dose of APAP was used to determine if hematotoxicity would occur at a concentration of APAP that would normally be associated with hepatotoxicity. The marked increase of ALT and AST activities in both wildtype and knockout mice receiving APAP indicated the presence of hepatocellular leakage [17]. This was suggestive of hepatocellular necrosis [17], a finding that was confirmed by microscopic examination of the livers. The decrease in ALT and AST activities in the 48 hour samples may have been due to either a decreased insult to the liver or loss of actively leaking cells due to cell death. There was a variation in the degree of hepatonecrosis between APAP-treated mice in our studies. This variability has been seen previously in *in vivo* trials in mice [18] and also occurs in APAP toxicity in humans and dogs [19,20].

The significantly higher PCV in both wildtype and knockout mice injected with APAP at 3 and 24 hours may have been due to dehydration, redistribution of fluids within the body or splenic contraction due to excitement [21]. A similar non-specific increase in the hematocrit has been reported within hours of APAP toxicosis in dogs [22].

The moderate anemia in the knockout mice receiving 250 mg/kg APAP at 48 hours in the first APAP experiment was similar to the anemia that can occur in dogs and cats at 48-72 hours post intoxication [1-3]. This anemia was not observed in the two subsequent experiments. A mild anemia due to APAP could have been masked by concurrent dehydration or splenic contraction. However, a moderate to severe anemia as was seen in the single group of mice would be unlikely to be hidden by simultaneous erythrocytosis. A difference in appetite resulting in lower concentrations of antioxidants such as GSH in the group of mice that developed anemia cannot be ruled out [23]. Diets high in antioxidants have been shown to have a protective effect against both APAP associated hepatotoxicity and hematotoxicity [24,25]. However, there was no change in the diet or other husbandry aspects of the mice between the different experiments. Therefore, it must be concluded that this was a spurious observation and does not reflect an increased susceptibility to APAP-induced anemia in knockout mice.

There was no significant APAP-induction of methemoglobin in either knockout or wildtype mice. The lack of hematotoxicity in the APAP treated knockout mice may have been due to species differences in APAP or PAP biotransformation or cytoprotective pathways, such as detoxification of

methemoglobin by methemoglobin reductase. Methemoglobin reductase activity is greater in mice than in cats and dogs [26,27] and therefore may have reduced the methemoglobin too efficiently to allow measurement of the methemoglobin that had been produced.

To avoid the effect of species differences of APAP metabolism to PAP, we treated mice directly with PAP. For these experiments, we measured the methemoglobin by co-oximetry at a local hospital because we felt that the precision and accuracy of this method would be better for low concentrations of methemoglobin than the alternative method we used in the APAP experiments. We used a five minute exposure time for the PAP experiments because of data from our initial time course as well as previous work by Smith et al that demonstrated a rapid peak of methemoglobinemia post injection of PAP, which the authors speculated to be due to highly efficient mouse methemoglobin reductase [28].

The significantly higher methemoglobin concentration in knockout mice compared to wildtype mice following injection with PAP supports that a deficiency of NAT enzymes contributes to the species sensitivity of dogs and cats to PAP-induced methemoglobinemia.

The methemoglobin concentration in PAP treated mice appeared to have a bimodal distribution, however higher numbers of treated mice would be required to confirm this pattern. A bimodal distribution could occur due to a genetic based individual variation in the reduction of methemoglobin or biotransformation of PAP, but this is unlikely in inbred mice. The GSH

concentration has been shown to vary with diet, gender or circadian rhythms in mice [29,30]. All mice in our experiments were fed the same diet for a period of two or more weeks prior to the procedures and all sampling was done in the morning to avoid dietary or diurnal variation of GSH in mice [23]. Female mice have been reported elsewhere to have lower concentrations of GSH than male mice [29]. In our experiments, the female mice did demonstrate significantly higher methemoglobin concentrations than male mice following injection of PAP. This may have been due to the GSH concentration, but further investigations into the effect of GSH on the methemoglobin induction by PAP using GSH inhibitors are necessary to confirm. The variation in the gender response to PAP was most obvious in the knockout females. A lower concentration of GSH or other factor involved in the biotransformation of PAP may be more prominent in mice deficient in the main biotransformation pathway of PAP, namely *N*-acetylation.

Mice were fasted overnight prior to treatment with PAP to determine the effect of fasting on PAP-induction of methemoglobin. Fasting has been shown to decrease the amount of reduced GSH as well as other biotransformation factors such as sulfhydryl groups [30]. Fasted wildtype mice developed significantly more methemoglobin following injection with PAP than non fasted wildtype mice. However the methemoglobin in fasted knockout mice did not significantly change from non fasted knockout mice. The majority of PAP has been shown to be biotransformed via *N*-acetylation in mice [8], but a significant amount of PAP is conjugated with GSH and small amounts are glucuronidated or sulfated [8,9]. A decrease in GSH, or sulfates due to fasting may delay the removal of PAP from

redox cycling however, further studies using GSH inhibitors such as L-buthionine-(S-R) sulfoxine are required. It was interesting to note that the fasted knockout mice, which presumably were already predisposed to prolonged PAP redox cycling, did not develop a higher methemoglobin concentration compared to non-fasted knockout mice. One possible explanation is that the efficient methemoglobin reductase in the mouse may limit the total amount of methemoglobin that can develop following exposure to PAP.

Our *in vivo* studies are in agreement with our *in vitro* findings. Both studies demonstrated that PAP is the metabolite most likely responsible for APAP-induced methemoglobinemia. They also suggest that while deficiency in NAT enzymes increases susceptibility to PAP-induced methemoglobinemia, this increase in susceptibility is relatively modest. Therefore, there are other important factors involved in determining susceptibility at the level of the erythrocyte. The available GSH, the limited glucuronidation in cats and poor methemoglobin reductase activity in dogs and cats may also play a role in species differences in susceptibility to APAP-hematotoxicity.

3.8 References

1. Aronson LR, Drobatz K. Acetaminophen toxicosis in 17 cats. J Vet Emerg Crit Care 1996;6:65-9.

2. MacNaughton SM. Acetaminophen toxicosis in a Dalmatian. Can Vet J 2003;44:142-4.

3. Mariani CL, Fulton RB. Atypical reaction to acetaminophen intoxication in a dog. J Vet Emerg Crit Care 2001;11:123-6.

4. Taylor NS, Dhupa N. Acetaminophen Toxicity in Dogs and Cats. Compend Contin Educ Pract Vet 2003;12:160-9.

5. Cullison RF. Acetaminophen toxicosis in small animals: Clinical signs, mode of action, and treatment. Compend Contin Educ Pract Vet 1984;6:315-21.

6. Kiese M. The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol Rev 1966;18:1091-161.

7. Kiese M, PEKIS M. The reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

8. Song H, Chen TS. p-Aminophenol-induced liver toxicity: tentative evidence of a role for acetaminophen. J Biochem Mol Toxicol 2001;15:34-40.

9. Dressler WE, Appelqvist T. Plasma/blood pharmacokinetics and metabolism after dermal exposure to para-aminophenol or para-phenylenediamine. Food Chem Toxicol 2006;44:371-9.

10. Yan Z, Nikelly JG, Killmer L, Jr., Tarloff JB. Metabolism of para-aminophenol by rat hepatocytes. Drug Metab Dispos 2000;28:880-6.

11. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol 1997;54:73-80.

12. Trepanier LA, Cribb AE, Spielberg SP, Ray K. Deficiency of cytosolic arylamine N-acetylation in the domestic cat and wild felids caused by the presence of a single NAT1-like gene. Pharmacogenetics 1998;8:169-79.

13. Sugamori KS, Wong S, Gaedigk A, et al. Generation and functional characterization of arylamine N-acetyltransferase Nat1/Nat2 double-knockout mice. Mol Pharmacol 2003;64:170-9.

14. Sugamori KS, Brenneman D, Wong S, et al. Effect of arylamine acetyltransferase Nat3 gene knockout on N-acetylation in the mouse. Drug Metab Dispos 2007.

15. Estrada-Rodgers L, Levy GN, Weber WW. Substrate selectivity of mouse N-acetyltransferases 1, 2, and 3 expressed in COS-1 cells. Drug Metab Dispos 1998;26:502-5.

16. Sugamori KS, Wong S, Gaedigk A, et al. Generation and functional characterization of arylamine N-acetyltransferase Nat1/Nat2 double-knockout mice. Mol Pharmacol 2003;64:170-9.

17. Willard MD, Twedt DC. Gastrointestinal, pancreatic, and hepatic disorders. In: Willard MD, Tvedten H, Turnwald GH, editors. Small Animal Clinical Diagnosis by Laboratory Methods. Philadelphia: W.B.Saunders; 1994. p.179-218.

18. Mitchell JR, Jollow DJ, Potter WZ, et al. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther 1973;187:185-94.

19. Prescott LF, Roscoe P, Wright N, Brown SS. Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet 1971;1:519-22.

20. Francavilla A, Makowka L, Polimeno L, et al. A dog model for acetaminophen-induced fulminant hepatic failure. Gastroenterol 1989;96:470-8.

21. Stockham SL, Scott AS. Erythrocytes. In: Stockham SL, Scott AS, editors. Fundamentals of Veterinary Clinical Pathology. First ed. Ames: Iowa Press; 2002. p.87-149.

22. Mariani L, Baratelli M. [Paracetamol: overdose and hepatic lesions]. ClinTher 1981;98:117-52.

23. Schnell RC, Bozigian HP, Davies MH, et al. Factors influencing circadian rhythms in acetaminophen lethality. Pharmacology 1984;29:149-57.

24. Weiss DJ, McClay CB, Christopher MM, Murphy M, Perman V. Effects of propylene glycol-containing diets on acetaminophen-induced methemoglobinemia in cats. J Am Vet Med Assoc 1990;196:1816-9.

25. Allison RW, Lassen ED, Burkhard MJ, Lappin MR. Effect of a bioflavonoid dietary supplement on acetaminophen-induced oxidative injury to feline erythrocytes. J Am Vet Med Assoc 2000;217:1157-61.

26. Srivastava S, Alhomida AS, Siddiqi NJ, Puri SK, Pandey VC. Methemoglobin reductase activity and in vitro sensitivity towards oxidant induced methemoglobinemia in swiss mice and beagle dogs erythrocytes. Mol Cell Biochem 2002;232:81-5.

27. Stolk JM, Smith RP. Species differences in methemoglobin reductase activity. Biochem Pharmacol 1966;15:343-51.

28. Smith RP, Alkaitis AA, Shafer PR. Chemically induced methemoglobinemias in the mouse. Biochem Pharmacol 1967;16:317-28.

29. Dai G, He L, Chou N, Wan YJ. Acetaminophen metabolism does not contribute to gender difference in its hepatotoxicity in mouse. Toxicol Sci 2006;92:33-41.

30. Walker RM, Massey TE, McElligott TF, Racz WJ. Acetaminophen toxicity in fed and fasted mice. Can J Physiol Pharmacol 1982;60:399-404.

4. CIMETIDINE INHIBITION OF *IN VITRO PARA*-AMINOPHENOL *N*-ACETYLATION IN CATS

4.1 Introduction

Acetaminophen (APAP) is a commonly used human analgesic and antipyretic. Although considered safe at therapeutic doses in humans, it is the most common drug overdose reported to poison control centers in the United States and Britain [1-3]. Veterinary poison control centers also report numerous cases of APAP toxicity in dogs and cats [4]. Acetaminophen toxicity is associated with hepatic centrilobular necrosis in humans and most laboratory species [5,6]. Dogs and cats are unique in that they develop methemoglobinemia and hemolytic anemia [7-9]. The primary therapy for APAP toxicity in humans, dogs and cats is *N*-acetylcysteine (Mucomyst®) [10-14]. Cimetidine was previously recommended in humans as an adjunct therapy to *N*-acetylcysteine for APAP toxicosis but its use is no longer advised [13,15-17]. Nevertheless, some veterinary articles recommend cimetidine for feline and canine APAP toxicosis [14,18].

Acetaminophen is metabolized primarily by sulfation and glucuronidation in the liver [19-22]. A small percentage of APAP is oxidized by cytochrome P450 (CYP) enzymes to the oxidative metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [22]. At therapeutic doses of APAP, NAPQI binds to glutathione (GSH) and is then excreted in the urine as cysteine and mercapturic acid metabolites [5]. At toxic doses, the production of NAPQI increases and GSH is depleted [5]. The NAPQI then binds to hepatocellular proteins, leading to hepatocellular death and

centrilobular necrosis [5,23,24]. Treatment of APAP toxicosis with *N*-acetylcysteine increases available GSH by providing the precursor cysteine for GSH production [25]. Initial *in vivo* and *in vitro* studies in mice, rats and humans indicated that cimetidine decreases NAPQI production by inhibition of CYP enzymes [15,16]. However, subsequent studies in humans have shown no significant effect of cimetidine on NAPQI production and thus cimetidine is no longer recommended as an adjunct therapy for human APAP toxicity [13,17,26,27].

The efficacy of cimetidine in the treatment of feline APAP toxicity has not been investigated. Its use in cats appears to be based on the early studies in humans and laboratory animals [16,28]. Feline APAP toxicity is characterized clinically by marked methemoglobinemia within 6 hours of exposure followed by hemolytic anemia due to oxidative changes in red blood cells within 48 hours [29]. Cats surviving the initial hematotoxicity can develop elevated liver enzymes, but histological examination has indicated that this is due to degenerative changes in the liver, likely as a result of hypoxia [30]. This is contrary to dogs, which can develop centrolobular hepatic necrosis if they survive the initial methemoglobinemia [31,32].

A minor pathway of APAP metabolism is de-acetylation of APAP by liver microsomal carboxyesterases [33]. Removal of the acetyl group from the benzene ring creates *para*-aminophenol (PAP). The metabolite PAP is known to induce methemoglobin in aniline dye toxicity by reacting with hemoglobin in a co-oxidation process (Figure 1.13) [34-36]. During a co-oxidation reaction, an electron is abstracted from the PAP hydroxyl group and from ferrous iron in the

oxyhemoglobin, creating oxidized PAP (benzoquinoneimine (BQI)) and methemoglobin. Both electrons are donated to oxygen resulting in a reactive oxygen species such as hydrogen peroxide [35]. The PAP is recycled within the erythrocyte through reduction of BQI to PAP by glutathione (GSH) [35]. Thus PAP will repeatedly redox cycle with oxyhemoglobin until it is removed by an alternative biotransformation pathway [35]. Our laboratory has demonstrated that PAP induces methemoglobin *in vitro* in feline erythrocytes while NAPQI, the oxidative metabolite responsible for the hepatic necrosis, does not (see Chapter 2). Thus PAP is the metabolite more likely responsible for APAP-induced methemoglobinemia.

In rats and mice, >70% of the active metabolite PAP is detoxified by *N*-acetylation to the parent compound APAP [37,38]. *N*-acetylation of drugs is catalysed by two closely related enzymes: *N*-acetyltransferase 1 and *N*-acetyltransferase 2 (NAT1 and NAT2; EC2.3.1.5) [39]. Although NAT1 and NAT2 show considerable homology, these phase II biotransformation enzymes differ in their preferred substrates and distribution in the body [40]. Human NAT1 is located in most tissues while NAT2 is mainly in the liver and small intestine [40]. Human NAT1 tends to catalyze acetylation of smaller molecules such as *para*-aminobenzoic acid (PABA) while larger molecules such as procainamide are *N*-acetylated by NAT2 [40]. Unlike other domestic species, cats only have NAT1 and dogs have no NAT enzymes [41,42]. Therefore, dogs are unable to *N*-acetylate and cats are limited in their ability [41,42].

Svensson and Tomilo have demonstrated that cimetidine inhibits *N*-acetylation of PABA and procainamide in rats [43]. This is of potential concern in cats. If cimetidine also inhibits feline *N*-acetylation, then it may inhibit PAP detoxification, both in the liver where PAP is produced and in erythrocytes where NAT1 may be instrumental in the removal of PAP from redox cycling. Inhibition of NAT1 in cats could therefore exacerbate APAP-induced methemoglobinemia through decreased clearance of PAP. Inhibition of NAPQI formation by cimetidine would offer no protective effect against methemoglobinemia since NAPQI is not responsible for the methemoglobinemia, and hepatotoxicity is not a major problem in cats [7]. Inhibition of *N*-acetylation by cimetidine is of no consequence in canine therapy for APAP toxicity as dogs have no NAT enzymes [42]. The following study presents the results of our work examining the ability of cimetidine to inhibit *N*-acetylation of PAP and PABA *in vitro* in cats.

4.2 Materials

Cimetidine, *para*-aminobenzoic acid (PABA), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 2,6-di-*tert*-butyl-4-methylphenol (BHT), acetyl DL-carnitine hydrochloride, carnitine acetyltransferase, acetyl coenzyme A (AcCoA), triethylamine (TEA), DL-dithiothreitol (DTT), potassium chloride (KCl), acetaminophen, ethylenediaminetetraacetic acid (EDTA), 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) and all chemicals for buffers were obtained from the Sigma Chemical Company (St Louis, MO). Perchloric acid

(PCA), acetonitrile, glacial acetic acid and anhydrous dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Fairhaven, NJ). Triethanolamine-HCl, bovine serum albumin (BSA), 4-acetamidobenzoic acid (Nac-PABA) and 4-aminophenol (PAP) were obtained from the Aldrich Chemical Company, Inc. (Milwaukee, WI). The Bio-Rad DC Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA).

4.3 Animals

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and with approval of the University of Prince Edward Island Animal Care Committee. Six healthy male Sprague-Dawley rats aged 10-12 weeks were obtained from Charles River (St. Hyacinthe, QC) and acclimatized for two weeks. Rats were anesthetized by CO_2 inhalation and exsanguinated by cardiac puncture. Four healthy male and seven healthy female adult cats deemed inappropriate for adoption were donated by a cat rescue society. The cats were euthanized with an overdose of intravenous (iv) pentobarbital following sedation with intramuscular 15 mg/kg ketamine (100 mg/ml Vetalar ®, Bioniche Animal Health Canada, Belleville, ON) and 0.7 mg/kg xylazine (20 mg/ml Rompun®, Bayer Animal Health, Toronto, ON).

Immediately following euthanasia, livers were removed and washed and perfused with cold phosphate buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, pH 7.4), frozen in liquid nitrogen and stored

at -80°C until use. Livers were homogenized on ice by a tissue homogenizer (Tissue Tearor, Model 398 Biospec Products Inc, Dremels, WI) in 4 volumes of TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) with 10 μM leupeptin, 100 μM PMSF, and 18 μM BHT. Initial differential centrifugation was at 9,000 g, for 20 minutes at 4°C (Allegra X-15R Centrifuge, Beckman Coulter). The supernatant was centrifuged at 115,000 g for one hour at 4°C (L8-60M Ultracentrifuge, Beckman Coulter). The cytosol supernatant was removed from the pellet and aliquots of cytosol were frozen at -80°C until use [44]. Protein concentrations were determined by Bio-Rad DC Protein Assay, a modified Bradford technique [45].

4.4 Enzyme assay

The assay for *N*-acetyltransferase was performed as previously described [46-48]. Cytosol was appropriately diluted to achieve final protein concentrations ensuring linear metabolism over the incubation period (approximately 1.0 mg protein/ml in cats, 0.3 mg protein/ml in rats). Rat cytosols were diluted with TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) containing 1 mg/ml bovine serum albumin (BSA) and cat cytosols were diluted with TEDK alone. A mixture consisting of 10 μl of substrate PAP or PABA (final concentration 50-100 μM in 1.38% DMSO), 10 μl of cimetidine (final concentration 250-2000 μM), 40 μl of 450 μM AcCoA (final concentration 100 μM) and 40 μl of an AcCoA regenerating system consisting of 5 mM acetyl DL-

carnitine and 0.22 U/ml carnitine acetyltransferase in a NAT assay buffer (225 mM triethanolamine-HCl, 4.5 mM EDTA, 4.5 mM DTT) were preincubated at 37°C for 5 minutes [47]. Unless otherwise stated, the reaction was initiated with the addition of 80 µl of appropriately diluted, separately preincubated cytosol (180 µl total final volume). . *para*-Aminobenzoic acid and PAP concentration ranges were 2.5-200 µM in studies to determine the Michaelis Menten values Incubations were carried out at 37°C for 10 and 15-20 minutes in rats and cats respectively and terminated by the addition of 20 µl of 15% perchloric acid, placement on ice and vortexing. The mixtures were then centrifuged at 15,000 rpm for 5 minutes and the supernatant removed for analysis. Every reaction was carried out in duplicate. Control reactions and reactions including cimetidine were carried out the same day.

Pre-incubation conditions were altered to further investigate the mechanism of inhibition by cimetidine on feline *N*-acetylation of PAP and PABA. Samples containing cytosol, AcCoA, the regenerating system and the vehicle were pre-incubated for 5 minutes and the reaction initiated with separately warmed substrate. The resulting velocities were compared to velocities of (1) samples containing cytosol, AcCoA and regenerating system with initiation of the reaction by separately warmed substrate and cimetidine, (2) samples containing cytosol, AcCoA, regenerating system and cimetidine with initiation of the reaction by addition of separately warmed substrate.

To determine if inhibition involves the binding of AcCoA, control samples containing the substrate, regeneration system, AcCoA and the vehicle were pre-

incubated for 5 minutes and the reaction was initiated by the addition of cytosol. These were compared to: (1) pre-incubated samples containing substrate, regeneration system, AcCoA and cimetidine with initiation of the reaction by addition of cytosol and (2) samples in which the cytosol was pre-incubated with AcCoA and the regeneration system and the reaction initiated by the addition of substrate and cimetidine.

4.5 Analytical procedures

Samples were analyzed for APAP or *N*-acetylated PABA (NacPABA) the same day as the enzyme assay by reverse phase high performance liquid chromatography (HPLC) using a Beckman Ultrasphere ODS C-18 column (150 X 4.6 mm, 5 µm particle size) (Beckman Coulter Canada Inc. Montreal, QC) and a Shimadzu SCL-10A solvent delivery system (Shimadzu Scientific, Columbia, MD) with UV detection at 270 nM [40]. The mobile phases consisted of water/acetonitrile/acetic acid/triethylamine at a ratio of 92.0:8.0:1.0:0.05 (volume/volume) (v/v) for Nac-PABA and 98.75:1.25:1.0:0.05 (v/v) for APAP. Both mobile phases were used isocratically at 1.25 ml/min. Fifty µl of supernatant obtained as stated previously were injected without further preparation. The retention times for APAP and Nac-PABA were 10.9 and 9.8 minutes, respectively. The limit of quantification for APAP was 0.156 µM and the limit for Nac-PABA was 0.078 µM with an injection of 50 µl of sample. Standard curves for APAP or Nac-PABA were performed each day of analyses. They were

linear over the range of concentrations measured (r^2= 0.99-1.0). The intraday coefficient of variation was < 5.0% and the interday coefficient of variation was <10.0% for both Nac-PABA and APAP.

4.6 Data analysis

A repeated measures one-way ANOVA followed by a Dunnett's test was used to determine the significance of concentration-dependent inhibition by cimetidine on *N*-acetylation of PAP and PABA in rats and cats. The K_m and V_{max} were derived by linear regression analysis of the Eadie-Hofstee plots. Paired two-tailed student's t-tests were used to compare feline *N*-acetylation velocities and K_m's with and without cimetidine in the Michaelis Menten studies and to compare the velocities in reactions with varying pre-incubation conditions. All statistical calculations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA). Unless otherwise stated, means are expressed ± SEM. In all cases, significance was taken to be $P<0.05$.

4.7 Results

Significant inhibition of feline PABA (100 µM) and PAP (100 µM) *N*-acetylation was present at ≥ 500 µM and ≥100 µM cimetidine respectively ($P<0.05$) (Figures 4.1, 4.2 and 4.3). Significant inhibition of rat *N*-acetylation was present at ≥1000 µM and ≥ 250 µM cimetidine for PABA (100 µM) and PAP (50 µM) respectively ($P<0.05$) (Figures 4.4 and 4.5).

Figure 4.1: Concentration-dependent inhibition of 100 µM PAP *N*-acetylation in cats by 0-2000 µM cimetidine

Each point represents the mean of 3 individual cats. **P*<0.05, ** *P*<0.01 as compared to *N*-acetylation of 100 µM PAP with 0 µM cimetidine.

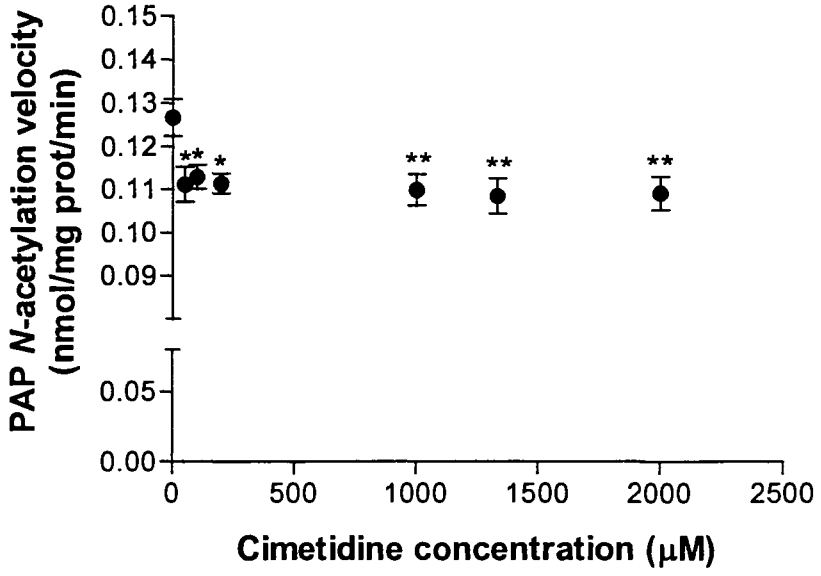


Figure 4.2: Inhibition of feline *N*-acetylation of 100 µM PAP *in vitro* by cimetidine

Each point represents the mean ± SEM of duplicate values from 3 cats at concentrations of cimetidine ranging from 100-2,000 µM.

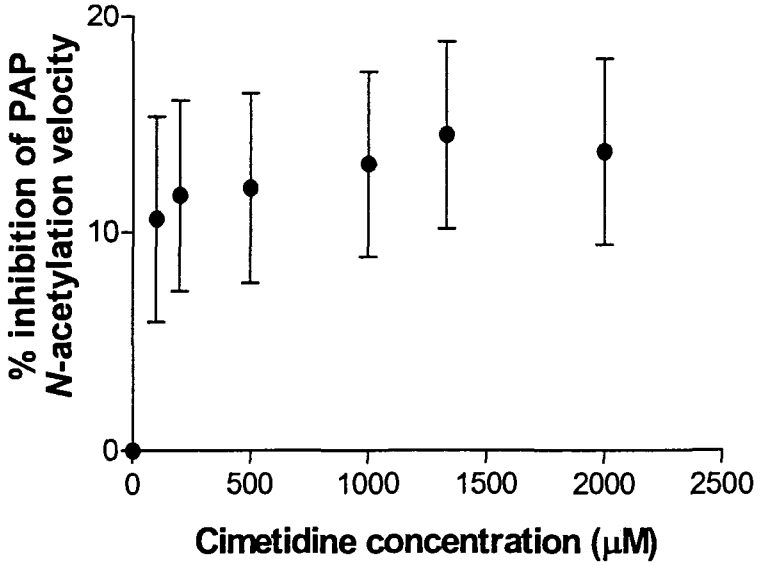


Figure 4.3: Concentration-dependent inhibition of 100 µM PABA *N*-acetylation in cats by 0-2000 µM cimetidine

Each point represents the mean ± SEM of 3 cats. **P*<0.05, ***P*<0.01 as compared to *N*-acetylation of 100 µM PABA with 0 µM cimetidine.

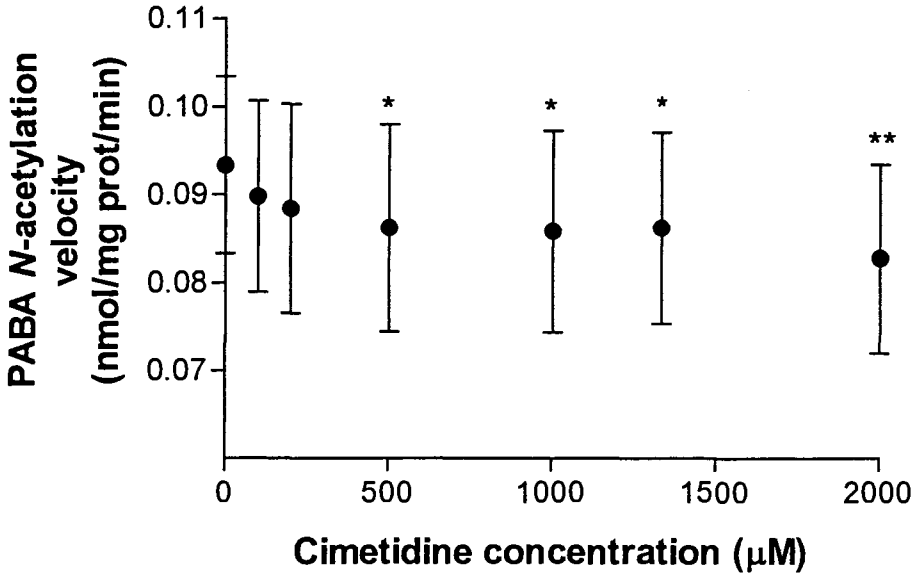


Figure 4.4: Concentration dependent inhibition of 50 µM PAP *N*-acetylation in rats by 0-1000 µM cimetidine

Each point represents the mean ± SEM of 3 rats. **P*<0.05, ***P*<0.01 as compared to *N*-acetylation of 50 µM PAP with 0 µM cimetidine.

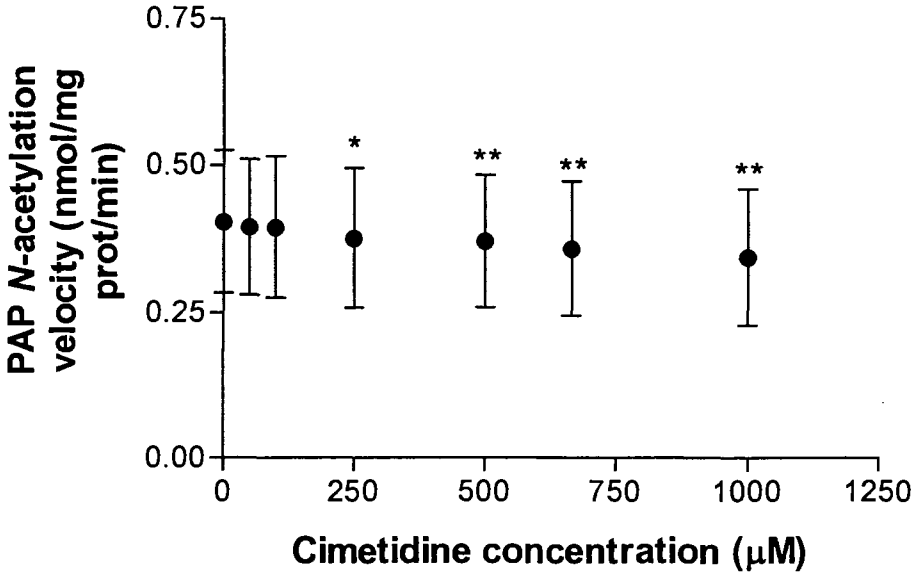
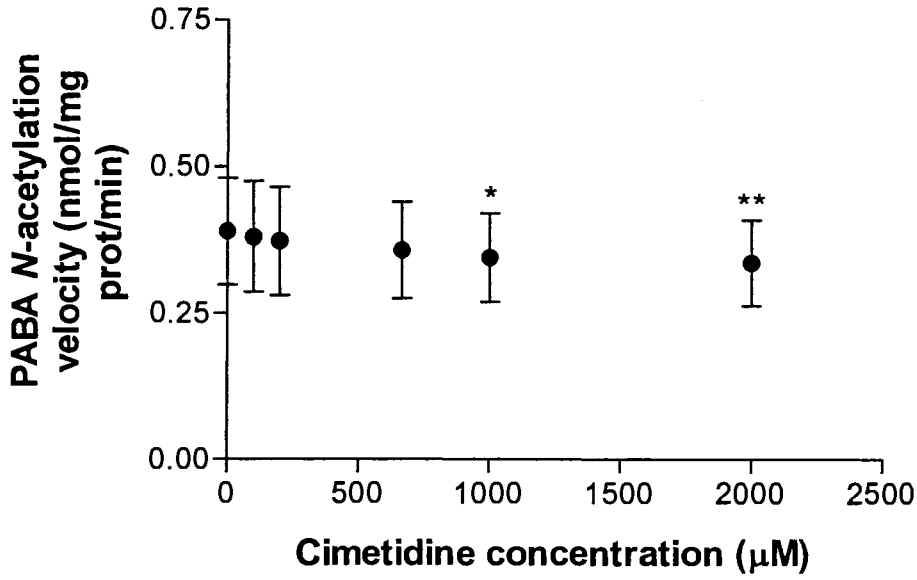


Figure 4.5: Concentration dependent inhibition of 100 µM PABA *N*-acetylation in rats by 0-2000 µM cimetidine

Each point represents the mean ± the SEM of 3 rats. **P*<0.05, ***P*<0.01 as compared to *N*-acetylation of 100 µM PABA with 0 µM cimetidine.



Michaelis Menten parameters for feline *N*-acetylation of PABA were determined with and without cimetidine (Figure 4.6) [49]. Cimetidine had no significant effect on PABA K_m values, but the V_{max} of PABA *N*-acetylation was significantly lower in reactions containing cimetidine (Table 4.1). The K_m and V_{max} of feline PAP *N*-acetylation were significantly lower in the presence of cimetidine (Figure 4.7) (Table 4.1). The intrinsic clearance is proportional to the overall clearance of the substrate and is represented by V_{max}/K_m. There was no significant difference (P=0.7) in the clearance of PABA without cimetidine (V_{max}/K_m = 0.046 ± 0.009) and with cimetidine (V_{max}/K_m = 0.043 ± 0.007). There was also no significant difference in the clearance of PAP with and without cimetidine (P=0.97) V_{max}/K_m = 0.009 ± 0.001 and V_{max}/K_m = 0.009 ± 0.001, respectively.

Pre-incubation conditions were altered to further characterize the mechanism of inhibition by cimetidine on feline *N*-acetylation of PAP and PABA. The *N*-acetylation activity of control reactions for both PAP and PABA (no cimetidine) were compared to reactions initiated with separately warmed substrate and cimetidine, and reactions with cimetidine pre-incubated with the cytosol (Table 4.2). There was significant inhibition of reactions by the addition of cimetidine. Pre-incubation of cimetidine with the cytosol inhibited the reactions significantly more than inhibition by separately warmed cimetidine. In a separate experiment, velocities of controls were compared to velocities of cytosol pre-incubated with or without AcCoA for 5 minutes (Table 4.3). In the PAP

Figure 4.6: Eadie Hofstee transformation of feline *N*-acetylation of PABA to NacPABA

Eadie Hofstee transformations of feline *N*-acetylation of PABA to NacPABA with (•) and without (○) 100 μM cimetidine are compared in a representative cat. Each point represents the mean of duplicate data. Seven-eight PABA concentrations (range of 2.5 - 250 μM), 100 μM AcCoA and a regenerating system to maintain a constant AcCoA were used.

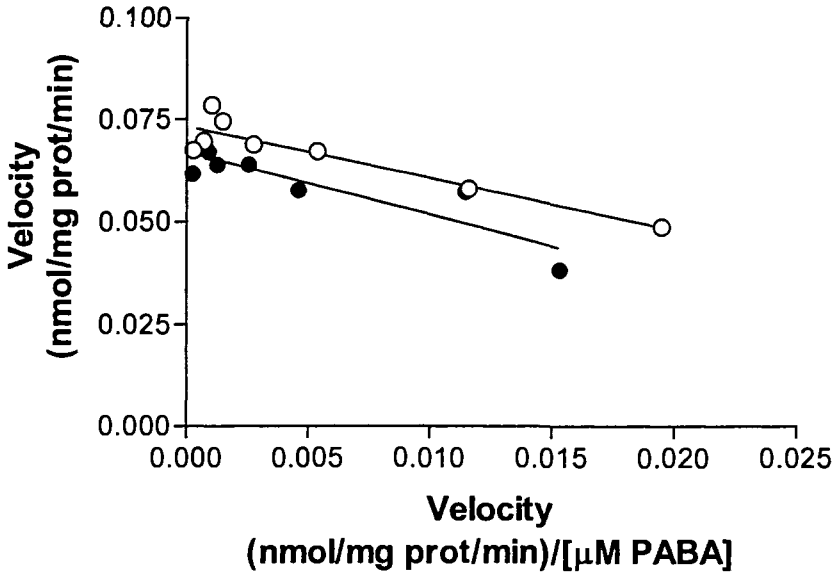


Figure 4.7: Eadie Hofstee transformation of feline *N*-acetylation of PAP to APAP

Eadie Hofstee transformations of feline *N*-acetylation of PAP to APAP with (•) and without (○) 100 µM cimetidine in a representative cat are compared. Each point represents the mean of duplicate data. Eight PAP concentrations (range of 2.5 µM - 250 µM), 100 µM AcCoA and a regenerating system to maintain a constant AcCoA were used

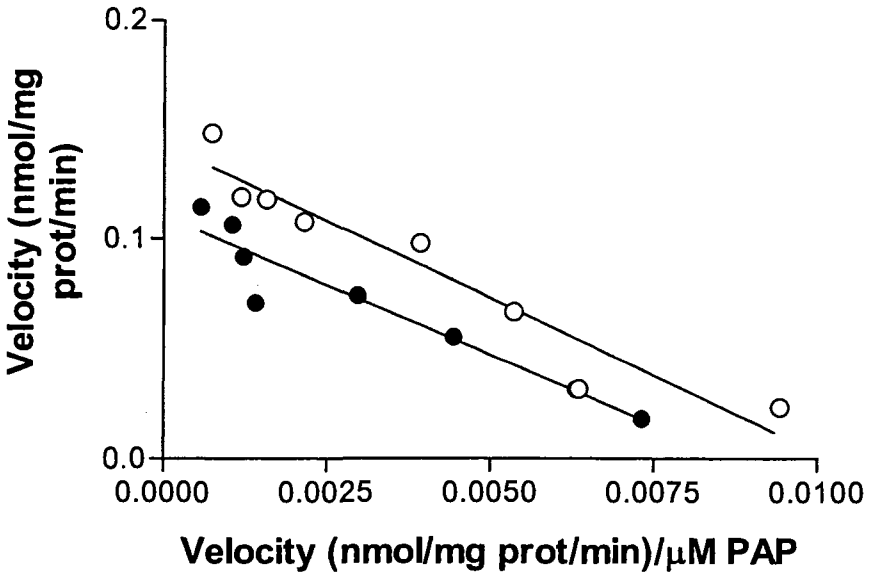


Table 4.1: Comparison of Michealis Menten parameters of feline *N*-acetylation of PAP and PABA with and without cimetidine

All reactions contained feline cytosol, 100μM AcCoA, a regenerating system, 100 μM PAP or PABA and the vehicle or cimetidine. The significantly lower V_{max} for feline *N*-acetylation of both PABA and PAP indicates that the inhibition may be non-competitive or uncompetitive. The additional significantly lower K_m for PAP is further suggestive of uncompetitive inhibition (n=3).

	Control		Cimetidine			Significance
	K_m (μM)	V_{max} (nmol/mg prot/min)	K_m (μM)	V_{max} (nmol/mg prot/min)	V_{max}% of control V_{max}	
N-acetylation of PABA	2.1±0.7	0.082±0.015	2.0±0.7	0.073±0.014	88.37±2.5	K_m *P*=0.61 V_{max} : *P*=0.02
N-acetylation of PAP	8.9±2.7	0.08±0.03	6.8±3.1	0.06±0.03	65.2±6.8	K_m *P*=0.03 V_{max} *P*=0.02

Table 4.2: Comparison of feline *N*-acetylation of PAP and PABA velocities in samples with cytosol pre-incubated with or without cimetidine

To better characterize the inhibition, the pre-incubation conditions were varied to determine if pre-incubation of cytosol with cimetidine would affect the inhibition. All reactions contained feline cytosol, 100μM AcCoA, a regenerating system, 100 μM PAP or PABA and the vehicle or cimetidine. All components were pre-incubated for 5 minutes prior to initiation of the reaction (n=3). The inhibition of PAP and PABA *N*-acetylation was significantly greater in reactions with pre-incubation of cytosol and cimetidine. This supports that the inhibition is not competitive.

	PAP		PABA	
	Velocity (nmol/mg prot/min)	Significance	Velocity (nmol/mg prot/min)	Significance
#1.Control: no cimetidine, initiation of reaction with substrate	0.031±0.008		0.041±0.011	
#2. Initiation of reaction with substrate and cimetidine	0.029±0.008	No significant inhibition of reaction (*P*=0.08)	0.036±0.010	Significant inhibition of reaction (*P*=0.02)
#3. Pre-incubation of cytosol with cimetidine, AcCoA and regenerating system, initiation of reaction with substrate	0.026±0.008	Significant inhibition (*P*=0.02) Significantly lower velocity than #2 (*P*=0.01)	0.034±0.010	Significantly more inhibition than #1 (*P*=0.03)

Table 4.3: Comparison of feline *N*-acetylation of PAP and PABA velocities in samples with cytosol pre-incubated with and without AcCoA

The pre-incubation conditions were varied to determine if pre-incubation of cytosol with AcCoA would affect the inhibition. All reactions contained feline cytosol, 100μM AcCoA, a regenerating system, 100 μM PAP or PABA and the vehicle or cimetidine. All components were pre-incubated for 5 minutes prior to initiation of the reaction.

#1. Control reactions contained no cimetidine. The reactions were initiated by the addition of pre-incubated cytosol to separately pre-incubated AcCoA, regenerating system, vehicle and PAP or PABA.
#2. Reactions were initiated by the addition of preincubated cytosol to separately pre-incubated PAP or PABA, 500 μM cimetidine, AcCoA and a regenerating system.
#3. Reactions were initiated by the addition of cytosol pre-incubated with AcCoA and a regenerating system to separately preincubated substrate and cimetidine.

	PAP		PABA	
	Velocity (nmol/mg prot/min)	Significance (*P*)	Velocity (nmol/mg prot/min)	Significance (*P*)
#1.Control: no cimetidine, initiation of reaction with cytosol	0.02±0.004		0.040±0.008	
#2.Co-incubation of substrate, cimetidine, AcCoA and regenerating system, initiation of reaction with cytosol	0.015±0.003	Significant inhibition of reaction (*P*=0.01)	0.039±0.010	Significant inhibition of reaction (*P*=0.04)
#3.Pre-incubation of cytosol with AcCoA and regenerating system, initiation of reaction with substrate and cimetidine	0.0099±0.002	Significantly more inhibition than #2 (*P*=0.04)	0.035±0.006	Inhibition not significantly different than #2 (*P*=0.07)

reactions, the velocities of reactions with pre-incubation of cytosol and AcCoA were significantly lower than velocities of reactions with no pre-incubation of cytosol with AcCoA. In PABA reactions, pre-incubation of cytosol with AcCOA had no significant effect on the inhibition.

4.8 Discussion

Approximately thirty percent of cats diagnosed with APAP toxicity and treated with *N*-acetylcysteine as currently recommended, still die despite treatment [7]. Thus, it is understandably tempting to adopt additional therapies such as cimetidine based on research in other species. However, biotransformation of drugs, resulting metabolites and enzyme inhibition can vary between species, making extrapolation between species inappropriate [50].

The metabolism of APAP is predominantly by sulfation and glucuronidation in most species, including humans, dogs, mice and rats [19,22]. Cats are primarily sulfators of APAP because of a pseudogene for the glucuronidation enzyme responsible for APAP metabolism (UGT1A6) [20,51]. All species appear to oxidize small quantities of APAP to the reactive metabolite NAPQI via CYP enzymes [5]. The increased production of this oxidative metabolite following a toxic overdose leads to the centrilobular hepatic necrosis seen in many species following APAP overdose [5,52]. The methemoglobinemia seen within hours of APAP overdose in cats has previously been presumed to be due to limited feline APAP glucuronidation resulting in higher production of

NAPQI with erythrocytes the target because of the greater numbers of exposed sulfhydryl groups in feline hemoglobin [14,53]. Our laboratory has demonstrated that NAPQI does not induce methemoglobin production *in vitro* (see Chapter 2). We have shown that the metabolite PAP induces marked amounts of methemoglobin and is more likely to be the metabolite involved in APAP-induced methemoglobinemia.

Cimetidine has been suggested as adjunct therapy to *N*-acetylcysteine for feline and canine APAP toxicity [14]. However, Svensson and Tomilo have demonstrated that cimetidine is an inhibitor of *N*-acetylation in rats. In their studies, cimetidine inhibited *N*-acetylation of procainamide by 48% at an inhibitor to substrate ratio of 2:1, and inhibited *N*-acetylation of PABA by 18% at an inhibitor to substrate ratio of 5:1 [43]. This raised the possibility that cimetidine might also inhibit the NAT1 enzyme found in cats and decrease detoxification of PAP in cats.

The results of our studies indicate that cimetidine significantly inhibits feline acetylation of PAP and PABA. The Eadie-Hofstee transformations of feline *N*-acetylation were linear, reflecting the acetylation of PAP and PABA by a single enzyme (NAT1). Our results for inhibition of PABA acetylation by cimetidine in rats were consistent with Svensson's previous results [43]. The degree of inhibition by cimetidine on the feline NAT activity was similar to the inhibition by cimetidine of PABA acetylation in the rat.

There was a significant decrease in the V_{max} for both PAP and PABA *N*-

acetylation which indicates that the inhibition is not competitive. The decreased K_m in the feline PAP *N*-acetylation reactions further suggests uncompetitive inhibition, however, as the K_m was not decreased for PABA *N*-acetylation, non-competitive inhibition must also be considered [54,55]. Uncompetitive inhibition is a reversible inhibition that occurs when an inhibitor only binds to an enzyme-substrate complex and not to a free enzyme whereas non-competitive inhibition involves the binding of an inhibitor to any site on the enzyme other than the active binding site [54]. The decrease in the V_{max} and K_m were proportional and therefore there was no significant change in the intrinsic clearance.

In order to better characterize the inhibition, we altered the pre-incubation conditions. Pre-incubation of cimetidine with cytosol caused significantly greater inhibition than initiation of the reaction by simultaneous addition of cimetidine and PAP in cats. This again indicates that the inhibition was not competitive. Pre-incubation of the cytosol with AcCoA inhibited the *N*-acetylation of PAP significantly more than pre-incubation of cytosol without AcCoA. This again suggests that there is uncompetitive inhibition. The concentration of enzyme-substrate complex (acetylated NAT) may increase with pre-incubation of the cytosol and AcCoA. This would give more opportunity for an uncompetitive inhibitor to bind and therefore potentially result in more inhibition. However, as the K_m of the feline *N*-acetylation of PABA reactions was not in agreement with the K_m from the PAP experiments, at this time we can only conclude that the inhibition of feline *N*-acetylation by cimetidine is not competitive.

Extrapolation of *in vitro* results to *in vivo* situations must always be done with caution. Our cimetidine concentrations were above expected plasma concentrations in treated cats. The C_{max} of cimetidine is approximately 45-70 μM at a dosage of 15 mg/kg BID orally in cats [56]. The cimetidine dosage for treatment of APAP toxicity is 5-10 mg/kg TID-QID intramuscularly, intravascularly or orally [14]. However, the concentration of drugs given orally can be higher in the portal vein than in the general body plasma [57]. The actual concentration of a drug within the hepatic cytosol where the inhibition occurs can in turn be higher than the portal vein [58]. Therefore, the cimetidine concentration within the hepatocyte cytosol is unknown, but likely higher than reported plasma concentrations [58]. The PAP concentration within the hepatocytes is also unknown. There was no change in the intrinsic clearance (V_{max}/K_m) and therefore there may be no effect of this inhibitor *in vivo.* However, our results demonstrate the potential for an *in vivo* interaction between cimetidine and *N*-acetylation of PAP. The actual extent of the potential interaction and the clinical ramifications are unknown.

The use of cimetidine in human APAP toxicosis has been closely examined and is no longer recommended [12]. Extrapolation of use of a CYP inhibitor between species is inappropriate as enzymes and substrate affinities may vary. In addition, cats have never been shown to develop APAP related centrilobular hepatic necrosis. In this paper, we have demonstrated that cimetidine has a significant inhibitory effect on feline PAP *N*-acetylation *in vitro.*

There does not appear to be any effect on the intrinsic clearance *in vitro*, but *in vivo* studies are required to determine the potential for clinical effects.

The use of cimetidine in feline APAP toxicosis is unlikely to be of any benefit and may prolong the redox cycling of PAP and oxyhemoglobin, thereby prolonging the methemoglobinemia. At this time, cimetidine should not be recommended as an additional therapeutic in the treatment of feline APAP toxicosis.

4.9 References

1. Dargan PI, Ladhani S, Jones AL. Measuring plasma paracetamol concentrations in all patients with drug overdose or altered consciousness: does it change outcome? Emerg Med J 2001;18:178-82.

2. Bialas MC, Reid PG, Beck P, et al. Changing patterns of self-poisoning in a UK health district. QJM 1996;89:893-901.

3. Watson WA, Litovitz TL, Klein-Schwartz W, et al. 2003 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2004;22:335-404.

4. Jones RD, Baynes RE, Nimitz CT. Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989-1990). J Am Vet Med Assoc 1992;201:475-7.

5. Davis DC, Potter WZ, Jollow DJ, Mitchell JR. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci 1974;14:2099-109.

6. Prescott LF, Roscoe P, Wright N, Brown SS. Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet 1971;1:519-22.

7. Aronson LR, Drobatz K. Acetaminophen toxicosis in 17 cats. J Vet Emerg Crit Care 1996;6:65-9.

8. Schlesinger DP. Methemoglobinemia and anemia in a dog with acetaminophen toxicity. Can Vet J 1995;36:515-7.

9. Nash SL, Savides MC, Oehme FW, Johnson DE. The effect of acetaminophen on methemoglobin and blood glutathione parameters in the cat. Toxicol 1984;31:329-34.

10. Bartlett D. Acetaminophen toxicity. J Emerg Nurs 2004;30:281-3.

11. Bahri LE. N-acetylcysteine. Compend Contin Educ Pract Vet 2003;15:276-9.

12. Dargan PI, Jones AL. Management of paracetamol poisoning. Trends Pharmacol Sci 2003;24:154-7.

13. Dart RC, Erdman AR, Olson KR, et al. Acetaminophen poisoning: an evidence-based consensus guideline for out-of-hospital management. Clin Toxicol (Phila) 2006;44:1-18.

14. Taylor NS, Dhupa N. Acetaminophen Toxicity in Dogs and Cats. Compend Contin Educ Pract Vet 2003;12:160-9.

15. Mitchell MC, Schenker S, Speeg KV, Jr. Selective inhibition of acetaminophen oxidation and toxicity by cimetidine and other histamine H2-receptor antagonists in vivo and in vitro in the rat and in man. J Clin Invest 1984;73:383-91.

16. Al Mustafa ZH, Al Ali AK, Qaw FS, Abdul-Cader Z. Cimetidine enhances the hepatoprotective action of N-acetylcysteine in mice treated with toxic doses of paracetamol. Toxicol 1997;121:223-8.

17. Hazai E, Vereczkey L, Monostory K. Reduction of toxic metabolite formation of acetaminophen. Biochem Biophys Res Commun 2002;291:1089-94.

18. MacNaughton SM. Acetaminophen toxicosis in a Dalmatian. Can Vet J 2003;44:142-4.

19. Gregus Z, Madhu C, Klaassen CD. Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. J Pharmacol Exp Ther 1988;244:91-9.

20. Savides MC, Oehme FW, Nash SL, Leipold HW. The toxicity and biotransformation of single doses of acetaminophen in dogs and cats. Toxicol Appl Pharmacol 1984;74:26-34.

21. Moldeus P. Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. Biochem Pharmacol 1978;27:2859-63.

22. Davis M, Simmons CJ, Harrison NG, Williams R. Paracetamol overdose in man: relationship between pattern of urinary metabolites and severity of liver damage. Q J Med 1976;45:181-91.

23. Landin JS, Cohen SD, Khairallah EA. Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol 1996;141:299-307.

24. Pumford NR, Roberts DW, Benson RW, Hinson JA. Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen protein adducts in subcellular liver fractions following a hepatotoxic dose of acetaminophen. Biochem Pharmacol 1990;40:573-9.

25. Rumbeiha WK, Lin YS, Oehme FW. Comparison of N-acetylcysteine and methylene blue, alone or in combination, for treatment of acetaminophen toxicosis in cats. Am J Vet Res 1995;56:1529-33.

26. Slattery JT, McRorie TI, Reynolds R, et al. Lack of effect of cimetidine on acetaminophen disposition in humans. Clin Pharmacol Ther 1989;46:591-7.

27. Zed PJ, Krenzelok EP. Treatment of acetaminophen overdose. Am J Health Syst Pharm 1999;56:1081-91.

28. Mitchell MC, Schenker S, Avant GR, Speeg KV, Jr. Cimetidine protects against acetaminophen hepatotoxicity in rats. Gastroenterol 1981;81:1052-60.

29. Finco DC, Duncan JR, Schall WD, Prasse KW. Acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1975;166:469-72.

30. St Omer VV, McKnight ED, III. Acetylcysteine for treatment of acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1980;176:911-3.

31. Kelly JH, Koussayer T, He DE, et al. An improved model of acetaminophen-induced fulminant hepatic failure in dogs. Hepatol 1992;15:329-35.

32. Francavilla A, Makowka L, Polimeno L, et al. A dog model for acetaminophen-induced fulminant hepatic failure. Gastroenterol 1989;96:470-8.

33. Newton JF, Kuo CH, Gemborys MW, Mudge GH, Hook JB. Nephrotoxicity of p-aminophenol, a metabolite of acetaminophen, in the fischer 344 rat. Toxicol Appl Pharmacol 1982;65:336-44.

34. Berheim F, Berheim MLC., Michel HO. The action of p-aminophenol on certain tissue oxidations. J Pharmacol Exp Ther 1937;61:311-20.

35. Kiese M., Pekis M. The reaction of p-aminophenol with hemoglobin and oxygen in vivo and in vitro. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1964;246:413-20.

36. Harrison JH, Jr., Jollow DJ. Contribution of aniline metabolites to aniline-induced methemoglobinemia. Mol Pharmacol 1987;32:423-31.

37. Klos C, Koob M, Kramer C, Dekant W. p-aminophenol nephrotoxicity: biosynthesis of toxic glutathione conjugates. Toxicol Appl Pharmacol 1992;115:98-106.

38. Song H, Chen TS. p-Aminophenol-induced liver toxicity: tentative evidence of a role for acetaminophen. J Biochem Mol Toxicol 2001;15:34-40.

39. Grant DM, Lottspeich F, Meyer UA. Evidence for two closely related isozymes of arylamine N-acetyltransferase in human liver. FEBS Lett

1989;244:203-7.

40. Grant DM, Blum M, Beer M, Meyer UA. Monomorphic and polymorphic human arylamine N-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. Mol Pharmacol 1991;39:184-91.

41. Trepanier LA, Cribb AE, Spielberg SP, Ray K. Deficiency of cytosolic arylamine N-acetylation in the domestic cat and wild felids caused by the presence of a single NAT1-like gene. Pharmacogenetics 1998;8:169-79.

42. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol 1997;54:73-80.

43. Svensson CK, Tomilo M. Effect of H2-receptor antagonists on rat liver cytosolic acetyl CoA:arylamine N-acetyltransferase activity. Drug Metab Dispos 1992;20:74-8.

44. Grant DM, Morike K, Eichelbaum M, Meyer UA. Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine N-acetyltransferase in human liver. J Clin Invest 1990;85:968-72.

45. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

46. Grant DM, Lottspeich F, Meyer UA. Evidence for two closely related isozymes of arylamine N-acetyltransferase in human liver. FEBS Lett 1989;244:203-7.

47. Cribb AE, Grant DM, Miller MA, Spielberg SP. Expression of monomorphic arylamine N-acetyltransferase (NAT1) in human leukocytes. J Pharmacol Exp Ther 1991;259:1241-6.

48. Andres HH, Klem AJ, Szabo SM, Weber WW. New spectrophotometric and radiochemical assays for acetyl-CoA: arylamine N-acetyltransferase applicable to a variety of arylamines. Anal Biochem 1985;145:367-75.

49. Hofstee B. On the evaluation of the constants Vmax and Km in enzyme reactions. Science 1952;116:329-31.

50. Drug elimination and pharmacokinetics In: Rang HP, Dale MM, Ritter JM, Moore PK, editors. Pharmacology. Fifth ed. Philadelphia: Churchill Livingston; 2003. p.106-19.

51. Court MH, Greenblatt DJ. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. Biochem Pharmacol 1997;53:1041-7.

52. Gibson JD, Pumford NR, Samokyszyn VM, Hinson JA. Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. Chem Res Toxicol 1996;9:580-5.

53. Desnoyers M. Anemias associated with heinz bodies. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm's Veterinary Hematology. Fifth ed. Philadelphia: Lippincott WIlliams & Wilkins; 2000. p.178-84.

54. Palmer T. Kinetics of multi-substrate enzyme-catalysed reactions. In: Palmer T, editor. Understanding Enzymes. Fourth ed. Hemel Hampstead: Prentice Hall/Ellis Horwood; 1991. p.155-74.

55. Guengerich FP. Inhibition of drug metabolizing enzymes: Molecular and biochemical aspects. In: Woolf TF, editor. Handbook of drug metabolism. New York: Marcel Dekker Inc.; 1999. p.203-28.

56. Heeb HL, Chun R, Koch DE, et al. Multiple dose pharmacokinetics and acute safety of piroxicam and cimetidine in the cat. J Vet Pharmacol Ther 2005;28:447-52.

57. Tabata K, Yamaoka K, Fukuyama T, Nakagawa T. Local absorption kinetics into the portal system using the portal-venous concentration difference after an oral dose of diclofenac in the awakening rat. Accelerative effect of bile on intestinal absorption of diclofenac. Drug Metab Dispos 1996;24:216-20.

58. Yamano K, Yamamoto K, Kotaki H, et al. Correlation between in vivo and in vitro hepatic uptake of metabolic inhibitors of cytochrome P-450 in rats. Drug Metab Dispos 1999;27:1225-31.

5. GENERAL DISCUSSION

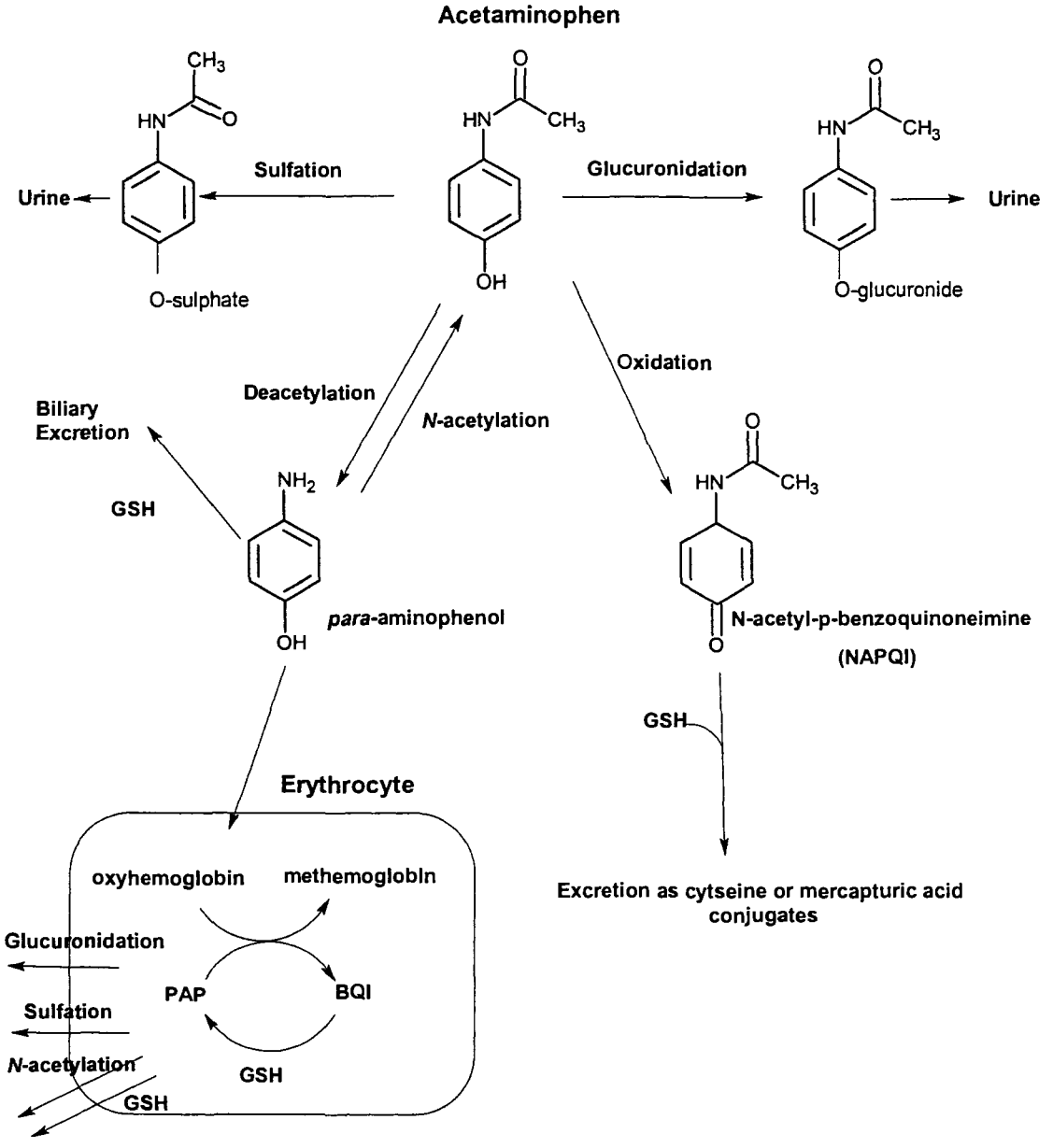
Dogs and cats are the only known species that develop APAP induced hematotoxicity consisting of methemoglobinemia and hemolysis [1-5]. The studies described in this thesis were undertaken to determine the underlying mechanisms for the development of methemoglobinemia in APAP toxicity in dogs and cats, and the basis for the species differences in target organ susceptibility.

We have demonstrated that APAP requires metabolism to a reactive metabolite to induce methemoglobinemia. Our results support the hypothesis that PAP is the metabolite responsible for the methemoglobinemia seen in APAP toxicity in dogs and cats (Figure 5.1).

While all species tested were able to deacetylate APAP to PAP, dogs were completely unable to re-acetylate PAP and cats showed a markedly reduced capacity. This supports that species-related deficiencies in NAT activity may be a pre-disposing factor to APAP-hematotoxicity. However, erythrocytes from *NAT1/NAT2* double knockout mouse were only slightly more susceptible to PAP-induced methemoglobinemia than those from wildtype mice, suggesting that other factors are also important in determining susceptibility.

Methemoglobin formation was compared in intact and lysed erythrocytes. Rats and wildtype mice had significantly higher methemoglobin formation in lysed erythrocytes than in intact red blood cells, while dogs and

Figure 5.1: Biotransformation and bioactivation of APAP



cats had significantly higher methemoglobin formation in the intact erythrocytes. This further supports differences between species at the level of the erythrocyte. There are at least two possible contributing factors to these results. Rats and mice have efficient NAT enzymes removing PAP from redox cycling with oxyhemoglobin, and highly active NADH methemoglobin reductase reducing the methemoglobin [6-10]. These two protective pathways are lost in rats and mice when erythrocytes are lysed, leading to a higher methemoglobin formation.

In contrast, dogs and cats would not be expected to have efficient removal of PAP from redox cycling in intact cells because they are deficient in NAT enzymes. In addition, canine and feline methemoglobin reductase is not as efficient as rat and mouse methemoglobin reductase [6,9,10]. The net result in lysed canine and feline erythrocytes is a reduction in the methemoglobin forming potential that is not outweighed by the loss of the protective pathways.

The conclusions from the *in vitro* results were supported by *in vivo* studies performed in wildtype and NAT double knockout mice. No methemoglobin was produced in either group following injection of a hepatotoxic dose of APAP. Therefore a *NAT1/NAT2* deficiency alone is not sufficient to induce susceptibility to APAP-induced hematotoxicity. Knockout mice were more susceptible to PAP-induction of methemoglobin, confirming that a NAT deficiency contributes to species susceptibility to PAP.

Multifactorial susceptibility is typical of pharmacogenetic syndromes in general. Acetaminophen is an arylamine. Other examples of arylamines include sulfamethazine and thiazolsulfone. It has been demonstrated that slow acetylators of sulfamethazine and thiazolsulfone produce more hydroxylated metabolites [11,12]. These hydroxylated active intermediates undergo co-oxidation with oxyhemoglobin, producing nitroso metabolites and reactive oxygen species which deplete glutathione and predispose to oxidative damage and hemolysis. Individuals who are both slow acetylators and deficient in glucose-6-phosphate-dehydrogenase (G6PD) are predisposed to acute hemolysis as they are unable to reduce oxidized GSH [11,12].

Further studies are necessary to identify the other contributing factors to APAP hematotoxicity in dogs and cats. The lack of glucuronidation in cats may contribute to the feline sensitivity to APAP but not, as previously thought, by resulting in increased NAPQI production, but rather by making more APAP available for deacetylation to PAP. The contribution of methemoglobin reductase and GSH concentration to the species sensitivity should be examined. In addition, the contribution of oxidation should also be explored, given that other arylamines require hydroxylation to an activated metabolite to cause adverse reactions such as methemoglobinemia and hemolysis.

Hematotoxicity is one of the most common types of adverse drug reactions in humans. It is important to understand species differences for testing new drugs and development of animal models. Acetaminophen

toxicity in dogs and cats is an excellent example of how species differences in biotransformation can markedly affect the response to a drug.

Acetaminophen toxicity in dogs and cats is not uncommon. The current treatment consists of *N*-acetylcysteine and supportive therapy [13]. Our studies indicate that additional treatment inhibiting APAP deacetylation or enhancing methemoglobin reduction should be explored. In addition, the practice of using cimetidine as adjunct therapy to *N*-acetylcysteine is not only unlikely to be of benefit, it may prolong the redox cycling of PAP and oxyhemoglobin and therefore is not advised at this time.

5.1 References

1. Finco DC, Duncan JR, Schall WD, Prasse KW. Acetaminophen toxicosis in the cat. J Am Vet Med Assoc 1975;166:469-72.

2. MacNaughton SM. Acetaminophen toxicosis in a Dalmatian. Can Vet J 2003;44:142-4.

3. Ilkiw JE, Ratcliffe RC. Paracetamol toxicity in a cat. Aust Vet J 1987;64:245-7.

4. Aronson LR, Drobatz K. Acetaminophen toxicosis in 17 cats. J Vet Emerg Crit Care 1996;6:65-9.

5. Mariani CL, Fulton RB. Atypical reaction to acetaminophen intoxication in a dog. J Vet Emerg Crit Care 2001;11:123-6.

6. Wyman JF, Gray BH, Lee LH, et al. Interspecies variability in propylene glycol dinitrate-induced methemoglobin formation. Toxicol Appl Pharmacol 1985;81:203-12.

7. Estrada-Rodgers L, Levy GN, Weber WW. Substrate selectivity of mouse N-acetyltransferases 1, 2, and 3 expressed in COS-1 cells. Drug Metab Dispos 1998;26:502-5.

8. Walraven JM, Doll MA, Hein DW. Identification and characterization of functional rat arylamine N-acetyltransferase 3: comparisons with rat arylamine N-acetyltransferases 1 and 2. J Pharmacol Exp Ther 2006;319:369-75.

9. Srivastava S, Alhomida AS, Siddiqi NJ, Puri SK, Pandey VC. Methemoglobin reductase activity and in vitro sensitivity towards oxidant induced methemoglobinemia in swiss mice and beagle dogs erythrocytes. Mol Cell Biochem 2002;232:81-5.

10. Stolk JM, Smith RP. Species differences in methemoglobin reductase activity. Biochem Pharmacol 1966;15:343-51.

11. Magon AM, Leipzig RM, Zannoni VG, Brewer GJ. Interactions of glucose-6-phosphate dehydrogenase deficiency with drug acetylation and hydroxylation reactions. J Lab Clin Med 1981;97:764-70.

12. Woolhouse NM, Atu-Taylor LC. Influence of double genetic polymorphism on response to sulfamethazine. Clin Pharmacol Ther 1982;31:377-83.

13. Rumbeiha WK, Lin YS, Oehme FW. Comparison of N-acetylcysteine and methylene blue, alone or in combination, for treatment of acetaminophen toxicosis in cats. Am J Vet Res 1995;56:1529-33.

Appendix A Results of APAP Experiments 1,2,3

	Time	PCV	ALT	AST	T bili	Met
Experiment #1						
WT-saline	0 hour	47.8±0.5	3±1	57±21	-	-
KO-saline	0 hour	49.6±2.8	38±14	115±35	-	-
WT-APAP 250	0 hour	40.7±3.2	235±206	140±24	-	-
KO-APAP 250	0 hour	47.7±1.9	52±10	411±137	-	-
WT-saline	3 hour	46.5±0.5	41±13	424±111	-	-
KO-saline	3 hour	49.8±0.5	39±16	279±132	-	-
WT-APAP 250	3 hour	46.7±3.0	537±255	***1286±461	-	-
KO-APAP 250	3 hour	45.5±1.0	**1319±755	***1331±362	-	-
WT-saline	24 hour	49.5±3.8	9±8	48±29	-	-
KO-saline	24 hour	47.3±1.2	21±13	97±52	-	-
WT-APAP 250	24 hour	47.0±1.2	24±11	47±36	-	-
KO-APAP 250	24 hour	51.3±2.6	890±493	389±217	-	-
WT-saline	48 hour	48.7±1.5	40±24	37±23	-	-

KO-saline	48 hour	44.7±2.0	74±31	100±28	-	-
WT-APAP 250	48 hour	48.0±1.5	77±32	551±137	-	-
KO-APAP 250	48 hour	**25.8±2.9	233±65	728±126	-	-

	Time	PCV	ALT	AST	T bili	Met
Experiment #2						
WT-saline	3 hour	49.3±0.3	48±12	139±45	3.5±0.5	0.6±0.6
KO-saline	3 hour	43.4±1.0	34±3	184±13	3.0±0.0	2.0±0.3
WT-APAP 250	3 hour	46.7±0.9	444±223	304±141	3.0±0.0	3.8±1.2
KO-APAP 250	3 hour	43.0±1.6	1564±445	**609±232	4.5±1.5	4.6±2.5
WT-APAP 400	3 hour	45.0±2.0	1704±905	**2176±983	7.5±0.9	2.4±1.9
KO APAP 400	3 hour	43.3±2.7	1646±660	2517±1118	7.3±1.3	4.6±1.2
WT-saline	48 hr	44.0±1.5	128±99	87±29	3.3±0.9	3.0±0.6
KO-saline	48 hr	45.0±1.2	50±4	157±26	3.8±0.9	1.1±0.5

KO APAP 250	48 hr	44.3±1.3	157±118	168±64	1.5±0.3	0.7±0.7
WT-APAP 400	48 hr	48.8±0.3	588±212	391±85	3.7±0.3	1.2±0.5
KO APAP 400	48 hr	48.3±0.3	613± 517	131±35	2.5±0.6	2.0±0.6

	Time	**PCV**	**ALT**	**AST**	**T bili**	**Met**
Experiment 3						
WT-saline	1 hour	45.7±0.7	149±56	116±38	3.0±0	5.1±1.0
KO-saline	1 hour	44.8±1.4	45±3	106±13	2.8±.5	5.7±1.2
WT-APAP 250	1 hour	49.3±0.6	55±19	194±43	3.3±1.3	10±1.6
KO-APAP 250	1 hour	49.3±1.1	84±41	307±133	3.8±1.5	6.0±0.6
WT-saline	24 hour	45.7±0.7	89±33	294±130	2.5±2.6	3.8±3.0
KO-saline	24 hour	44.8±1.4	51±5	150±18	3.3±1.3	4.8±2.3
WT-APAP 250	24 hour	49.7±0.6	***11895 ±899	***9962 ±1316	3.0±1.2	5.5±0.1

KO-APAP 250	24 hour	50.0±0.7	***11907 ±6765	***14317 ±4415	4.7±1.2	4.6±1.0
WT-saline	48 hour	43.0±1.1	93±24	101±27	2.0±1.8	3.0±2.2
KO-saline	48 hour	46.8±0.6	31±6	86±9	3.0±0	1.6±0.6
WT-APAP 250	48 hour	46.5±1.7	1089±516	833±279	3.3±0.9	10.8±0.9***
KO-APAP 250	48 hour	49.0±1.7	677±169	536±156	3.5±1.9	7.8±3.2**WT

Appendix A

* $P<0.05$
** $P<0.01$
*** $P<0.001$