THE MECHANISM OF AMANTADINE ACETYLATION

BY

ALVARO PEREIRA MARQUES BRAS

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS

FACULTY OF MEDICINE

UNIVERSITY OF MANITOBA

WINNIPEG, MANITOBA

CANADA

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Alvaro Pereira Marques Bras

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

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ABSTRACT

Amantadine is an important drug in the treatment of and prophylaxis against influenza A virus infection and in the treatment of Parkinson's disease. Amantadine was approved for the preceding diseases in the late 1960s and was initially reported not to be subject to metabolism. However, in 1985, N-acetylation was identified as a minor metabolic pathway for amantadine. No further study in this area was conducted. Therefore the mechanism that mediates the acetylation of amantadine remained to be described. This thesis work has investigated how amantadine is acetylated. The hypotheses of this project were divided into two parts: 1. The N-acetyltransferases, NAT1 and NAT2, do not catalyze the acetylation of amantadine; 2. The enzyme, spermidine/spermine N^1-acetyltransferase (SSAT), that catalyzes the acetylation of spermidine and spermine is responsible for catalyzing the acetylation of amantadine when this enzyme is induced or overexpressed. The first study used NAT1 and NAT2 enzymes from three different sources, rat liver, rabbit liver, and human recombinant wild type NAT1 and NAT2, incubated with their selective substrates *p*-aminobenzoic acid (PABA) and sulfamethazine (SMZ), respectively. The addition of amantadine to the selective subtrates in the same incubation medium did not inhibit their acetylation. Amantadine incubated in the absence of the substrates also was not acetylated. These results suggested that neither NAT1 nor NAT2 were responsible for amantadine acetylation. The second study involved transgenic mice overexpressing SSAT and injected with amantadine. All mice excreted acetylamantadine in their urine. Controls of the same strain not overexpressing SSAT did not excrete acetylamantadine. In vitro experiments using the cytosolic liver fraction as the source of SSAT demonstrated that amantadine

could inhibit spermidine acetylation competitively. Incubation of amantadine with an acetyl-coenzyme A regenerating system and transgenic mouse liver as the cytosolic source of SSAT produced modest amounts of acetylamantadine. Co-incubation of amantadine and the NAT2 selective substrate SMZ inhibited production of acetylamantadine. The selective NAT2 substrate, SMZ, but not the NAT1-selective substrate PABA, modestly inhibited spermidine acetylation. In conclusion, the data support the hypothesis that amantadine acetylation is catalyzed by SSAT and may be a specific substrate for this enzyme. These findings indicate that SSAT may be a drug acetylation pathway when induced or overexpressed and may contribute to NAT2 selective substrate acetylation.

ACKNOWLEDGEMENTS

First of all I would like to express my sincere thanks to Dr. Daniel Sitar for providing me the opportunity to pursue a scientific carrier. As a supervisor, he offered excellent mentorship, guidance, and shared his insights into doing science. I greatly appreciated his generous availability of time to discuss the project and all its complexities. His enthusiasm for science always amazed me and provided the motivation to continue the pursuit of scientific insight. As a friend, he appreciated some of the hurdles that I faced in everyday life and always tried to help if he could, and these gestures will never be forgotten.

The Department of Pharmacology and Therapeutics as a whole provided a great atmosphere for learning and personal development. All professors are praise worthy in their generous availability of time for the students when they require it. Time is the greatest gift a professor can give a student and I greatly appreciated all the discussions that I had with different professors.

I am grateful to the members of my examining committee Dr. Gary Glavin, Dr. Fred Aoki and Dr. Alastair Cribb for agreeing to be the external examiner for this thesis.

I am indebted to the following people who donated time and materials for this project; Dr. Dennis Grant and Geoffrey Goodfellow at Sick Children's Hospital in Toronto, Dr. Carl Porter at the Grace Cancer Drug Center, Buffalo, New York, and locally Dr. Elliot Scott at the Faculty of Dentistry, and Dr. Hope Weiler at the Faculty of Human Ecology.

I am grateful to James Sherwin for his technical assistance without which this project would have been much more difficult. I would like to thank former students Chao Han, Miguel Escobar, and Kerry Goralski for their willingness to share their knowledge, be it from computers to methods of analysis. I thank Jodi Schoen for her taking the time to demonstrate techniques that I required for my project.

I would like to thank Dr. Deepak Bose and Dr. Ratna Bose for their willingness to share their knowledge and wisdom. I thank Dr. Fiona Parkinson, Dr. D. Smyth, and Dr. Wayne Lautt for the discussions that I had with them over the years. Financial support for this study was provided by the Health Sciences Centre Research Foundation. I would also like to thank Dr. Kevin Hall and my colleagues Helen, Stacey, Beata, and Wendy for their support at the Health Sciences Centre Pharmacy Department.

I wish to dedicate this thesis to my family, my wife Teresa and my children James, Philipe, and Cristina. My wife provided me with the emotional support and encouragement necessary to persevere. When my life was hanging by a thread she stood by my side and comforted me. She continually nurtures my spirit and provides a positive outlook on life. She has enriched my life with her friendship and love. Thank you.

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ABBREVIATIONS

Acetyl-CoA	Acetyl-coenzyme A
AcPABA	N-Acetyl-p-aminobenzoic acid
AcSMZ	N-Acetylsulfamethazine
ANOVA	Analysis of variance
DDT	Dithiothreitol
DDW	Distilled deionized water
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GLC	Gas liquid chromatography
HPLC	High performance liquid chormatography
IC_{50}	Concentration of inhibitor required to inhibit acetylation by 50%
INH	Isoniazid
KCl	Potassium chloride
K_i	Constant for inhibition
K_m	Apparent affinity constant for acetylation
MGBG	Methylglyoxal bis-(guanylhydrazone)
min	minute

NAT	N-Acetyltransferase
NAT1	N-Acetyltransferase-1
NAT2	N-Acetyltransferase-2
NMDA	N-methyl-D-aspartic acid
ODC	Ornithine decarboxylase
PABA	p-Aminobenzoic acid
PAO	Polamine oxidase
PAS	p-Amino-salicylic acid
PBS	Phosphate buffered saline
SAMDC	S-adenosyl methionine decarboxylase
SMZ	Sulfamethazine
SSAT	Spermidine/spermine N^1-acetyltransferase
TEDK	Homogenizing buffer composed of triethanolamine-HCl, EDTA, DDT, KCl, leupeptin, phenylmethylsulfonyl flouride, butylated hydroxytoluene
Tris	Tris[hydroxymethyl]aminomethane
V_{max}	Apparent maximal acetylation capacity

1.INTRODUCTION

1.1. AMANTADINE

1.1.1. Approved Clinical Uses

Amantadine (Fig I-1) was synthesized at the Du Pont laboratories in the 1960s (Davies *et al.*, 1964) and has a unique polycyclic aliphatic structure, with an achiral primary amine that makes it a weak base (pKa = 10.1). At physiological pH, it exists mainly in the cationic form. Amantadine has been used as an adjunct in the symptomatic relief of Parkinson's disease and for the prophylaxis and treatment of influenza A virus infection (Aoki & Sitar, 1988; Oxford & Galbraith, 1980; Schwab *et al.*, 1969), the two approved clinical indications for amantadine in Canada.

1.1.2. Mechanism of Action

The exact mechanism of amantadine's action against influenza A virus replication is not known. Initial studies, using cultures of chick-embryo cells suggested that amantadine (12.5 mcg/ml) blocked or slowed the virus penetration into cells, but did not inhibit adsorption of virus to the cell surface or cause release of virus from cells (Hoffmann *et al.*, 1965). This finding indicated that the effect of amantadine was at the cell surface. Kato and Eggers, using fowl plague virus, demonstrated that amantadine had only a slight effect on virus penetration of chick embryo cells, and concluded that the drug affected viral uncoating (Kato & Eggers *et al.*, 1969). Recent evidence suggests that amantadine blocks pH-sensitive cation channels formed by the influenza A M2 integral membrane protein. These cation channels are thought to play an essential role in

the uncoating of the influenza A virus (Wang *et al.*, 1994; Duff *et al.*, 1994). In a study by Herrmann *et al.*, amantadine affected the fluidity of human erythrocyte membranes (Herrmann *et al.*, 1985). Amantadine's cationic charge at physiologic pH interacted with the opposite charge of the phospholipid head group of the lipid bilayer and became incorporated into the membrane itself. These changes in surface charge of the lipid bilayer caused fluidity and mechanical bending properties to change in a manner that prevented vesicle release (Herrmann *et al.*, 1985). One could speculate that as the influenza A virus attaches to a cell surface receptor, it initiates internalization of the virus. However, the presence of amantadine on the lipid bilayer could prevent vesicle release, or subsequent fusion of virus lipid with that of the cell plasma membrane to release viral nucleic acids into the cytoplasm. Also, within the vesicle, the amantadine concentration could be sufficient to affect the influenza A M2 cation channel, preventing the uncoating of the virus (Wang *et al.*, 1994). Amantadine's inhibition of virus uncoating reduces viral replication has the beneficial side effect of reducing the production of the chemokine RANTES, thus decreasing airway inflammation and minimizing exacerbation of bronchial asthma (Asai *et al.*, 2001).

Amantadine's exact mechanism of action in Parkinson's disease remains undefined. Schwab *et al.*, were the first to report the beneficial effects of amantadine in Parkinson's disease (Schwab *et al.*, 1969). Bailey & Stone's review of initial studies on the mechanism of action of amantadine in Parkinson's disease suggests that the drug causes dopamine release or direct stimulation of dopamine receptors. They also reported that dopamine re-uptake inhibition by amantadine was far too weak to contribute substantially to amantadine's action in vivo (Bailey & Stone, 1975). The very weak re-

uptake inhibition by amantadine was demonstrated by Heikkila & Cohen, using neostriatum slices from male rat and rabbit (Heikkila & Cohen, 1972). The very weak re-uptake inhibition only occurred at concentrations (0.1 M) that would not be seen in vivo. Soon after Bailey and Stone's review (Bailey & Stone, 1975), evidence was published that suggested that amantadine did not have a direct effect on dopamine receptors. Brown and Redfern demonstrated, by measuring dopamine levels in the brains of male Sprague-Dawley rats, that amantadine had no effect on dopamine turnover. However, α-methyl-*p*-tyrosine methyl ester, a direct dopamine receptor agonist, altered dopamine levels. Based on these findings, they suggested that amantadine caused no significant activation of postsynaptic dopamine receptors (Brown & Redfern, 1976). Heikkila & Cohen predicted that amantadine did not act directly to release dopamine but rather acted synergistically to increase the amount liberated during the nerve impulse (Heikkila & Cohen, 1972). This interpretation is supported by the review of Tilley and Kramer which concluded the bulk of evidence suggested that amantadine facilitates the release of dopamine in response to nerve stimulation (Tilley & Kramer, 1981). However, Jackisch *et al.* demonstrated, using male rabbit caudate nucleus, that basal and evoked enhancement of dopaminergic transmission occurred only at concentrations of amantadine (> 50 μM) higher than those seen *in vivo* so far. They suggested that amantadine's anti-Parkinson effect was unrelated to enhancement of dopaminergic transmission, but instead was probably related to amantadine's inhibitory effect on striatal N-methyl-D-aspartate (NMDA) receptor mediated acetylcholine release (Jackisch *et al.*, 1992). This mechanism was demonstrated by Stoof *et al.*, using male Wistar rat brain neostriatum; they found that amantadine inhibited the NMDA evoked release of

acetylcholine from this tissue in a non-competitive manner. They found the concentration of amantadine (3 μM) required to inhibit NMDA-evoked release of acetylcholine was approximately 100 times lower then that required to cause release of dopamine (Stoof *et al.*, 1992). However, Matsubayashi *et al.*, using hippocampal cultured neurons from Sprague-Dawley rat fetuses, demonstrated that amantadine (10 μM) was a potent non-competitive inhibitor of nicotinic acetylcholine mediated receptor function. They suggested that nicotinic receptors in the nigrostriatal pathway have the potential to affect the symptoms of Parkinson's disease (Matsubayashi *et al.*, 1997). Based on the studies of Matsubayashi *et al.*, and Stoof *et al.*, one could suggest that both mechanisms may be working in conjunction to alleviate Parkinson's disease symptoms.

1.1.3 Experimental Therapeutic Uses

Currently, new therapeutic uses for amantadine are being explored based on its ability to cause NMDA receptor inhibition and its lysosomotropism. Recently, amantadine has been shown to reduce neuropathic pain. Pud *et al.*, in a double blind controlled trial, demonstrated that amantadine relieved surgical neuropathic pain in patients with cancer. Patients reported a 40% reduction in pain when compared with placebo. They suggest that amantadine can effectively and safely reduce spontaneous and possibly evoked neuropathic pain (Pud *et al.*, 1998). Amantadine has been used in treating cognitive and neurobehavioral problems following brain injury. Nickels *et al.*, in a retrospective study, reported that amantadine improved cognitive and behavioral outcomes. The most consistent areas of improvement included focused and sustained

attention, concentration, orientation, alertness, arousal, vocalization, psychomotor speed, mobility and increased participation in therapy. These results were all judged to be beneficial effects in the recovery of these subjects (Nickels *et al.*, 1994).

The lysosomotropic nature of amantadine has also been explored in the treatment of malaria. Evans & Havlik, using *Plasmodium falciparum* in an *in vitro* experiment, showed amantadine's potential to be an effective antimalarial drug. More importantly, it was more potent against strains resistant to antimalarial therapy than against sensitive strains. Amantadine's effect was also slightly synergistic with chloroquine. One of the likely mechanisms of action reported was the accumulation of amantadine (lysosomotropic) within the food vacuole of the parasite. Amantadine accumulation causes an increase in pH within the organelle, inhibiting enzymes that function at low pH optima. This effect blocks vacuolar digestion of macromolecules and of the host's haemoglobin, resulting in the starvation of the parasite (Evans & Havlik, 1993).

1.1.4 Absorption and distribution

Amantadine is available only in oral formulations in North America, and its absorption is relatively complete, varying from 55 to 90%, depending upon age and dose administered (Aoki & Sitar, 1988). Amantadine's time to peak plasma concentration ranges between 1 and 7 hours after ingestion, and is dependent on age. It is more rapidly absorbed in healthy young adults than in the elderly (Aoki & Sitar, 1988). Amantadine's apparent volume of distribution exceeds body volume, suggesting that it is sequestered in tissue. Bleidner *et al.*, examined different tissues, and reported higher amantadine

concentrations in mouse lung and heart than in blood (Bleidner *et al.*, 1965). Uchiyama and Shibuya, using tritiated amantadine, also showed increased drug distribution to heart, lung, liver, kidney and spleen in male mice (Uchiyama & Shibuya, 1969). The data from both of these studies were reanalyzed in a manner such that they could be compared, and showed that the highest concentrations of amantadine were in the lung and kidney, followed by the liver (Aoki & Sitar, 1988). They also reported that the liver represented the most important organ for the sequestration of amantadine, due to its mass (Aoki & Sitar, 1988). Amantadine distributes to the central nervous system where concentrations are higher then in the circulation. Kornhuber *et al.*, using postmortem brains of patients on therapeutic doses of amantadine, measured amantadine concentrations in various brain regions. The concentrations appeared to be homogeneously distributed over the different anatomical regions and ranged from 48.2 to 386 μM. In contrast, the concentration of amantadine in the cerebrospinal and serum fluid was less then 17 μM. This concentration in brain tissue was speculated to occur because of accumulation of the drug in acidic intracellular compartments (Kornhuber *et al.*, 1995). This accumulation may or may not be relevant to the mechanisms of action discussed above.

Two *in vitro* studies provide some evidence of amantadine's intracellular distribution and its lysosomotropic nature. Using bovine chromaffin cells, Johnson *et al.*, demonstrated that tritiated amantadine, at concentrations that did not alter pH, accumulated inside these cells. The accumulation inside the cells paralleled the difference in pH between the chromaffin granule and the cytosol. The pH on the inside of the chromaffin cell was 5.5 compared with the cytosol pH 7.4. If amantadine distributed according to the difference in pH, an approximately 100 fold increase in

amantadine concentration within the chromaffin granules when compared to the cytosol is predicted (Johnson *et al.*, 1981). Richman *et al.*, also provided evidence that dog kidney cells accumulated amantadine in the cytosol and lysosomes, and that this concentration was more then 100 fold higher than the extracellular medium (Richman *et al.*, 1981). Both of these studies suggest that amantadine can easily cross the plasma membranes of cells, and Johnson *et al.*, suggested that permeation by amantadine occurs in uncharged form such that at equilibrium its distribution approaches the magnitude of the difference in pH between cytosol and plasma (Johnson *et al.*, 1981). However, recent evidence demonstrated the presence of organic cationic transporters ubiquitously distributed in several human tissues, suggesting that they may contribute to the observed cellular permeation of amantadine in its charged state (Nishiwaki *et al.*, 1998; Zhang *et al.*, 1998).

1.1.5 Excretion

The clearance of amantadine by the kidneys has been well documented. Bleidner *et al.*, reported that an average of 86% ± 9% of an orally administered dose is recovered in the urine unchanged in a collection period that lasted 96 hr (Bleidner *et al.*, 1965). Aoki *et al.*, showed that healthy young men, 19 to 36 years of age on chronic amantadine dosing, excreted an average of 90 to 97% of an ingested dose in the urine. They reported an amantadine plasma half-life that ranged from 10.2 to 31.4 hr with a mean half-life of 14.7 hr. However, in healthy elderly men 60 to 76 years of age, although 88% of single doses was recovered in the urine, the period of collection was 120 hr. The plasma half-life ranged from 18.5 to 45 hr with a mean of 28.9 hr (Aoki *et al.*, 1979). These

observations indicated that renal clearance was greater in younger subjects and declined with increasing age. Similarly, with impaired renal function and in haemodialysis patients, the half-life of amantadine is greatly increased. Horadam *et al.*, demonstrated that the half-life of amantadine depended on the degree of renal impairment. They showed that in patients with renal insufficiency and a creatinine clearance between 43.1 to 5.9 ml/min/1.73 m^2, the half-life of amantadine ranged from 18.5 hr to 33.8 days, respectively. In patients on chronic haemodialysis, the amantadine half-life was 8.3 days (range 7 to 10.3) (Horadam *et al.*, 1981). In all these reports not all of the administered amantadine is recovered, and the variation in the amount recovered can be quite wide. One could suspect that complete absorption is not occurring and that amantadine is being excreted in the stools. However, Wu *et al.*, reported that in patients with various degrees of renal failure and in controls, the total amount of amantadine recovered from stools during the first 72 hr was always less then 1mg (less then 1% of administered dose) (Wu *et al.*, 1982). Alternatively, amantadine could be undergoing metabolism, and thus contributing to the variation seen in the amount collected unchanged after its administration. From the late 1960s the consensus was that amantadine was not metabolized in humans. Therefore, these variations were not suspected to occur because of metabolism. These researchers were therefore not looking for amantadine metabolites that could account for most of the observed variation.

1.1.6 Metabolism

The first report that addressed amantadine metabolism was by Bleidner *et al.*. They stated that there was no evidence of acetylated or methylated forms of amantadine in human urine or other extraneous peaks that could be attributed to metabolites of the drug (Bleidner *et al.*, 1965). This view was accepted and formed the basis of opinion concerning amantadine metabolism and the assumption that incomplete oral absorption could account for some of the unrecovered dose.

Koppel and Tenczer, provided the first evidence for metabolic disposition of amantadine in humans. These researchers reported that 5 to 15% of the administered dose was recovered in the urine as acetylamantadine. Furthermore, they reported the existence of other minor metabolites, but these were not quantified (Fig I-1). They suggested that other minor metabolic pathways may be involved in N-methylation, formation of Schiff bases and N-formiates. There was no evidence for oxidation of the adamantane ring (Koppel & Tenczer, 1985). This observation was extended by Sitar *et al.*, when they reported that acetylation of amantadine was not correlated with the NAT2 acetylator phenotype. This observation suggested that NAT2 was not the acetyltransferase enzyme that catalyzed this conjugation reaction (Sitar *et al.*, 1991).

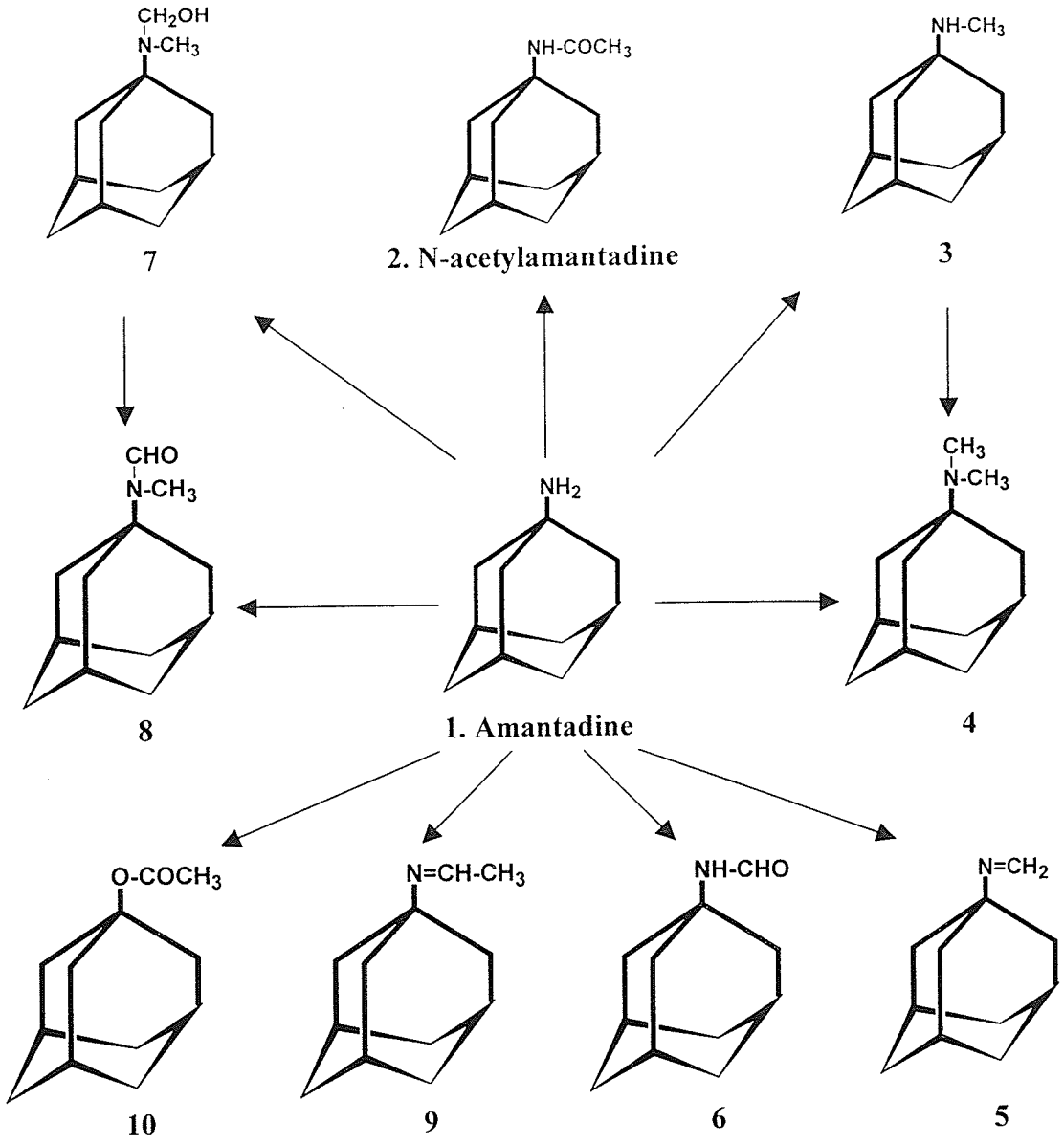


Figure I-1. Adapted from Köppel and Tenczer (1985), representing the metabolic disposition of amantadine. 1. Amantadine 2. **Acetylamantadine** (major metabolite) 3. N-methylamantadine 4. N,N[1]-dimethylamantadine 5. N-methyleneamantadine 6. N-formylamantadine 7.and 8. possible stepwise oxidation of N-dimethylamantadine (4) 9. N-ethylideneamantadine 10. 1-adamantol acetate.

1.2 ENZYMES

Many biological functions depend on complex proteins known as enzymes. Mammalian enzymes possess a high degree of specificity for their substrates and function in aqueous conditions with mild temperatures and pH (Lehninger, 1982). One of the enzyme's most important facets is its ability to catalyze chemical reactions at extremely high rates (Alberts *et al.*, 1994). The enzymes accomplish these high rates of reaction by holding the specific substrate in an orientation that facilitates the subsequent reaction. This function was first recognized by Linus Pauling, who suggested that a substrate molecule is attracted to the enzyme, and caused by the forces of attraction to assume the strained state that favors the chemical reaction. That is, the activation energy of the reaction is decreased by the enzyme (Pauling, 1946). Therefore, the substrate passes through a transition state where its geometry and electron distribution are altered by the enzyme, thereby lowering the activation energy required for a reaction to proceed (Alberts *et al.*, 1994). This process is also known as transition state theory. However, a second theory has emerged which suggests that the pathway from reactants to products might not need to pass over the potential energy barrier but could pass through it by quantum tunneling (Sutcliffe & Scrutton, 2000). Recent experimental findings provide support for this alternate theory, at least for some enzymes (Sutcliffe & Scrutton, 2000; Basran *et al.*, 2000).

Biologically there are more than 3000 known enzymes that mediate specific chemical reactions (Koeller & Wong, 2001; Lehninger, 1982). This thesis, will focus on acetylation reactions carried out by acetyltransferases. Here too, there are different acetyltransferases such as histone acetyltransferase (Kuo & Allis, 1998), choline

acetyltransferase (Cronin, 1998), carnitine acetyltransferase (Cronin, 1998), serotonin N-acetyltransferase (Klein *et al.*, 1992), N-acetyltransferase-1, 2 and 3 (Hein *et al.*, 2000), and spermidine/spermine N^1-acetyltransferase (Casero & Pegg, 1993), spermidine N^8-acetyltransferase (also known as histone acetyltransferase) (Desiderio *et al.*, 1993) and many others. This thesis will focus on only three amino acetyltransferases: N-acetyltraferase 1 and 2 (NAT1 and NAT2), and spermidine/spermine N^1-acetyltransferase (SSAT). NAT1 and NAT2 are characteristically involved in the metabolism of xenobiotics. The substrates for NAT1 and NAT2 include arylamines, hydrazines, and other primary amines. SSAT maintains polyamine homeostasis within cells. Each of these enzyme systems will be individually discussed below.

1.3 N-ACETYLTRANFERASES 1 and 2

1.3.1 Discovery

The initial discovery of an amino acetyltransferase enzyme arose from an interest in its role in metabolizing the hydrazine containing compound, isoniazid (INH). INH was first synthesized in 1912 by two Prague chemists, Hans Meyer and Josef Mally, to satisfy requirements for their doctorates in chemistry (Ryan, 1992). However, they did not realize the anti-tubercular properties that their drug possessed, and it took another 34 years before isoniazid was resynthesized by the chemist Robert Behnisch and its properties evaluated by Gerhard Domagk in Elberfeld, Germany (Evans, 1989; Ryan, 1992). Clinical trials were carried out to evaluate the antitubercular properties of INH

(Contenben) by Gerhard Domagk (from Bayer) and collegues throughout Germany, but these results were not published. The Roche Chemical Co. was also investigating and synthesizing new antitubercular drugs. Its testing of INH (Rimifon) at Sea View Hospital in New York led to the first publication by Robitzek and Selikoff of the dramatic efficacy of the drug (Robitzek & Selikoff, 1952). As more patients were included in clinical trials interindividual differences in toxicity of INH became apparent. Hughes *et al.*, showed an association between the occurrence of peripheral neuropathies and impairment of INH acetylation. The patients who excreted the least amount of acetyl INH and the most unchanged isoniazid were noted to have the highest incidence of peripheral neuritis (Hughes *et al.*, 1954).

Biehl showed a bimodal frequency distribution histogram of the percentage of the INH dose excreted in urine (Biehl, 1957). Following this report, Mitchell and Bell proposed that, based on variations of isoniazid metabolism, patients could be classified into slow, rapid and intermediate inactivators based on their INH plasma levels. Those with low plasma levels would be classified as rapid inactivators and those with high plasma levels would be classified as slow inactivators (Mitchell & Bell, 1957). Further studies revealed that INH acetylation was under genetic control (Price Evans *et al.*, 1960). They showed that slow inactivation was an autosomal recessive trait and that rapid inactivators can be heterozygous or homozygous dominants. Studies also confirmed the existence of inter-ethnic acetylator phenotype differences. Differences in the proportion of rapid and slow acetylator phenotypes varied based on the ethnic and geographic origin (Evans, 1989). The terms slow and fast inactivators were changed to slow and fast acetylators. In North America and Europe slow acetylators make up

between 40 to 70 % of the population, whereas the Pacific Asia littoral (Japanese, Chinese, Korean, Thai etc) populations have only 10 to 30% of slow acetylators (Meyer & Zanger, 1997). The Inuit as a population are different in that more then 90% are fast acetyltors, and they are also unique in that more than 46% are homozygous fast acetylators compared to only 5% in the Caucasian population (Kalow, 1982).

These early studies clearly showed that the bimodal distribution of isoniazid was caused by a difference in the activity of the cytosolic N-acetyltransferase. Experiments in rabbits showed bimodal frequency distribution histograms of the half-lives of sulfadiazine and INH; however *p*-amino-salicylic acid (PAS) and *p*-aminobenzoic acid (PABA) frequency distributions for acetylation were unimodal (Frymoyer & Jacox, 1963; Weber *et al.*, 1976). Based on these observations, simple arylamines such as PAS and PABA were termed monomorphic. In contrast sulfadiazine, sulfamethazine (SMZ), INH and others were termed polymorphic. Jenne, using human liver tissue from fast and slow acetylators, was the first to suggest that two acetylation pathways (2 different enzymes) existed in man. These two enzymes were responsible for the metabolism of the monomorphic and polymorphic substrates. Jenne also suggested that the differences in activity of these two groups (fast and slow acetylators) were due to differences in the amount of enzyme present in slow acetylators (Jenne, 1965). Subsequently,Grant *et al.*, showed that the slow acetylator phenotype was caused by decreased or absent NAT in human liver. Using phenotyped human liver from surgical patients, they were able to show that slow acetylation both in vivo and in vitro was the result of a decrease in the quantity of immunodetectable NAT protein relative to that seen from rapid acetylators (Grant *et al.*, 1990).

Although, Jenne had suggested the concept of two distinct enzymes that were responsible for substrate specificity, the genetic basis for the two enzymes, N-acetyltransferase-1 (NAT1) (monomorphic, now known to be polymorphic) and N-acetyltransferase-2 (NAT2) (polymorphic), was not discovered until much later. Hein *et al.*, confirmed the concept of two distinct enzymes. By using inbred Syrian hamsters of each acetylator genotype, they were able to identify two distinct enzymes; one exhibiting polymorphic activity and the second showing apparently monomorphic activity (Hein *et al.*, 1985). Subsequently, Grant *et al.*, separated NAT1 and NAT2 by anion-exchange chromatography from human livers. NAT1 and NAT2 were shown to be functionally distinguishable by their different apparent affinities for SMZ. However, antibodies raised against NAT1 recognized both enzymes, showing antigenic relatedness, and inability to distinguish between the proteins (Grant *et al.*, 1989). Definitive proof was soon to follow that showed the two distinct proteins were coded for by two different genes. Blum *et al.*, cloned three human NAT genes from human leukocyte DNA. The genes designated *NAT1* and *NAT2* were transfected into monkey kidney cell COS-1 and transiently expressed. The *NAT1* and *NAT2* genes gave rise to functional NAT proteins, as determined by their activity toward the arylamine substrates. The third gene yielded a non-functional protein and was labeled pseudo N-acetyltransferase (NATP) (Blum *et al.*, 1990). Furthermore, Grant *et al.*, using the same process as Blum *et al.*, compared the expressed products of the two cloned genes, NAT1 and NAT2, with native human liver N-acetyltransferases. They showed that both native and expressed NAT1 and NAT2 proteins had the same characteristic selectivity for their respective substrates, PABA and

SMZ respectively (Grant *et al.*, 1991). These results also provided strong evidence that the NAT2 gene is a locus for acetylation polymorphism.

NAT1 enzyme was initially described as being monomorphic because it metabolized PABA, PAS, and other arylamines that phenotypically displayed a monomodal distribution in the population (Evans, 1989; Grant *et al.*, 1991; Jenne, 1965). Recent evidence showed that human NAT1 is polymorphic. Grant *et al.*, using phenotyped human livers, demonstrated variation in NAT1 content and activity among individuals (Grant *et al.*, 1991). This observation was further extended by Cribb *et al.*, when they showed a 7-fold variation in activity among human individuals using the cytosolic fraction of mononuclear leukocytes, of unknown phenotype (Cribb *et al.*, 1991). The results of Vatsis and Weber, gave evidence that the Caucasian NAT1 gene locus was polymorphic. They compared the NAT1 gene sequence from 13 Caucasian individuals with established phenotype and NAT2 genotype and demonstrated structural variants among the individuals (Vatsis & Weber, 1993). The functional significance of these variants remains unknown, however the results show that the NAT1 gene has a polymorphic locus.

1.3.2 Molecular Genetics of the N-acetyltransferase Enzymes

As a generalization, the human hepatic arylamine N-acetyltransferase enzyme phenotypically divides populations into fast and slow acetylators. Blum *et al.*, were the first to localize and clone the human NAT1 and NAT2 genes. The NAT1 and NAT2 genes have an intronless coding exon with an open reading frame of 870 bp (Blum *et al.*,

1990). The NAT1 gene produces the entire transcript from a single exon. In contrast the NAT2 gene is separated into two exons. The NAT2 gene mRNA is derived from both the protein coding exon and a second noncoding exon of 100 bp located about 8 kb uptream of the translation start site (Blum *et al.*, 1990; Ebisawa & Deguchi, 1991). The 870 bp encode for a 290 amino acid protein, with NAT1 having an apparent molecular mass of 33 kDa and NAT2 31 kDa (Grant *et al.*, 1991). Grant *et al.*, found under their experimental conditions that human NAT2 enzyme was more heat stable at 37°C with a $t_{1/2} = 61$ hr. In contrast the human NAT1 $t_{1/2} = 3.5$ hr under the same conditions (Grant *et al.*, 1991).

Blum *et al.*, also cloned a third sequence and designated it NATP. The clone showed insertions, deletions and detrimental mutations in the sequence range and therefore was considered as a pseudogene. The NAT1, NAT2 and NATP genes were localized to chromosome 8p21.3-23.1 (Blum *et al.*, 1990; Hickman *et al.*, 1994; Thygesen *et al.*, 1999). The NAT1 and NAT2 protein coding regions are separated by approximately 30 kilobases (Grant *et al.*, 1991). The three genes were found to be highly homologous. The nucleotide homology between NAT1 and NAT2 is 87% in the coding region, and the homology between these two genes and NATP is 79 and 80% respectively (Blum *et al.*, 1990).

The polymorphic NAT2 gene has many allelic variants; currently 27 alleles have been described (Table 1) (Grant *et al.*, 2000). This allelic variation at the NAT2 gene locus is responsible for the classical phenotypic acetylation polymorphism observed in the population for drugs such as SMZ and INH (Grant *et al.*, 2000). The *NAT2*4* allele is considered the wildtype because it lacks the substitutions in its coding region that have

been identified to be associated with slow acetylator phenotype (Hein *et al.*, 2000). The *NAT2*4* wild type allele is associated with the fast acetylator phenotype and its occurrence depends on the ethinic group (Grant *et al.*, 1997). In most ethnic groups this allele is not the most common one (Hein *et al.*, 2000). For example, 83% of Egyptians are slow acetylators and this figure rises to 90% in the Morrocan population (Weber & Hein, 1985). In contrast, Europeans are divided approximately equally between fast and slow acetylator phenotypes, but among the Inuit, 95% are fast acetylators (Weber & Hein, 1985). Even though there are a large number of allelic variants, many of them have mutations in common (Grant *et al.*, 2000). A total of 11 sites within the NAT2 gene coding exon region have been identified to produce the observed multitude of alleles (Grant *et al.*, 2000). Seven are missense and four are silent substitutions that have been identified (Hein *et al.*, 2000). The four most common alleles that impart the slow acetylator phenotype are *NAT2*5*, *NAT2*6*, *NAT2*7* and *NAT2*14* (Table 1). The resulting amino acid substitutions in the NAT2 coding sequence occur at nucleotide positions 341 ($Ile^{114}\rightarrow Thr$), 590 ($Arg^{197}\rightarrow Gln$), 857 ($Gly^{268}\rightarrow Glu$), and 191 ($Arg^{64}\rightarrow Gln$) (Grant *et al.*, 2000). These mutations usually result in the production of defective NAT2 enzyme or decreased levels. Grant *et al.*, showed using phenotyped human livers that slow acetylation both in vitro and in vivo was associated with decreased levels of immunodetectable NAT protein (Grant *et al.*, 1990). Using bacterial expression systems, Hein *et al.*, expressed 15 NAT2 allelic variants with nucleic acid substitutions at positions 191, 341, and 590 either alone or in combination that resulted in NAT2 enzyme with significant reductions in acetylation activity. Furthermore, they showed that some of the NAT2 allelic variant enzymes showed intrinsic instability when incubated at 37°C

when compared to the wildtype (Grant *et al.*, 1990). Therefore these allelic variations could cause the slow acetylator phenotype observed in the population.

NAT1, long considered monomorphic, with ubiquitous tissue distribution in humans and kinetic selectivity for compounds such as PABA and PAS (Evans, 1989; Pacifici *et al.*, 1986), was revealed to be coded for by a separate gene (Blum *et al.*, 1990; Grant *et al.*, 1991). *In vitro* studies revealed that NAT1 expression may be variable in the human population, but the variation seen in acetylation of NAT1 selective substrates was thought to be unrelated to classical acetylation polymorphism (Cribb *et al.*, 1991; Grant *et al.*, 1991). Vatsis and Weber, using liver and leukocyte DNA from subjects with established acetylator phenotype and genotype, demonstrated that NAT1 had a polymorphic locus. They also were the first to describe the NAT1 wild type gene, known as the allele *NAT1*4* (Vatsis & Weber, 1993). The *NAT1*4* allele codes for a region of 870 nucleotides, and all other allelic variants are referenced to the wild type nucleotide sequence. The polymorphic NAT1 gene has many allelic variants, and currently 26 have been described (Table 2). The allelic variants observed and their attendant nucleotide variations tend to occur in non-coding regions, especially the 3´ untranslated region (Grant *et al.*, 2000). The most common allele in the populations that have been examined is the wild type *NAT1*4* (Hein *et al.*, 2000). Interethnic variability in allelic frequencies has been observed. Probst-Hensch *et al.*, observed that the *NAT1*10* frequency was highest among Asians (50%) and African Americans (42.6%) and lowest in Caucasians (20.9%) and Latino (30.5%) in a southern California population (Probst-Hensch *et al.*, 1996). The correlation of *NAT1*10* and *NAT1*11* genotype and phenotype has been

ambigous. The contribution of these allelic variants to acetylation remains contraversial. Below is a brief overview of the literature that discusses these issues.

Bell *et al.*, using bladder and colon tissue, showed that the *NAT1*10* allelic variants (heterozygotes) had a 2 fold greater activity compared to *NAT1*4* wild type (homozygotes) (Bell *et al.*, 1995). This situation was also observed by Yang *et al.* In Japanese female subjects, they found that *NAT1*4* homozygotes had slower activity than *NAT1*10* heterozygotes (Yang *et al.*, 2000). Both of these studies support the conclusion that the *NAT1*10* allelic variant is a rapid phenotype having higher activity then the wild type allele. However, other studies do not support this viewpoint. Bruhn *et al.*, using venous blood from healthy German male volunteers who were both genoptyped and phenotyped, found no trend toward increase in activity from NAT1*4/*4 (homozygotes) to *NAT1*4/*10* (heterozygotes) and *NAT1*10/*10* (homozygotes)(Bruhn *et al.*, 1999). Hughes *et al.*, using blood and urine from volunteers phenotyped using the NAT1 selective substrate PAS, found that there was no increase NAT1 activity in individuals who were genotyped *NAT1*10* (Hughes *et al.*, 1998). Bruhn *et al.*, suggested that the studies that found increased activity did not have sufficient power or that the samples sizes used were variable, compared to the relatively homogeneous blood samples used by their own laboratory and by Hughes *et al* (Bruhn *et al.*, 1999; Hughes *et al.*, 1998). Bruhn *et al.*, could not rule out variability of *NAT1*10* expression in different tissues due to differences in transcription factors or RNA-degrading enzymes (Bruhn *et al.*, 1999). More recently Vaziri *et al.*, found no differences in NAT1 activity between subjects with the *NAT1*4* (wild type) and NAT1*10 allelic variant (Vaziri *et al.*, 2001).

Other NAT1 allelic variants that occur at low frequencies in the population have significant effects on their phenotypic expression. The three allelic variants are *NAT1*11*, *NAT1*14* and *NAT1*15*, which occur at a frequency of 0.021, 0.028 and 0.014 respectively in a sample of mixed ethnic origin from the Toronto population (Hughes *et al.*, 1998). In a Caucasian population the frequency is low but significant, and ranges from 0.01 to 0.03 (Grant *et al.*, 1997). The *NAT1*11* allelic variant was first described by Vatsis and Weber, and has an amino acid change from Ser^{214}→Ala (Vatsis & Weber, 1993). Hughes *et al.*, observed that individuals that were heterozygous for the *NAT1*11* allelic variant were phenotypically similar to homozygous wildtype and that the expressed allelic variant behaved similarly to the wild-type enzyme. They also suggested that *NAT1*11* variant probably only produced a minor effect on the expressed enzyme activity (Hughes *et al.*, 1998). Bruhn *et al.*, however, demonstrated using human blood cells (described above) that when they compared the homozygous wild type *NAT1*4/*4* with the activity of homozygous *NAT1*11/*11*, and hetorozygous *NAT1*11/*10* and *NAT1*11/*4* genotypes, the activity was reduced by 20.7%, 35.7% and 31.5% respectively (Bruhn *et al.*, 1999). Hughes *et al.*, using urine and blood ratios of acetyl-PAS:PAS, that genotyped individuals heterozygous for *NAT1*14* or *NAT1*15* alleles, had four-fold lower ratios when compared to homozygous *NAT1*4* wild type subjects (Hughes *et al.*, 1998). Bruhn *et al.*, confirmed these results showing that *NAT1*10/*14* and *NAT1*4/*14* heterozygotes resulted in a reduction of 49.5% and 55.6% in activity when compared to the homozygoes wild type (Bruhn *et al.*, 1999). The *NAT1*14* allele contains 1 point mutation that results in an amino acid change Arg^{187}→Gln and 2 mutations in the 3´ non-coding region (Hughes *et al.*, 1998). The *NAT1*15* contains a

single point mutation producing a premature stop codon ($Arg^{187}\rightarrow$Stop) with an expressed protein of only 23 kDa (Grant *et al.*, 1997; Hughes *et al.*, 1998). NAT1*19 allele also produces a truncated protein, which is also devoid of NAT1 activity (Lin *et al.*, 1998). The heterozygous combination of *NAT1*14* and *NAT1*15* produces a phenotype with dramatically reduced activity (Hughes *et al.*, 1998). The homozygous *NAT1*15* allele is devoid of any detectable enzyme activity as expected since the protein is truncated, producing an incomplete enzyme (Bruhn *et al.*, 1999; Hughes *et al.*, 1998). The allelic variant *NAT1*3* confers the highest enzymatic activity in homozygous *NAT1*3/*3* with a frequency of 3.03% in German subjects (Bruhn *et al.*, 1999). The allelic variation observed above controls the expression of the NAT1 acetylation phenotype. Individuals that are heterozygous and have a recessive allele could display a decrease in activity phenotypically toward a selective substrate. However, although one allele may be recessive, *in vivo* the second allele may code for a high activity NAT1 variant that may be able to compensate for the loss of function of the other allele.

1.3.3 Structure-Function of NAT1 and NAT2

The acetyl-Coenzyme A dependent N-acetyltransferases, NAT1 and NAT2, transfer the acetyl moiety to compounds bearing primary aromatic amino, hydroxylamine and hydrazino functional groups (Dupret *et al.*, 1994; Hein *et al.*, 2000). The sequencing of the *NAT1* and *NAT2* genes by Blum *et al.*, revealed that 2 different genes encoded for the two enzymes, but also showed that the human wild-type genes *NAT1*4* and *NAT2*4* share 87% genomic sequence (Blum *et al.*, 1990). The 290 amino acid protein sequence

of the enzymes differ by only 55 amino acids, and of these only 28 are non-conservative changes, in other words they are 81% identical (Dupret *et al.*, 1994). The difference in amino acids between the NAT enzymes imparts the characteristic patterns of selectivity observed between NAT1 and NAT2 for arylamine substrates.

To identify the relevant linear amino acid sequences that impart enzyme selectivity and stability Dupret *et al.*, constructed a panel of NAT1 and NAT2 chimeric genes which were expressed in *Escherichia coli.* Using selective substrates PAS and SMZ for NAT1 and NAT2 respectively, they determined the kinetic characteristics of the mutant proteins on catalytic function and compared them to wild type NAT enzymes. Distinct amino acid sequences imparted significant differences in both K_m and V_{max}. The enzymes with amino acid sequence 112-210 in the central region derived from *NAT1*4* (wild type) possessed low apparent K_m for PAS. However, those enzymes with central regions derived from NAT2 had high apparent K_m for PAS (Dupret *et al.*, 1994). This central region of NAT1 is associated with high V_{max} values for both PAS and SMZ; therefore this central region plays an important role in imparting substrate selectivity for NAT1 and NAT2 (Dupret *et al.*, 1994). For NAT2, the sequence from amino acid 211-255 is required for contributing to low apparent K_m of SMZ. The active site region that encompasses the amino acid sequence from 47-111, seems to play a more important role for determining PAS affinity for NAT1 than for determining sulfamethazine affinity for NAT2 (Dupret *et al.*, 1994). The region that correlated with imparting intrinsic stability to the NAT2 enzyme was from amino acids 210-250, increasing the half life of the enzyme several fold when compared to the NAT1 enzyme without this sequence. NAT1

enzymes with this region derived from NAT2 had dramatically increased half-lives, 25 ±15 hr compared to 1.8 ± 2.7 hr; $p = 0.0001$ (Dupret *et al.*, 1994).

Studies using chimera construction and site directed mutagenesis by Goodfellow *et al.*, identified three amino acid residues at positions 125, 127, 129 from the central region (112-210) that may confer substrate selectivity to NAT1 and NAT2. The NAT1*4 (wild type) at positions 125, 127, and 129 encodes for phenylalanine, arginine and tyrosine respectively, whereas NAT2*4 encodes for serine in all three positions. Modification at position 127 where arginine is substituted for serine produced a 42-fold decrease in NAT1 affinity for PAS. The replacement at position 125 of serine for phenylalanine caused a two fold decrease in SMZ affinity for NAT2. The substitution of residue 129 (tyrosine) in NAT1 is permissive with respect to PAS acetylation, but is required for SMZ acetylation. The removal of either arginine (127) or tyrosine (129) decreases the stability of NAT1. These three amino acid residues in a three dimensional protein structure of NAT1 and NAT2, may contribute to the formation of the active site crypt surrounding the catalytic residue cysteine at position 68 (see below), and determine substrate selectivity (Goodfellow *et al.*, 2000).

Acetyl-CoA is the essential acetyl donor required in N-acetylation by NATs. This enzymatic reaction involves a two step classical ping-pong Bi-Bi mechanism that involves the acetylation of the enzyme followed by the release of CoA. The enzyme is then ready to mediate the transfer of the acetyl group to the acceptor substrate (Weber & Cohen, 1967). This insight remained as the paradigm in the explanation of NATs catalytic activity. Structure-function studies have recently allowed the identification of the active site residue that participates in the catalytic mechanism of NATs, refining our

understanding of the acetylation process. Dupret and Grant, using site directed mutagenesis of recombinant human NAT2 gene, determined which of 3 highly conserved cysteine residues was participating in the enzyme's catalytic mechanism. The recombinant genes were expressed in *Escherichia coli*. Three highly conserved residues of cysteine at positions 44, 68, and 223 on the 290 amino acid protein, were suspected to be important in the enzyme's catalytic mechanism. Substitution of cysteine with glycine at either position 44 or 223 produced enzymatically active proteins, but with reduced *in vitro* stability, suggesting these residues had a crucial role in the stability of the tertiary structure of the enzyme. Substitution at position 68 resulted in the production of normal quantities of immunoreactive NAT2 devoid of catalytic activity (Dupret & Grant, 1992). In studies using *Salmonella typhimurium*, Watanabe *et al.*, 1992, using site directed mutagenesis, indicated that the mutant O-acetyltransferase which contained alanine at position 69 instead of cysteine did not show any N-or O-acetyltransferase activity. These confirmatory results with the corresponding cysteine at position 69 suggest that this residue is essential for the enzyme's catalytic activity as the acetyl-CoA binding site (Watanabe *et al.*, 1992).

1.3.4 Arylamine N-Acetyltransferases in Metabolic Activation and Deactivation of Arylamines

NAT conjugation may deactivate arylamines and heterocyclic amines that produce non-toxic and stable acetates (Grant *et al.*, 1997). However, N-oxidation of arylamines by cytochrome P4501A2 or by peroxidase function of prostaglandin H synthase produces

hydroxyl and hydroxamic acids respectively, both more reactive species (Eling & Curtis, 1992; Grant *et al.*, 1997; Guengerich, 1992). These species can be further N-acetylated, O-acetylated, or undergo intramolecular N,O-acetyltransfer mediated by NATs, producing unstable acetoxy esters and N-acetyl-acetoxy esters which can decompose to nitrenium ions that can form adducts by binding to DNA or proteins (Grant *et al.*, 1997; Hein *et al.*, 1993; Minchin *et al.*, 1992). Human NAT1 and NAT2 polymorphisms influence both the extent of activation and deactivation of O-acetylation and N-acetylation of arylamine carcinogens, thus potentially altering the risk for a variety of disorders including cancer (Grant *et al.*, 2000; Hein *et al.*, 1993).

1.3.5 Epidemiological Studies

Epidemiological studies have examined the relationship between polymorphism of NAT1 and NAT2 in the predisposition to disease, especially cancer after exposure to aromatic amines (Evans, 1989; Hein *et al.*, 1994). The NAT2 slow acetylator phenotype has been associated with increased urinary bladder cancer risk (Cartwright *et al.*, 1982; Risch *et al.*, 1995). However, others have found no association between NAT2 phenotype and increased incidence of bladder cancer (Hayes *et al.*, 1993; Hirvonen *et al.*, 1994). More recently Taylor *et al.*, reported increased bladder cancer risk from smoking for those possessing both the NAT2 slow alleles in combination with one or two copies of the *NAT1*10* alleles (Taylor *et al.*, 1998). However, Vaziri *et al.*, showed that the *NAT1*10* allele did not occur more freqently in bladder cancer patients compared to controls (Vaziri *et al.*, 2001). This area of study remains controversial; the level and type of

exposure to xenobiotics may play a significant role in the development of bladder cancer, causing the inconsistencies observed in some of the studies (Grant *et al.*, 2000).

Epidemiological studies have also reported an association between colorectal cancer and NAT2 fast acetylator phenotypes when compared to slow acetylator phenotypes (Ilett *et al.*, 1987; Wohlleb *et al.*, 1990). This association has also been reported in situations where documented consumption of well-cooked meat that can contain significant levels of heterocyclic amines resulted in increased risk for colorectal cancer (Chen *et al.*, 1998; Kampman *et al.*, 1999).

Studies have examined the allelic variations of NAT1 and NAT2 and how they alter the risk for a variety of disorders. The epidemiological studies have focused on how NAT1 and NAT2 acetylation polymorphism modify the risk for breast cancer, lung cancer, head and neck cancer, and prostate cancer. The results of these studies have been inconsistent, and the associated risk has been relatively weak (Grant *et al.*, 2000; Hein *et al.*, 2000).

1.3.6 NAT1 Endogenous Substrate

NAT1 and NAT2 catalyze the acetylation of arylamines and heterocyclic amine xenobiotics, but until recently no candidate endogenous substrate had been reported. The endogenous substrate, *p*-aminobenzoylglutamate is a catabolite of folic acid metabolism, formed when the C_9-N_{10} bond of folic acid is cleaved (Minchin, 1995). Using human recombinant NAT1 expressed in monkey kidney COS-1, Minchin demonstrated that *p*-aminobenzoylglutamate was acetylated by NAT1 with aan apparent K_m = 130 μM.

However, using the same procedures with NAT2, it was shown that *p*-aminobenzoylglutamate was not a substrate for that enzyme. In the same study, using the pro-monocytic cell-line, U937, which has a constitutive expression of NAT1 and little or no NAT2, the apparent Km = 333 μM was slightly higher (Minchin, 1995). Similarly, Ward *et al.*, using human recombinant NAT1 expressed in *Escherichia coli* also showed that *p*-aminobenzoylglutamate was a substrate of NAT1 with an apparent K_m value of 262 μM (Ward *et al.*, 1995). Both studies demonstrate a possible role of NAT1 in cellular folate metabolism.

1.3.7 Factors Influencing NAT Acetylation

It is generally accepted that NATs are constitutive enzymes, and no induction is necessary to enhance enzyme concentration before 100,000 x g tissue supernatant is used for *in vitro* experiments. However, studies have attempted to demonstrate induction under the influence of various treatments. du Souich and Courteau, were the first to demonstrate and increase in acetylation of sulfamethazine. Using male and female New Zealand white rabbits treated with Freund's adjuvant, these investigators demonstrated that rabbits excreted greater amounts (60%) of acetylsulfamethazine in their urine compared to controls (du Souich & Courteau, 1981). However, studies by Zidek *et al.*, using male and female random bred albino rats that were treated with Freund's adjuvant showed a different pattern of results. The *in vivo* studies demonstrated an increase in acetylsulphadimidine in the urine compared to controls. However, the *in vitro* experiments, using a cytosolic liver fraction demonstrated no increase in sulphadimidine

acetylation activity when compared to controls (Zidek *et al.*, 1977). This finding suggests that no induction occurred for the arylamine N-acetyltransferase enzymes in the liver when animals are treated with Freund's adjuvant.

Similarly, Reeves *et al.*, using male New Zealand white rabbits and injecting them with hydrocortisone, found that the treated rabbits excreted more acetylsulfamethazine in their urine compared to controls. In contrast, i*n vitro* experiments, using the liver as the source of NAT enzymes, found no increase in sulfamethazine acetylation activity when compared to controls. The increase observed *in vivo* could be attributed to an increase in the mass of the liver (69%) compared to controls (Reeves *et al.*, 1988).

However, Zaher and Svensson, using male Sprague-Dawley rats injected with glucocorticoids, demonstrated a modest induction of NAT activity using rat liver cytosolic fraction when compared to controls (Zaher & Svensson, 1994).

Interestingly, in male mice testosterone seems to modulate an increase in kidney NAT2 activity (Smolen *et al.*, 1993). Within the NAT2 gene, in the 5′-flanking region, one of the regulatory elements is a hormone response element, indicating that androgen regulation may occur in the kidney, although it does not apperar to occur in liver (Estrada-Rodgers *et al.*, 1998).

Ethanol has also been implicated in the increased acetylation of NAT2 selective substrates. Olsen and Morland, showed that ingestion of alcohol by humans had an acute effect in both fast and slow acetylators, causing an increase in the amount of acetylated sulphadimidine found in blood and urine compared to controls. The drug's apparent half-life was also decreased by 20% (Olsen & Morland, 1978). Similarly, isoniazid acetylation has been shown to increase and its apparent half-life to decrease in humans

who consume alcohol and in rats given ethanol in their diet (Lester, 1964; Thomas & Solomonraj, 1977). In these studies with ethanol, the increase in acetylated selective drug metabolites that are observed is never directly associated with increased levels of NAT protein. The associations between increased acetylation activity and the various treatment modalities have in general been relatively weak, with the exception of the hormone response element studies in the mouse kidney (Estrada-Rodgers *et al.*, 1998). However, one cannot rule out a different acetylation pathway that is induced in some form and causes the increased acetylation of the selective substrates in the above studies.

1.3.8 Differences in Animal Models

NAT activity is widely distributed in different species and shows a high degree of conservation among them. Nevertheless, NAT species variations occur in their ability to catalyze acetylation of arylamine, hydrazines, and heterocylic amines substrates. Species like the *Suncus murinus*, a mole like creature and the dog lack NAT enzymes. These various animal models have contributed greatly to the characterization of the NAT enzymes, not only from the point of view of the species differences, but also by providing insight into the human NATs.

Different animal models have been used in the characterization of NAT enzymes over the years. Interspecies variation in NAT enzyme affinity and capacity for xenobiotic substrates occur and have been reported in the literature (see Table 3). Differences between laboratories can occur because of differences in methodologies used to characterize the NAT enzymes. However, differences between species can occur due to

differences in expression of the enzyme or lack of expression. Genetic differences in the sequence of the NAT genes also occur.

The mouse model is unique; it is the only species that contains a third NAT gene (NAT3). Kelly and Sim, were the first to identify this particular characteristic. The NAT3 enzyme has catalytic activity towards 2-aminfluorene substrate, and 2 fold less activity toward anisidine. No enzymatic activity is detected towards SMZ and barely detectable activity with PABA. The mouse NAT2 enzyme is also peculiar in that its preferred selective substrate is PABA and for the NAT1 enzyme the SMZ substrate is preferred (Kelly & Sim, 1994). This is a reversal of what is commonly observed in humans where the NAT1 and NAT2 selective substrates are PABA and SMZ respectively. The reversal in substrate selectivity is plausible because mouse NAT2 amino acid sequence is 80% homologous with human NAT1, and only 74% with human NAT2 (Vatsis *et al.*, 1995). Acetylation polymorphism has been described in mouse and in inbred strains of fast and slow acetylator mice (Fretland *et al.*, 1997).

Similarly, acetylation polymorphism has also been demonstrated in inbred hamster (Hein *et al.*, 1985). Like the mouse, hamster NAT1 exhibits selectivity for catalyzing the acetylation of procainamide; however NAT2 catalyzes the acetylation of PABA and 2-aminoflourene (Wagner *et al.*, 1996). Large differences in acetylation capacity have been observed to occur with PABA and PAS but not with SMZ and procainamide substrates (Hein *et al.*, 1985).

In rats the NAT coding sequence is the same for Wistar, Sprague-Dawley and Fisher rats (Jones *et al.*, 1996). However, the only expressed arylamine N-acetyltransferase enzyme is NAT1, and it is capable of N-acetylation, O-acetylation, and

N,O-acetylation (Land *et al.*, 1993). The NAT2 gene does code for a functional protein when cloned and expressed in *Escherichia coli*. The properties are similar to hamster liver NAT2. Therefore in these rats the gene seems to be turned off and does not express the NAT2 enzyme (Jones *et al.*, 1996). However, Feng *et al.*, found that rats exhibit polymorphism in NAT2 capacity, yielding rapid and slow acetylator phenotypes. In rats the selective substrates for NAT1 and NAT2 are sulfamethazine (procainamide) and PABA respectively, the opposite selectivity of humans (Feng, *et al.*, 1997, Drobitch, *et al.*, 1998)

Certain species lack NATs and genes coding for the enzymes are undectable. The *Suncus murinus*, the mole like creature used in emesis research does not have genes that code for NATs (Nakura *et al.*, 1995). Similarly, dog and other canids lack the NAT genes, and therefore produce no detectable cytosolic NAT (Trepanier *et al.*, 1997).

The New Zealand white rabbits used in our experiments have been widely used as a model for the human acetylation polymorphism. The New Zealand white rabbit exhibits both *in vivo* and *in vitro* patterns of N-acetylation in slow acetylator rabbits that closely resemble that in humans. The NAT1 and NAT2 enzymes have a preference for the selective substrates PABA and SMZ, respectively, that is similar to humans. However, the slow acetylator phenotype rabbit model is caused by a deletion of the *NAT2* gene encoding the polymorphic liver acetyltransferase resulting in the absence of the enzyme (Blum *et al.*, 1989).

TABLE 1

Human NAT2 alleles

Allele	Nucleotide change	Amino acid change
NAT2*4	None	None
NAT2*5A	T341C,C481T	Ile^{114}→Thr
NAT2*5B	T341C,C481T,A803G	Ile^{114}→Thr, Lys^{268}→Arg
NAT2*5C	T342C,A803G	Ile^{114}→Thr, Lys^{268}→Arg
NAT2*5D	T341C	Ile^{114}→Thr
NAT2*5E	T341C,G590A	Ile^{114}→Thr,Arg^{197}→Gln
NAT2*5F	T341C,C481T,C758T,A803G	Ile^{114}→Thr, Lys^{268}→Arg
NAT2*6A	C282T,G590A	Arg^{197}→Gln
NAT2*6B	G590A	Arg^{197}→Gln
NAT2*6C	C282T,G590A,A803G	Arg^{197}→Gln, Lys^{268}→Arg
NAT2*6D	T111C,C282T,G590A	Arg^{197}→Gln
NAT2*7A	G857A	Gly^{286}→Glu
NAT2*7B	C282T,G857A	Gly^{286}→Glu
NAT2*12A	A803G	Lys^{268}→Arg
NAT2*12B	C282T,A803G	Lys^{268}→Arg
NAT2*12C	C481T,A803G	Lys^{268}→Arg
NAT2*13	C282T	None
NAT2*14A	G191A	Arg^{64}→Gln
NAT2*14B	G191A,C282T	Arg^{64}→Gln
NAT2*14C	G191A,T341C,C481T,A803G	Arg^{64}→Gln,Ile^{114}→Thr, Lys^{268}→Arg
NAT2*14D	G191A,C282T,G590A	Arg^{64}→Gln, Arg^{197}→Gln
NAT2*14E	G191A,A803G	Arg^{64}→Gln, Lys^{268}→Arg
NAT2*14F	G191A,T341C,A803G	Arg^{64}→Gln,Ile^{114}→Thr, Lys^{268}→Arg
NAT2*14G	G191A,C282T,A803G	Arg^{64}→Gln, Lys^{268}→Arg
NAT2*17	A434C	Gln^{145}→Pro
NAT2*18	A845C	Lys^{282}→Thr
NAT2*19	C190T	Arg^{64}→Trp

Adapted from Hein *et al.*(Hein *et al.*, 2000). and NAT nomenclature accessible on the websitewww.louisville.edu/medschool/pharmacology/NAT.html

TABLE 2

Human NAT1 alleles

Allele	Nucleotide change	Amino acid change
NAT1*3	C1095A	None
NAT1*4	None	None
NAT1*5	G350,351C,G497-499C, A884G,*976,*1105	Arg^{117}→Thr,Arg166→Thr, Glu^{167}→Gln
NAT1*10	T1088A,C1095A	None
NAT1*11	C-344T,A-40T,G445A,G459A,T640G,*9 between 1065-1090,C1095A	Val^{149}→Ile,Ser^{214}→Ala
NAT1*11B	C-344T,A-40T,G445A,G459A,T640G,*9 between 1065-1090,C1095A	Val^{149}→Ile,Ser^{214}→Ala
NAT1*11C	C-344T,A-40T,G445A,G459A, T640G,*9 between 1065-1090,C1095A	Ser^{214}→Ala
NAT1*14	AG560A,T1088A,C1095A	Arg^{187}→Gln
NAT1*14B	G560A	Arg^{187}→Gln
NAT1*15	C559T	Arg^{187}→Stop
NAT1*16	[AAA]immediately after 1091,C1095A	None
NAT1*17	C190T	Arg^{64}→Trp
NAT1*18A	*3 between 1064-1087,T1088A,C1095A	None
NAT1*18B	*3 between 1064-1091	None
NAT1*19	C97T	Arg^{33}→Stop
NAT1*20	T402C	None
NAT1*21	A613G	Met^{205}→Val
NAT1*22	A752T	Asp^{251}→Val
NAT1*23	T777C	None
NAT1*24	G781A	Glu^{261}→Lys
NAT1*25	A787G	Ile^{263}→Val
NAT1*26A	[TAA] insertion between 1066 and 1091, C1095A	None
NAT1*26B	[TAA] insertion between 1066 and 1091	None
NAT1*27	T21G,T777C	None
NAT1*28	[TAATAA] deletion between 1085 and 1090	None
NAT1*29	T1088A,C1095A,*1025	None

Adapted from Hein *et al.* (Hein *et al.*, 2000). and NAT nomenclature accessible on the website www.louisville.edu/medschool/pharmacology/NAT.html

TABLE 3. Animal models used to characterize NAT enzymes

	SPECIES, ORGAN	Acetyl-CoA (μM)	SUBSTRATE	Km (μM)	Vmax (nmol/min/mgprotein)	Reference
NAT1[a]	Rabbit, fast acetylator, liver	100	PABA	105 ± 21	9.34 ± 1.05	(Andres & Weber, 1986)
NAT1[a]	Rabbit, fast acetylator, liver	100	PAS	74 ± 16	5.02 ± 0.77	(Andres & Weber, 1986)
NAT2[a]	Rabbit, fast acetylator, liver	100	PA	67 ± 28	4.43 ± 0.77	(Andres & Weber, 1986)
NAT2[a]	Rabbit, fast Acetyltor, liver	100	SMZ	90 ± 19	4.78 ± 0.70	(Andres & Weber, 1986)
NAT1[a]	Human, liver	100	SMZ	118		(Grant *et al.*, 1989)
NAT2[a]	Human, liver	100	SMZ	62		(Grant *et al.*, 1989)
NAT2[a]	Rabbit, slow acetylator, liver	100	SMZ	182 & 154	0.29 & 0.2	(Blum *et al.*, 1989)
NAT1	*H. pylori*	500*	2-AF	1100 ± 80	2.34 ± 0.14	(Chung *et al.*, 1997)
NAT1	*H. pylori*	500*	PABA	920 ± 90	2.08 ± 0.16	(Chung *et al.*, 1997)
NAT1	Mononuclear leukocytes	100	PABA	13.2 ± 0.6	6.3 ± 0.7	(Cribb *et al.*, 1995)
NAT1	Mononuclear leukocytes	100	SMX	966 ± 54	0.21 ± 0.02	(Cribb *et al.*, 1995)
NAT1	Human placenta	100	PABA	19.1 ± 0.97	3.84 ± 0.32	(Derewlany *et al.*, 1994)
NAT2	Human placenta	100	SMZ	3623 ± 331	0.148 ± 0.012	(Derewlany *et al.*, 1994)
NAT1	HR NAT in *E. coli*	100	SMZ	3500	360	(Dupret & Grant, 1992)
NAT1	HR NAT in *E. coli*	100	PABA	13	6050	(Dupret & Grant, 1992)
NAT2	HR NAT in *E. coli*	100	SMZ	116	11.5	(Dupret & Grant, 1992)
NAT2	HR NAT in *E. coli*	100	PABA	0	0	(Dupret & Grant, 1992)
NAT1	HR NAT in *E. coli*	100	PAS	11	3400	(Dupret & Grant, 1992)
NAT2	HR NAT in *E. coli*	100	PAS	6100	3.5	(Dupret & Grant, 1992)

a. Assumed to be NAT1 and NAT2 enzymes according to substrates used
HR NAT stands for human recombinant N-acetyltransferase
* Acetyl-CoA regenerating system not used

TABLE 3 (Continued)

	SPECIES, ORGAN	Acetyl-CoA (μM)	SUBSTRATE	Km (μM)	Vmax (nmol/min/mgprotein)	Reference
NAT1	Human liver	100	SMZ	1530	0.42	(Grant *et al.*, 1991)
NAT1	Human liver	100	PABA	12	13.85	(Grant *et al.*, 1991)
NAT2a	Human liver	100	SMZ	117	5.50	(Grant *et al.*, 1991)
NAT2a	Human liver	100	PABA	0	0	(Grant *et al.*, 1991)
NAT1	Fischer rat, inbred, liver	2000*	PABA	97 ± 7	17.9 ± 4	(Hein *et al.*, 1991)
NAT1	Fischer rat, inbred, liver	2000*	2-AF	195 ± 32	2.14 ± 0.22	(Hein *et al.*, 1991)
NAT1	WKY rat, inbred, liver	2000*	PABA	132 ± 8	4.87 ±2	(Hein *et al.*, 1991)
NAT1	WKY rat, inbred, liver	2000*	2-AF	136 ± 17	1.59 ± 0.11	(Hein *et al.*, 1991)
NAT2	Rabbit, fast Acetyltor, liver	2200*	INH	---	17.5 ± 0.72	(Hein *et al.*, 1982)
NAT2	Rabbit, slow acetylator, liver	2200*	INH	---	0.12 ± 0.03	(Hein *et al.*, 1982)
NAT1	Rabbit, fast Acetyltor, liver	2200*	PABA	---	4.86 ± 0.20	(Hein *et al.*, 1982)
NAT1	Rabbit, slow acetylator, liver	2200*	PABA	---	2.66 ± 0.27	(Hein *et al.*, 1982)
NAT2 w	HR NAT in *E. coli*	100	SMZ	123	11.5	(Hickman *et al.*, 1995)
NAT2 w	HR NAT in *E. coli*	100	DAPSONE	117	0.84	(Hickman *et al.*, 1995)
NAT2 w	HR NAT in *E. coli*	100	INH	374	16.3	(Hickman *et al.*, 1995)
NAT2 w	HR NAT in *E. coli*	100	PA	3220	11.7	(Hickman *et al.*, 1995)
NAT2 w	HR NAT in *E. coli*	100	ACETYL-CoA & SMZ#	92	11.7	(Hickman *et al.*, 1995)
NAT1	SD rat, liver	200*	SMZ	---	0.98 ± 0.67	(Tannen & Weber, 1979)
NAT2	SD rat, liver	200*	PABA	---	0.80 ± 0.40	(Tannen & Weber, 1979)

NAT2a – a type of phenotype in the human population
W enzyme expressed is wild type
Determination of Acetyl-CoA kinetic parameters

TABLE 3 (Continued)

	SPECIES, ORGAN	Acetyl-CoA (μM)	SUBSTRATE	Km (μM)	Vmax (nmol/min/mgprotein)	Reference
NAT1	Wistar rat, liver	200	SMZ	---	2.01 ± 1.38	(Tannen & Weber, 1979)
NAT2	Wistar rat, liver	200	PABA	---	1.32 ± 0.1	(Tannen & Weber, 1979)
NAT1	Balb/c, liver	200	PABA	---	7.29 ± 1.28	(Tannen & Weber, 1979)
NAT1 &2	Frog, liver, male	100	AF	---	0.932 ± 0.567	(Ho *et al.*, 1996)
NAT1 &2	Frog, liver, female	100	AF	---	0.1.270 ± 0.499	(Ho *et al.*, 1996)
NAT1	Frog, liver, male	100	PABA	---	0.320 ± 0.146	(Ho *et al.*, 1996)
NAT1	Frog, liver, female	100	PABA	---	0.479 ± 0.210	(Ho *et al.*, 1996)

NAT1&2, substrate has affinity for both enzymes

1.4. INTRODUCTION TO POLYAMINES

A preliminary discussion on the polyamine system is given before the introduction to the spermidine/spermine N^1-acetyltransferase enzyme that is the major focus of this thesis. The preliminary discussion will briefly examine various enzymes that participate in polyamine biosynthesis (Fig IP-1). A brief historical perspective is also given along with details on the polyamines and their participation in cellular homeostasis. This background information will enhance the understanding of how spermidine/spermine N^1-acetyltransferase participates in the maintenance of polyamine homeostasis in cells. Although spermidine/spermine N^1-acetyltransferase is present in very small amounts in cells, a number of factors (see Table 4) including increased levels of polyamines may cause induction of spermidine/spermine N^1-acetyltransferase enzyme. While spermidine/spermine N^1-acetyltransferase is in an induced state, it may participate in the acetylation of drugs, in particular the acetylation of amantadine, the focus of this thesis.

1.4.1. POLYAMINES

Polyamines are natural compounds ubiquitously distributed in nature that are present in every living cell (Morgan, 1999). In 1678, Antonii van Leewenhoeck described mysterious crystals that formed when human semen samples were allowed to cool (Leewenhoeck, 1678). These crystals are now known to be spermine phosphate (Harrison, 1931; Tabor & Tabor, 1964). Over the next two hundred years, spermine

crystals in human semen were rediscovered and described by Vauquelin (Vauquelin, 1791), Charcot and Robin (Charcot & Robin, 1853), Boettcher (Boettcher, 1865), Schreiner (Schreiner, 1878) and others. However the discoverers were unaware of the previous work, and by the 19th century there were 10 names describing the spermine phosphate crystals (Harrison, 1931; Morgan, 1999). Investigators originally believed that spermine was uniquely present in human semen, but as early as 1878 it was found to be widely distributed in mammalian tissue (Harrison, 1931; Cohen, 1998). The formal term spermine was first applied to describe the crystals in 1888 (Landenburg & Abel, 1888).

The polyamines are aliphatic amine containing molecules that occur in a linear form and at physiological pH they are cations (Morgan, 1999). They are among the major polycations in the cell along with calcium and magnesium, and can bind to polyanion molecules and structures affecting their function (Igarashi & Kashiwagi, 1999). The most common structures observed in nature are putrescine, spermidine, and spermine. All three occur in eukaryotes, but spermine rarely occurs in prokaryotes (Morgan, 1999). The polyamines are required for eukaryotic and prokaryotic cellular growth and differentiation (Casero & Pegg, 1993; Tabor & Tabor, 1964). The depletion of polyamines or inhibition of their biosynthesis causes cessation of cellular growth that can be restored upon the removal of inhibitors of the polyamine biosynthesis or the addition of exogenous polyamines (Morgan, 1990). Polyamine concentrations and adjustments within cells are highly regulated to meet physiological requirements (Seiler & Dezeure, 1990). Cells maintain homeostasis of polyamines by regulating their biosynthesis, degradation and transport (Igarashi & Kashiwagi, 1999).

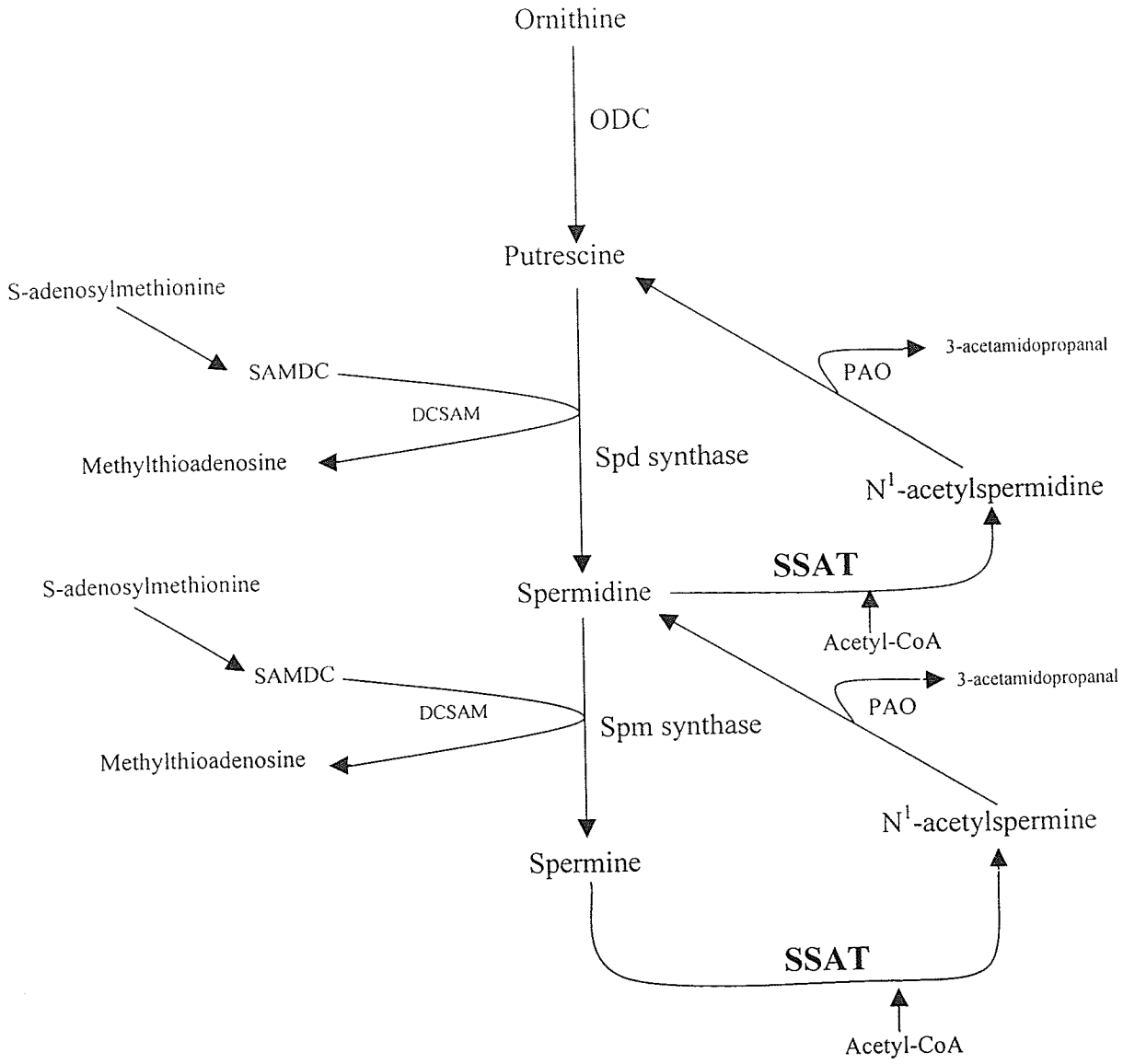


Figure IP-1 The polyamine biosynthetic and degradation pathway. The inducible spermidine/spermine N^1-acetyltransferase (SSAT) enzyme is highlighted to emphasize its importance in the degradation pathway. ODC, ornithine decarboxylase; Spd synthase, spermidine synthase; Spm synthase, spermine synthase; SAMDC, S-adenosylmethionine decarboxylase; DCSAM, decarboxylated S-adenosylmethionine; PAO, polyamine oxidase.

1.4.2. POLYAMINE BIOSYNTHESIS AND DEGRADATION

Polyamine homeostasis is maintained under strong regulation reflecting its importance in cellular function (Cohen, 1998). The importance of maintaining polyamine homeostasis can be demonstrated by the experimental approach where specific inhibitors of polyamine synthesis block cellular proliferation (Heby & Persson, 1990). However, the addition of polyamines to polyamine depleted cells causes them to resume normal growth (Heby & Persson, 1990).

Intracellular polyamine levels are maintained by three key enzymes that regulate polyamine biosynthesis and their initial degradation. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) participate in key points in regulating polyamine biosynthesis, whereas spermidine/spermine N^1-acetyltransferase (SSAT) is the initial step in polyamine degradation (Morgan, 1998). A key feature of all three enzymes is that they have extremely short half-lives (Pegg, 1986), allowing new levels of enzyme to be reached when appropriate stimuli for growth or inhibition are received by a mammalian cell. In the following description of these three key enzymes, the thesis will include other enzymes that participate in the polyamine biosynthetic pathway; these are spermidine synthase, spermine synthase and polyamine oxidase.

1.4.2.1 ORNITHINE DECARBOXYLASE (ODC)

ODC catalyzes the first step in the metabolic pathway for polyamine biosynthesis, and represents the initial and rate limiting step for decarboxylation of ornithine to putrescine (Heby & Persson, 1990; Murakami *et al.*, 2000; Pegg, 1986; Persson *et al.*, 1998). ODC is induced dramatically by stimuli that lead to cell growth. The induction occurs in response to stimuli by growth hormones, drugs, regenerative stimuli, and tumour promoters (Pegg, 1986). The half-life of ODC is the shortest of any currently known enzyme (Murakami *et al.*, 2000). Depending on species, the half-life of ODC can range from 10 to 60 min. (Russell & Snyder, 1969; Morgan, 1998; Murakami *et al.*, 2000). The levels of ODC in mammalian cells, even after growth stimuli which can cause several hundred fold increase in activity, remain very low, representing less then 0.0001% of the total cellular protein (Heby & Persson, 1990).

The decrease in polyamines causes cells to dramatically upregulate the production of ODC to replenish their polyamine levels. In humans, there appears to be two ODC genes situated on chromosomes 2 and 7; one may be a pseudogene (Heby & Persson, 1990). In the mouse, the ODC enzyme is composed of two identical subunits of 461 amino acids (Coleman *et al.*, 1994; Murakami *et al.*, 2000). The homology of the amino acid sequence of ODC monomer between mouse, rat and human is greater than 90% (Heby & Persson, 1990).

Regulatory mechanisms of ODC prevent excess production of polyamines. As the intracellular concentration of polyamines increases, they augment the instability of ODC that is mediated by the binding of antizyme, an inhibitory protein induced by the

increase in levels of polyamines (Canellakis *et al.*, 1979; Murakami *et al.*, 2000). The protein has now been characterised as a 26.5 kDa molecule that can reversibly bind to ODC (Murakami *et al.*, 2000). The antizyme's second function is to inhibit polyamine uptake by polyamine transporters (Mitchell *et al.*, 1994).

Antizyme also has an inhibitor, antizyme-inhibitor, that binds to antizyme more firmly than antizyme to ODC, thus causing the release of ODC from ODC-antizyme complex (Murakami *et al.*, 1996). The antizyme-inhibitor also plays a role in stabilizing newly synthesized ODC during growth phase stimuli (Murakami *et al.*, 2000). The presence of antizyme-inhibitor occurs when cells are growing where there is a need for increased polyamine biosynthesis. In summary, ODC is a highly regulated enzyme, responsive to polyamine levels within mammalian cells with rapid upregulation and degradation.

Subsequent steps are required to convert putrescine into the higher polyamines. The enzymes required for conversion are SAMDC, spermidine synthase and spermine synthase. These enzymes work in concert and in sequence for the synthesis of polyamines to occur.

1.4.2.2 S-ADENOSYLMETHIONINE DECARBOXYLASE (SAMDC)

SAMDC, the second rate limiting enzyme, is highly regulated and plays a key role by responding to stimuli that change the polyamine content and polyamine pools (Pegg *et al.*, 1998). SAMDC catalyzes the decarboxylation of S-adenosylmethionine, forming S-5'-deoxyadenosyl-(5')-3-methylthiopropylamine, that provides the

aminopropyl group required by spermidine and spermine synthase for synthesis of higher polyamines (Pegg, 1986). Therefore, SAMDC is essential for mammalian biosynthesis of spermidine and spermine (Morgan, 1998).

The SAMDC gene in humans is located on chromosome 6 with a pseudogene located on chromosome X (Maric *et al.*, 1992). The SAMDC protein sequence is highly conserved between species that include rat, mouse, hamster, and human with homology greater then 90% (Maric *et al.*, 1992; Morgan, 1998; Nishimura *et al.*, 1999).

The mammalian SAMDC protein is synthesized as a proenzyme that cleaves autocatalytically forming two non-identical subunits known as α and β subunits. The human proenzyme is composed of 334 amino acid residues with a molecular mass of 38,331 (Pajunen *et al.*, 1988). SAMDC in humans is a cytosolic enzyme, and functions as hetero-tetramer composed of two pairs of non-identical subunits, $\alpha_2\beta_2$ (Cohen, 1998; Pegg *et al.*, 1998).

The regulation and activity of SAMDC responds to growth factors and polyamine levels (Pegg, 1988; Shantz & Pegg, 1999). The synthesis of SAMDC is reduced by spermidine and spermine when their levels go beyond homeostasis (Kameji & Pegg, 1987) Putrescine, in contrast, favors the processing activity (autocatalysis) and formation of the active enzyme (Pegg & McCann, 1992). This action by putrescine, when its levels are high, causes an increase in the availability of decarboxylated S-adenosylmethionine, that is required for the next enzyme, spermidine synthase, in the biosynthetic pathway (Shantz & Pegg, 1999).

1.4.2.3 SPERMIDINE AND SPERMINE SYNTHASE

Spermidine and spermine are formed by the action of spermidine and spermine synthase respectively, that catalyze the sequential addition of aminopropyl groups donated by decarboxylated S-adenosylmethionine (Pegg, 1986).

Spermidine and spermine synthase are constitutively expressed enzymes that are much more stable than either ODC or SAMDC (Heby & Persson, 1990; Morgan, 1998). Both enzymes lack the regulatory complexity observed in ODC and SAMDC; their activities are instead dependent on the availability of their respective substrates (Morgan, 1998; Pegg, 1988).

Spermidine synthase and spermine synthase are each composed of two identical proteins forming a homodimer, each with a different molecular weight (Kajander *et al.*, 1989). The spermidine synthase gene was localized to human chromosome 1 (Winqvist *et al.*, 1993), whereas the spermine synthase gene was localized to chromosome X (Grieff *et al.*, 1997).

The apparent activities of spermidine and spermine synthase appear to be regulated by the availability of their substrates. They lack a regulatory or rate limiting role in the polyamine biosynthetic pathway (Heby & Persson, 1990; Morgan, 1998).

1.4.3 POLYAMINE TRANSPORT

The transport and efflux of polyamines may play an important role in maintaining polyamine homeostasis. The depletion or inhibition of polyamine biosynthesis and rapid growth of cells can promote uptake of polyamines from plasma to maintain their

homeostasis (Morgan, 1990). Polyamine uptake is carrier-mediated with characteristics of being saturable, temperature-, and energy-dependent (Seiler & Dezeure, 1990). The transport of polyamines into cells serves to meet the polyamine requirement of cells experiencing enhanced growth rates, that may not be met by *de novo* polyamine synthesis alone (Seiler & Dezeure, 1990). Hormones and growth factors can enhance polyamine uptake to meet cellular needs as they arise, such as in transformed cells. Cells entering cell cycle division G1 phase also express transporters to meet increased cellular requirements (DeBenedette *et al.*, 1993). The needs and therefore the intracellular concentration of polyamines vary through out the cell cycle. Differences in polyamine concentration also occur within different cell types. In resting fibroblasts, the calculated concentration of polyamines is approximately 29 μM putrescine, 159 μM spermidine, and 635 μM spermine (Morgan, 1990). However, for resting transformed and tumour cells the concentrations are generally higher; for ascites cells polyamine levels are 43 μM putrescine, 430 μM spermidine, and 692 μM spermine (Morgan, 1990). In simian virus-40 transformed Swiss mouse fibroblasts (SV-3T3), the levels are 229 μM putrescine, 1835 μM spermidine, and 694 μM spermine (Morgan, 1990). During normal cellular growth the polyamine requirements may increase less than 2 fold the calculated values above (Morgan, 1990). However, during active division of tumour cells, the polyamine requirements may be greater than de novo synthesis can accommodate. Then polyamine transport acquires polyamines from plasma, where their concentration can range from 0.03 to 0.488 nmol/ml, with spermine being in the lower concentration range when compared to spermidine and putrescine (Morgan, 1990).

1.4.4 EFFLUX OF POLYAMINES

Efflux of polyamines is one of two methods that mammalian cells have to diminish intracellular concentrations. The second method will be discussed in the spermidine/spermine N^1-acetyltransferase section to follow.

Polyamine diffusion is unlikely to occur due to the polycationic nature of the polyamines at physiological pH (Seiler *et al.*, 1996; Wallace & Mackarel, 1998). Current evidence suggests that efflux of polyamines is carrier-mediated (Mackarel & Wallace, 1994).

The majority of effluxed polyamines are in the form of spermidine and N^1-acetylspermidine, and have been shown to occur in mammalian cell types such as human colon tumour (HT 29) cells (Pegg *et al.*, 1989), and Chinese-hamster ovary cells (Pegg *et al.*, 1990).

Wallace and Mackarel, 1998, using human colonic carcinoma cells (HT115), observed that other polyamines effluxed were minor in amount when compared to N^1-acetylspermidine. They suggested that the secretion was specific for N^1-acetylspermidine, thus making it the major excretory product.

In summary, efflux of polyamines in mammalian cells plays a role in the regulation of polyamine content. The extent to which this pathway is a major contributor to polyamine homeostasis in normal cells remains to be further explored.

1.4.5 SPERMIDINE/SPERMINE ACETYLTRANSFERASE (SSAT)

SSAT, the third rate limiting enzyme, is ubiquitously distributed in mammalian

tissues and plays a role in catabolism and elimination of polyamines from cells (Cohen, 1998; Morgan, 1998). However, in normal or uninduced mammalian tissues SSAT is present at very low levels (Casero & Pegg, 1993; Cohen, 1998). SSAT is an inducible enzyme that catalyzes the transfer of an acetyl group from acetyl-coenzyme A to the aminopropyl moiety of polyamines. This action by SSAT facilitates polyamine degradation, excretion, cycling and/or intracellular cycling (Casero & Pegg, 1993). In this manner SSAT participates in the maintenance of polyamine homeostasis in mammalian cells.

Interest in acetylation of polyamines began in earnest when reports started to appear in the literature of acetylspermidine in urine of normal and cancer patients (Abdel-Monem *et al.*, 1975; Blankenship & Walle, 1977; Tsuji *et al.*, 1975). Acetylputrescine was also reported to occur in various mammalian tissues, including brain (Blankenship & Walle, 1977; Perry *et al.*, 1967).

Seiler and al-Therib, using tissues of Wistar rat in homogenate or subcellular fractions, were the first to use an in vitro system to describe putrescine acetylation. The brain, liver microsomal and nuclear fractions, but not liver homogenate, were the most active in catalyzing putrescine acetylation. The nuclear fractions from brain and liver had the highest putrescine acetylation activity; however the apparent K_m of 3 mM was high. Spermine but not spermidine was able to inhibit acetylation of putrescine, suggesting a separate enzyme for putrescine acetylase (Cohen, 1998; Seiler & al-Therib, 1974).

Extending the above findings, Blankenship and Walle, using chromatin prepared from albino rat liver and kidney, showed that the incubation medium catalyzed the acetyl-coenzyme A dependent acetylation of putrescine, spermidine, and spermine. However,

both acetylation and metabolism of acetylated polyamines were observed in the study raising the question as to whether significant levels of polyamine acetylation occurred *in vivo* (Blankenship & Walle, 1977).

Libby, using the nuclear fraction from calf liver, was able to separate two N-acetyltransferases. Both enzymes could transfer acetate from acetyl coenzyme A to either histone or spermidine. The two purified enzymes had small differences that distinguished between them. Acetyltransferase A had a molecular weight of 150,000 versus 175,000 for B. The acetyltransferases also differed in their histone fraction preference. Both enzymes preferentially acetylated histones before polyamines (spermidine, spermine and diaminodipropylamine) and polyamines before diamines (Libby, 1978).

The above research pointed to the possibility of two enzymes being present that could catalyze the acetylation of polyamines. Furthermore, two isomeric forms of acetylspermidine were known to occur, denoted as N^1-acetylspermidine and N^8-acetylspermidine (Blankenship & Walle, 1977). These two isomeric forms of acetylspermidine were isolated from urine in both normal and cancer patients (Abdel-Monem *et al.*, 1975). The ratio of N^1-acetylspermidine to N^8-acetylspermidine was shown to be considerably higher in cancer patients' urine than in normal subjects (Abdel-Monem & Ohno, 1977). This result also had the effect of demonstrating that significant levels of polyamine acetylation had to occur *in vivo* for it to be present in the urine.

Additional studies also showed the presence of acetylspermidine deacetylase, confirming the above observations that metabolism of acetylated polyamines was occurring. Libby, using rat liver extracts and partially purified fractions, showed that

they deacetylated N^8-acetylspermidine and N^1-acetylspermine. However diacetylspermidine, and N^1-acetylputrescine were not deacetylated (Libby, 1978). *In vitro* experiments by Blankenship, using male albino rat liver, spleen, kidney, and lung showed that N^8-acetylspermidine deacetylation occurred in the cytoplasmic fractions, with the highest activity in the liver. The *in vitro* experiments also showed that the metabolism of N^1-acetylspermidine differed from N^8-acetylspermidine. The result suggested that N^8-acetylspermidine metabolism was a straightforward deacetylation to spermidine and radiolabeled acetate. The metabolism of N^1-acetylspermidine differed. The loss in radiolabelled acetate could not be accounted for, suggesting that deacetylation was not the primary metabolic process (Blankenship, 1978). Holtta showed, using rat liver cytosolic and mitochondrial fractions, that N^1-acetylspermidine could be metabolized via a novel enzyme, polyamine oxidase (Holtta, 1977).

Blankenship and Walle, suggested that the acetylation and deacetylation activity in tissues could play a role in regulating and maintaining the concentration of polyamines (Blankenship & Walle, 1977). The fact remained that although progress had been made, the routes of acetylpolyamine formation and metabolism (deacetylation) remained unknown (Blankenship, 1978).

A key publication that suggested the interconversion between spermidine and putrescine required prior acetylation of spermidine was reported by Matsui and Pegg. Using female Sprague-Dawley rats treated with carbon tetrachloride, they demonstrated an increase of putrescine with concomitant decrease of spermidine, that amounted to 50 to 60% of controls in the liver within 6 to 12 hours after treatment. Furthermore, using radiolabeled [^{14}C]spermidine injected intraperitoneally followed by treatment with carbon

tetrachloride, they showed enhanced production of [^{14}C]putrescine in the liver, suggesting that the decline of spermidine over the same time period was due to the conversion to putrescine. *In vitro* experiments, using liver cytosol from carbon tetrachloride-treated rats, demonstrated that this treatment led to a substantial increase in the ability to catalyze the accumulation of N^1-acetylspermidine and acetylspermine compared to controls (Matsui & Pegg, 1980). These experiments suggested that SSAT acetylation was the rate limiting step in the interconversion pathway, and induction of SSAT had to occur for there to be sufficient SSAT protein so that N^1-acetylspermidine formation could be observed *in vitro*.

Subsequently Matsui and Pegg, using *in vitro* methods, showed that comparable increases in SSAT activity could be achieved with another liver damaging agent, thioacetamide, in male Sprague-Dawley rats. Smaller increases of SSAT activity were also brought about with partial hepatectomy, and by treatment with growth hormone. Peak activity of SSAT in rat liver treated with thioacetamide occurred 18-24 hours after injection, with an associated increase in putrescine content (Matsui & Pegg, 1980).

Further characterization of the SSAT enzyme was made possible by the ability of carbon tetrachloride to cause substantial increases in the enzyme within 6 hours of administration (Matsui & Pegg, 1981). The induction of SSAT caused by carbon tretrachloride could be abolished by the introduction of protein synthesis and mRNA inhibitors. By first administering the protein synthesis inhibitors cycloheximide or puromycin to female Sprague-Dawley rats, they showed that carbon tetrachloride dependent induction of SSAT was abolished in the liver. Actinomycin D, an inhibitor of mRNA synthesis also abolished the SSAT increase. Taken together, these results

demonstrated that new protein synthesis rather then release of the enzyme from a cryptic inactive form was occurring. The conversion of spermidine to putrescine seen in the liver with the carbon tetrachloride treatment was abolished, confirming the importance of the induction of SSAT for the conversion to occur (Matsui & Pegg, 1981). The SSAT in the presence of acetyl coenzyme A was able to acetylate spermidine, spermine, norspermine, norspermidine and at lower rate 1,3 diaminopropane. However it was not able to acetylate putrescine, cadaverine, homospermidine, or histones. The selectivity of polyamine acetylation further distinguished SSAT from chromatin associated N-acetyltransferases that acetylate histones and other polyamines including putrescine (Matsui *et al.*, 1981). The high levels of SSAT present in rats treated with carbon tretrachloride allowed the purification and further characterization of the enzyme. Ragione and Pegg, using livers from female Sprague-Dawley rats treated with carbon tetrachloride, purified the SSAT protein 112,000 fold. It had an apparent molecular weight of about 115,000 and appeared to be made up of two subunits. Using the purified enzyme, pharmacokinetic characterization showed that the apparent K_m for acetyl coenzyme A was 1.5 μM. The apparent K_m for spermidine, spermine, and N^1-acetylspermine (with a free aminopropyl group on the opposite end) were 130 μM, 35 μM, 30 μM and apparent V_{max} was 8 μmol/min/mg, 1.8 μmol/min/mg, 1.2 μmol/min/mg, respectively (Ragione & Pegg, 1982). The coenzyme A was inhibitory with an apparent K_i of 40 μM. They also showed that SSAT had no deacetylase activity effectively making the reaction irreversible. The name spermidine/spermine N^1-acetyltransferase (SSAT) was suggested by Ragione and Pegg, to describe the enzyme (Ragione & Pegg, 1982).

Ragione and Pegg, using purified SSAT protein (18,000 fold) from livers of female Sprague-Dawley rats treated with carbon tetrachloride, showed that the acetylation mechanism of SSAT acts via a ordered Bi Bi mechanism reaction (to be discussed in the kinetics section) (Della Ragione & Pegg, 1983).

The amount of SSAT enzyme in un-induced rat liver hepatocyte is thought to be less then 1000 molecules per cell. In contrast, the induced hepatocyte (assuming it is restricted to these cell types in the liver) is thought to be about 60,000 molecules per cell (Matsui & Pegg, 1981; Pegg *et al.*, 1982). To determine if the changes observed in acetylation activity were due to increase in SSAT Persson and Pegg raised SSAT specific antiserum (Persson & Pegg, 1984). Using specific antibodies to rat liver SSAT (raised in New Zealand white rabbits with purified rat SSAT), they showed that the increase in SSAT activity could be precipitated by the antibody, suggesting the increase was due to the amount of enzyme. Although the exact basal level of SSAT was difficult to determine, the induction by carbon tetrachloride caused about a 250 fold increase in the enzyme after 6 hr, about 25 fold for spermidine and thioacetamide after 6 and 24 hr respectively, and approximately 300 fold for methylglyoxal bis(guanylhydrazone) after 24 hr. The SSAT antibody detected increased SSAT in not only liver extracts, but also in rat kidney, lung, and pancreas, and mouse liver. However, no increase was detected in other tissues examined that included the intestine, prostate, and brain, which suggested a lack of uptake by the inducer or lack of sensitivity for detection. The SSAT protein was found to turn over very rapidly with a half-life of 15 min. This half-life increased in carbon tetrachloride treated rats to 180 min. The results suggested that part of the increase observed in SSAT activity is due to the stabilization of the enzyme by the

inducer. The SSAT protein purified to homogeneity had a molecular weight of approximately 65,000 (Persson & Pegg, 1984). The molecular weight was different from the previous description above because the methodology used in this instance changed. Here Brij 35, a nonionic detergent, was used for part of the purification procedures (Libby *et al.*, 1991). Under these experimental conditions, the results suggested that SSAT had 3 to 4 subunits each with a molecular weight of 18,000 (Persson & Pegg, 1984).

A second species that was examined for SSAT was the White Leghorn cockerel chick. Shinki and Suda used 1α-,25-hydroxycholecalciferol (calcitriol) to induce SSAT in chick duodenum after the chicks were deprived of this vitamin in their diet for one month. Purifying the protein 63,000 fold, they isolated an SSAT protein with a molecular weight of 36 kDa. The results suggested that it was a dimer composed of two subunits with a kDa of 18 each. The apparent K_m values for spermidine, spermine, and *sym*-norspermidine were 139 μM, 64 μM, and 12 μM, respectively (Shinki & Suda, 1989). Although the pharmacokinetic results are similar to the rat described above, the chick SSAT seems to be composed of a dimer compared to a trimer or tetramer in the rat.

Therefore in the two species described above, rat and chick, SSAT was purified to homogeneity and characterized. The next step was to characterize the human SSAT. A novel approach to characterizing human SSAT was taken by Libby *et al.*; they used fast dividing human melanoma cells as their source of SSAT protein. Treatment of human melanoma cells (MALME-3) in culture with N^1,N^{11}-bis(ethyl)norspermine for 48 to 72 hr induced SSAT activity 1000 to 4000 fold (Libby *et al.*, 1991). The increase in SSAT

protein enabled its purification to homogeneity. The undissociated enzyme had a molecular weight of approximately 80,000 with the subunit weight of 20,300, suggesting the makeup of the human SSAT to be a tetramer. The kinetic characterization showed some differences compared to rat (described above). The apparent K_m for acetyl coenzyme A was 6 μM, and for spermidine, spermine and N^1-acetylspermine the K_m was 55 μM, 5 μM, and 36 μM, respectively. The activity of human SSAT with spermidine as a substrate has an apparent V_{max} 285 μmole/min/mg protein. Although the values for spermidine, spermine, and N^1-acetylspermine are lower then rat and chick, the temperatures used for the incubation in the *in vitro* experiments were different (Libby *et al.*, 1991). The temperature for the in vitro incubations were the following: for the chick it was 25° C (Shinki & Suda, 1989); for rat it was 30° C (Persson & Pegg, 1984); and for human 37° C (Libby *et al.*, 1991). Although, SSAT is very heat labile, a variety of in vitro temperatures have been used in experiments, with 37° C representing the native temperature in humans and possibly reflecting a more accurate representation of function.

Similar results for SSAT subunit molecular weight were also reported by (Casero *et al.*) However, they used a human large cell lung carcinoma cell line (NCI H157) induced with N^1,N^{12}-bis(ethyl)spermine for their source of SSAT protein. The increase in SSAT was 600 fold compared to uninduced cells. The SSAT subunit size had a molecular weight of approximately 20,000. The apparent K_m for spermidine and spermine were 70 μM and 20 μM, and the V_{max} 38 μmole/min/mg protein and 9.9 μmole/min/mg protein, respectively (Casero *et al.*, 1990).

The large amounts of SSAT protein produced by induction of the large cell lung carcinoma cell line (NCI H157) with N^1,N^{12}-bis(ethyl)spermine was an excellent source

for purification of the protein to homogeneity and its use in amino acid sequence analysis. They fractionated the SSAT protein and performed N-terminal sequence analysis of the resultant peptides that yielded a 7 amino acid sequence. This sequence was used to generate an oligomer of 20 nucleotides that could be used to screen a cDNA library for cDNA corresponding to the SSAT protein. Isolated mRNA from the induced cells was translated in rabbit reticulocyte lysates and produced SSAT that was precipitated by using anti SSAT antiserum (as described above). They converted crude mRNA from the induced NCI H157 cells into a cDNA library to screen for SSAT coding cDNA using the 20 nucleotide oligomer. The screen identified a 972 base pair cDNA, with a 513 base open reading frame that coded for a 171 amino acid protein with a predicted molecular weight of 20,023 (Casero *et al.*, 1990). To confirm that this cDNA coded for authentic SSAT, an *in vitro* translation of the mRNA produced from the cDNA resulted in active enzyme that precipitated with SSAT antibodies. However it was not known at this time if the 972 base pair corresponded to the entire coding region of the SSAT gene (Casero *et al.*, 1991). Xiao *et al.*, using the same methodology as above cloned a cDNA of 1,060 base pairs with a 513 base open reading frame coding for a 171 amino acid protein. This cDNA clone represented the full length of the SSAT message (Xiao *et al.*, 1991). Subsequent work by the same investigators showed that the overall length of the SSAT genomic sequence is 4,095 base pairs, that span the entire cDNA coding region. The gene is located on the human X chromosome, localized to p22.1 and is comprised of 6 exons and 5 introns. The SSAT cDNA showed no homology with any other sequence in the GenBank data base that suggests there is only one gene copy in the human genome (Xiao *et al.*, 1992).

The human cDNA sequence was subsequently used to isolate the cDNA clones for the Balb-C mouse (*Mus domesticus*) and the Syrian golden hamster. The Syrian golden hamster cDNA consisted of 1016 base pairs with an open reading frame of 516 nucleotides that coded for 171 amino acids (Pegg *et al.*, 1992), the same as in humans. There were only 8 amino acid differences between hamster and human SSAT. The hamster SSAT coding region cDNA has 91% homology with human cDNA. For the mouse, a gene sequence of 4066 base pairs was isolated using the human SSAT cDNA. A 940 base pair mouse liver SSAT cDNA was isolated containing an open reading frame of 513 nucleotides (same as humans) that coded for 171 amino acids. The human and mouse SSAT gene are very similar. Both contain 6 exons and 5 introns that are very similar in terms of size. The mouse SSAT differed from the human by only 6 amino acids. The changes occurred in the amino acid sequence at positions 5, 12, 32, 42, 119, and 169. Most of the differences in the sequence were conservative substitutions. The SSAT amino acid sequence for mouse, hamster and human had a homology that was greater then 96%. The mouse may also have two separate gene copies for the SSAT. This differs from the human genome, where it appears that only one gene copy is present. However this suggestion remains to be confirmed (Fogel-Petrovic *et al.*, 1993). The high interspecies homology observed for SSAT, confirms its importance to cellular function in helping to maintain polyamine homeostasis

The cloning of the human SSAT gene and the sequencing of the purified human protein permitted further investigation of the amino acid residues required for enzyme activity. Coleman *et al* used a reticulocyte-lysate system for the transcription/translation of mutant or control SSAT cDNA to determine the effect of amino acid changes on

SSAT enzyme activity. They examined eight arginine, five histidine and four cysteine residues and individually replaced them with alanine in the SSAT protein. Three cysteines replaced by alanine decreased SSAT protein accumulation between 57-63%, suggesting that they may be required for protein stability. However cysteine at position 122, when replaced by alanine, caused a 64% reduction in SSAT activity. The single histidine to alanine change at position 126 caused a fifteen fold increase in the apparent K_m for spermidine (1100 μM versus wild type 70 μM) and 88% decrease in the SSAT activity, suggesting the crucial role this amino acid has in binding spermidine. Similarly the change in arginine to alanine at position 155 also caused a ten fold increase in apparent K_m for spermidine (690 μM). Taken together, these two amino acids are crucial for binding spermidine in a proper orientation that facilitates interaction with the acetyl-coenzyme A site. The mutations of arginine to alanine at positions 101, 142, 143, caused an increase of the apparent K_m for acetyl-coenzyme A by 150, 7, and 20 fold respectively. Concurrent double mutation of arginine with alanine at position 142 and 143 caused complete loss in SSAT activity. These results then suggest that three arginine residues are crucial for forming the acetyl-coenzyme A catalytic site (Coleman *et al.*, 1996).

Lu *et al.*, using site directed mutagenesis, also identified some similar amino acid residues that are required for SSAT activity. The most conserved amino acid residues occurred in a region defined as domain I that encompasses amino acids from tyrosine at position 90 to leucine at position 110, a total of 20 amino acids. Nucleotide mutations at positions Val-96, Arg-101, Gly-102 and Gly-106 that changed these amino acid residues to Asp-96, Ala-101, Asp-102 and Asp-106 produced a protein with no measurable

acitivity. Glycine at position 102 that was changed to aspartic acid caused a 23% decrease in SSAT activity compared to wild type SSAT. In a second region, defined as domain II, that is 22 amino acids downstream form domain I, change of glycine to aspartic acid position 144 reduced SSAT activity by more than 95%. This mutation also caused a significant increase in the apparent K_m for spermidine (data not shown by authors). The affinity of acetyl-coenzyme A was also affected by the mutations that occurred at position 69, 102 and 144 that changed His to Ala, Gly to Asp and Gly to Asp, respectively. These changes caused the apparent K_m to increase to 10, 35.7 and 41.9 μM, respectively compared to wild type apparent K_m of 3 μM. The mutation at position 144 seems to indicate that it causes a disruption of the enzyme structure, leading to a decreased affinity for both acetyl-coenzyme A and spermidine (Lu *et al.*, 1996).

Lu *et al.*, also compared the amino acid sequence of domain I of human SSAT with 14 microbial antibiotic acetyltransferases. The greatest homology was found with *Compylobacter coli* streptothricin acetyltransferase *sat4* gene, where 10 amino aids were identical and 6 were conserved residues that occur in the 22 amino acid stretch (Lu *et al.*, 1996). Furthermore, the cloning of sheep N-serotonin acetyltransferase showed that it contained a domain I and II (Coon *et al.*, 1995). These comparisons suggested that human SSAT protein was an evolutionary member of a conserved superfamily of N-acetyltransferases (Lu *et al.*, 1996). Although these studies indicate the critical residues and sections that are important in the enzyme, they still lack the three dimensional conformation of the enzyme that is required for the enzyme to function. However, these investigations do provide the first information on the topology of the enzyme crypt required for catalysis to occur.

The instability that was observed in the site directed mutagenesis above is one type of instability dealing with the residues required for activity and stability of SSAT. The second type of instability of SSAT occurs in the absence of polyamines, and can be prevented by the presence of polyamines and polyamine analogue N^1,N^{12}-bis(ethyl)spermine. Using site directed mutagenesis and expression of SSAT protein in a coupled transcription/translation assay, they showed that the residue glutamic acid at position 152 and MATEE (M, methionine; A, aspartic acid; T, threonine; E, glutamic acid) amino acid sequence at residues 167-171 at the carboxy terminal end are required for the binding of polyamines. The binding of polyamines or the polyamine analogue at these sites causes a conformational change that increases the affinity of the polyamine substrates and protects the protein from protease digestion by decreasing the exposure of the carboxy terminal tail (Coleman *et al.*, 1995).

The mechanism for rapid cellular degradation of SSAT was unkown until Coleman and Pegg showed that it was mediated by ubiquitination and the proteasomal system. Using ^{35}S-labeled SSAT in a reticulocyte lysate system, they showed the appearance of two molecular weight complexes that were consistent with the predicted weight of mono- and di-ubiquitinated SSAT. Inhibition of SSAT degradation using specific inhibitors of the proteasomal system, calpain I and human S5a protein, caused a persistence in the SSAT-ubiquitin protein bands, suggesting the dependence on proteasomal degradation. The degradation could also be progressively decreased with the increasing concentrations of spermidine and spermine, with spermine decreasing degradation by 73% compared to spermidine which decreased it by 36% in a two hour incubation. Site directed mutagenesis showed that for rapid degradation the carboxyl-

terminal amino acid region MATEE is required. Within the sequence, two glutamic acid residues at positions 170 and 171 were found to be of critical importance for rapid degradation of SSAT. Change of the glutamic acid residues to lysine prevented SSAT degradation by the proteasome. Although the MATEE motif lacks some of the amino acids that are present in PEST sequences, the amino acid sequence may act as a pseudo-pest, facilitating proteasomal degradation (Coleman & Pegg, 1997).

1.4.5.1 REGULATION OF SSAT

Induction of SSAT can be caused by different drugs, toxic substances, hormones, and physiological stimuli (Casero & Pegg, 1993) (see Table 4). Discussed above were toxic substances such as carbon tetrachloride, thioacetamide as well as polyamines and their analogues. All could cause induction, but the induction occurred at different times for each individual compound. The regulation of SSAT expression occurs at the levels of transcription, mRNA stability, mRNA translation and protein stability (Fogel-Petrovic *et al.*, 1997).

Discussed above was the example of increased SSAT protein stability observed upon the addition of spermidine, spermine, and N^1,N^{12}-bis(ethyl)spermine (Coleman *et al.*, 1995). The polyamine analogues, N^1,N^{12}-bis(ethyl)spermine causes an increase in SSAT half-life from 20 min to more than 13 hr (Parry *et al.*, 1995). This may be the early effect that the polyamines and the analogues have on enzyme activity when added to cells in *in vitro* systems (Coleman *et al.*, 1995).

The polyamine analogues including, N^1,N^{12}-bis(ethyl)spermine and N^1,N^{11}-diethylnorspemine, are extremely powerful inducers of SSAT that can increase the activity by more than 100 fold within a few hr (Parry *et al.*, 1995). The increased activity is not only caused by increased protein stability, but also by increased transcription and mRNA stabilization. Fogel-Petrovic *et al.*, showed that treatment of melanoma cells with spermine or N^1,N^{12}-bis(ethyl)spermine causes a 5.5 fold and 22 fold increase in mRNA by 24 hours, respectively. The treatment with N^1,N^{12}-bis(ethyl)spermine resulted in a time-dependent accumulation of SSAT-specific mRNA. The increase in mRNA did not occur at the same rate or extent as the SSAT activity.

Table 4
Substances and stimuli that cause increase in SSAT activity

SUBSTANCE	DOSE	TIME TO ASSESSMENT	Fold Increase[3]	TISSUE SPECIES	REFERENCE
Carbon tetrachloride	2 ml/kg	6 hr	10	Liver SD-rats	(Matsui & Pegg, 1980)
Calcitriol[1]	625 ng	2 hr	150	Duodenum Chick	(Shinki & Suda, 1989)
Phorbo 12-mysristat-13-acetate	50 ng/ml	24 hr	2	Lymphocytes Bovine	(Matsui-Yuasa *et al.*, 1984)
Bovine growth Hormone	10 mg/kg	6 hr	2-3	Liver SD-rats	(Matsui & Pegg, 1980)
2/3 partial hepatectomy		8 hr	2-3	Liver SD-rats	(Matsui & Pegg, 1980)
Thioacetamide	150 mg/kg	18-24 hr	15	Liver SD-rats	(Matsui & Pegg, 1980)
N^1,N^{12}-bis(ethyl)spermine	10 μM	0-48 hr	200	Melanoma cells human	(Fogel-Petrovic *et al.*, 1993)
N^1,N^{11}-diethylnorspermine[2]	10 μM	48 hr	980	Melanoma cells human	(Fogel-Petrovic *et al.*, 1997)
Spermidine	100 mg/kg	6 hr	40 90 14 9 22 1.5 -2	Liver, Kidney, Lung, Pancreas, Prostate, Brain, Intestine, SD-rats	(Persson & Pegg, 1984)
Methylglyoxal bis(guanylhydrazone)	80 mg/kg	24 hr	300	Liver SD-rats	(Persson & Pegg, 1984)
Carbon tetrachloride	2 ml/kg	6 hr	250	Liver SD-rats	(Persson & Pegg, 1984)
Liver cancer, Initiated with diethylnitrosamine	200 mg/kg	35 days 8-12 months	5.5 6.2	Nodules Hepatomas	(Sessa & Perin, 1991)
Paraquat	1.2 mmol/kg	6 hr	20 26 36 26	Spleen, Lung, Kidney, Liver, male mouse	(Sugimoto *et al.*, 1988)

1. White Leghorn cockerels maintained on a calcitriol deficient diet for four weeks.
2. Reference contains many polyamine analogues but with decreased activity.
3. Fold increase in SSAT activity
4. Spague-Dawley rats (SD)

Table 4 (continued)

SUBSTANCE	DOSE	TIME TO ASSESSMENT	Fold Increase[3]	TISSUE SPECIES	REFERENCE
Lipopolysacharide	4 mcg/kg	6 hr	3.5 4.5 16	Spleen Kidney Liver, male mouse	(Sugimoto *et al.*, 1988)
Halothane	10 nmols/ kg	6 hr	10	Liver Male mouse	(Sugimoto *et al.*, 1988)
Acetaminophen Isoniazid	3, 1.5, mmols/ kg	18 hr 6 hr	120 13	Liver Male mouse	(Sugimoto *et al.*, 1988)
Ethanol[5]	36% calories in diet	4 months	2	Liver SD male rats	(Perin & Sessa, 1993)
Sodium arsenite	100 μM	2 hr	3	Ehrlich Ascites tumor cells	(Obayashi *et al.*, 1992)
Heat stress	43°C	20 min	3	Ehrlich Ascites tumor cells	(Obayashi *et al.*, 1992)
Ethanol	5%	1 hr	2	Ehrlich Ascites tumor cells	(Obayashi *et al.*, 1992)
Heat stress	45°C	20 min	10 7	Melanoma Derived cells, Lung cancer cells	(Gerner *et al.*, 1993)

5. Caused 230 fold increase in N^1-spermidine content in liver.

SSAT activity increased 280 fold over a 48 hour period compared to 45 fold increase in SSAT specific mRNA over the same period. In contrast, inhibition of polyamine synthesis with α-difluoromethylornithine caused a decrease in polyamine pools within the cells and a decrease in SSAT specific mRNA compared to basal levels. The stabilization of mRNA also plays a role in causing increase in SSAT activity. Fogel-Petrovic *et al.* (1993) showed that melanoma cells treated with N^1,N^{12}-bis(ethyl)spermine extended the SSAT specific mRNA half life to 64 hr compared to 17 hr for controls.

Further confirmation was given by Fogel-Petrovic *et al.*, using N^1,N^{11}-diethylnorspemine; it also confirmed the above observations in which there is increased transcription of SSAT gene occurring, increased SSAT specific mRNA and mRNA stabilization (Fogel-Petrovic *et al.*, 1996).

The increase in SSAT transcription that occurs when spermine or polyamine analogue N^1,N^{12}-bis(ethyl)spermine is added to sensitive cells can range from 1 to 7 fold. Although the increase is small, it is required for downstream events that result in up to 1000 fold increase in SSAT activity (Fogel-Petrovic *et al.*, 1993; Wang *et al.*, 1998).

The SSAT gene contains a polyamine responsive element located in a region that occurs at –1522 to –1492 with respect to the SSAT transcriptional start site (Wang *et al.*, 1998). Within this 31 base pair sequence, the polyamine response element was identified as a 9 base pair sequence. The polyamine response element mediates transcriptional induction of SSAT by the polyamine analogue N^1,N^{12}-bis(ethyl)spermine or natural polyamines (Wang *et al.*, 1998). The increased transcription is associated with a constitutively expressed nuclear protein Nrf2 (the acronym stands for Nuclear factor erythroid 2 = Nf-E2, Nf-E2-related factor 2 = Nrf2) that is constitutively bound to the

polyamine response element and its activity is altered in the presence of natural polyamines or polyamine analogue (Wang *et al.*, 1999; Wang *et al.*, 1998). In tumor cells that are capable of highly expressing SSAT when treated with polyamines or polyamine analogues, the transacting factor Nrf2, appears to be constitutively expressed and bound to the polyamine response element (Wang *et al.*, 1999). However, Nrf2 does not initiate transcription. The regulation of the expression requires a second transcriptional cofactor, polyamine-modulating factor-1 (PMF-1). The PMF-1 in conjunction with Nrf2 regulates the polyamine analogue-induced transcription of SSAT (Wang *et al.*, 2001; Wang *et al.*, 1999). The highest expression of PMF-1 was observed by Wang *et al.*, to occur in normal tissues that are highly differentiated such as heart, skeletal muscle, kidney and liver (Wang *et al.*, 2001). These two factors then play an important role in the regulation of the SSAT expression and thus the maintenance of polyamine levels.

1.5 POLYAMINE OXIDASE (PAO)

The increase of polyamines spermidine and spermine intracellularly can cause an increase in the expression of SSAT as described above. SSAT catalyzes the acetylation of spermine and spermidine resulting in the formation of N^1-acetylspermine and N^1-acetylspermidine. The N^1-acetylspermidine can be excreted by the cell or along with N^1-acetylspermine can undergo further metabolism by flavin adenine dinucleotide dependent PAO (Morgan, 1998). The activity of PAO in normal cells is so high that the levels of N^1-acetylspermine or N^1-acetylspermidine are virtually undetectable. However, when

SSAT is induced, both N^1-acetylspermine and N^1-acetylspermidine form a greater part of the polyamine pool within the cells, but this may be only transient in nature because of the excretion and metabolism by PAO (Casero & Pegg, 1993). PAO cleaves N^1-acetylspermine to form 3-acetamidopropanal and spermidine. Similarly N^1-acetylspermidine can be cleaved by PAO to form 3-acetamidopropanal and putrescine completing the back conversion of polyamines and thus recycling of spermine and spermidine back to putrescine (Morgan, 1998; Pegg, 1986) (see Figure IP-1). The copper containing polyamine oxidase also oxidizes polyamines, polyamines with aminopropyl moieties being more readily attacked than putrescine and primary amines. The copper containing oxidases occur in plasma, not intracellularly as with PAO (Morgan, 1998).

1.6 SPERMIDINE N^8-ACETYLTRANSFERASES (Histone N-acetyltransferases)

The spermidine N^8-acetyltransferase while not part of the polyamine metabolic pathway, plays a role in acetylation of histones and nuclear spermidine and spermine (Cohen, 1998; Morgan, 1998). Spermidine contains three nitrogens joined by a three and four carbon aliphatic chain (Wang *et al.*, 1999). The spermidine N^8-acetyltransferase catalyzes the transfer of the acetyl-group from acetyl-coenzyme A and places it on the terminal nitrogen adjacent to the 4 carbon chain, whereas the SSAT places the acetyl group on the terminal nitrogen adjacent to the 3 carbon chain (Blankenship, 1998; Blankenship & Walle, 1977).

The initial findings by Blankenship and Walle, using chromatin prepared from albino rat liver and kidney, showed that the nuclear fraction catalyzed the acetyl-

coenzyme A dependent acetylation of putrescine, spermidine, and spermine (Blankenship & Walle, 1977). Subsequently, Libby, isolated the nuclear fraction from calf liver and separated two N-acetyltransferases. Both enzymes could catalyze the acetylation of histones or spermidine. The two purified enzymes denoted as A and B differed in their molecular weight, sensitivity to heat, and histone fraction selectivity. The above experiments showed that the enzymes were predominantly nuclear, but lacked data on specificity of substrates (Libby, 1978). Using adult female Sprague-Dawley rat liver nuclei, he separated two similar histone acetyltransferases (Libby, 1980). The results indicated that both N-acetytransferase enzymes, A and B, had the highest affinity for spermidine, followed by spermine with both being efficiently acetylated. However, very low or no acetylation was observed for putrescine and other diamines (1,3-propanediamine, 1,4-butanediamine). Since acetylation occurs in the presence of DNA, Libby incubated the enzymes A and B with DNA (200 μg/ml) and showed that it had no effect on spermidine acetylation. However, histone acetylation by enzyme A is progressively inhibited with increases in DNA concentration; 25 μg/ml of DNA causes 50% inhibition. In contrast, enzyme B inhibition is much less marked. Even when DNA concentration reaches 200 μg/ml inhibition does not reach a 50% level (Libby, 1980).

The nuclear spermidine N^8-acetyltransferase was shown to be immunologically distinct from the cytosolic SSAT. Erwin *et al.*, using rabbit antiserum raised against homogenous rat liver SSAT, showed that it did not inhibit the activity of crude nuclear extract from male Sprague-Dawley rat liver induced with carbon tetrachloride. In contrast, the cytosolic fraction from the carbon tetrachloride treated rats when treated with antiserum raised against the SSAT caused 95% decrease in the enzyme and its

activity. This indicated that the acetylating ability of the nuclear spermidine N^8-acetyltransferase is not related to cytosolic SSAT. Furthermore, the product formed from the nuclear extract was mainly N^8-acetylspermidine, with about 15% being N^1-acetylspermine. However, some N^1-acetylspermidine was also seen (Erwin *et al.*, 1984).

The use of carbon tetrachloride to enhance SSAT production apparently has very little effect on the nuclear spermidine N^8-acetyltransferase. Matsui and Pegg showed that the liver nuclear fraction from carbon tetratchloride-treated female Sprague-Dawley rats increased nuclear spermidine acetylase activity marginally when compared to the 12 fold increase observed from the cytosolic fraction (Matsui & Pegg, 1980). The regulation of the nuclear spermidine N^8-acetyltransferase responds to stimuli differently than SSAT. Desiderio *et al.*, using fibroblast cell (NIH 3T3) cultures showed that refeeding of serum starved cultures or addition of spermidine causes increase in SSAT activity whereas N^8-acetyltransferase activity remained unchanged (Desiderio *et al.*, 1993).

The N^8-acetylspermidine, once formed, is exported from the nucleus to the cytosol where it is deacetylated by N-acetylspermidine deacetylase (Morgan, 1998). The deacetylation yields spermidine and acetic acid (Blankenship, 1978). The initial studies describing the deacetylation of N^8-acetylspermidine were discussed above. A refinement of the previously discussed studies was performed by Marchant *et al* to demonstrate the preferred substrate for N-acetylspermidine deacetylase. Using the cytosolic fraction prepared from livers of male Wistar rats and buffer more conducive to the enzyme, they showed that the preferred substrate for the enzyme was N^8-acetylspermidine. In fact, N^1-acetylspermidine was not deacetylated under the conditions of the study, suggesting that the appropriate substrate is the N^8-acetylspermidine (Marchant *et al.*, 1986).

Spermidine is thought to play a role in the stabilization and folding of DNA and mRNA in the nucleus (Blankenship *et al.*, 1987; Morgan *et al.*, 1987). The acetylation of spermidine in the nucleus potentially destabilizes the nucleosome structure in tandem with histone acetylation, facilitating cell transcription and proliferation (Blankenship *et al.*, 1987; Wang *et al.*, 1999). The significance of N^8-acetylspermidine increase to cellular growth was not fully understood until Wang *et al.*, described its impact. Using leukemia cells (L1210) in culture, they showed that selective inhibition of N^8-spermidine deacetylase with 7-[-N-(3-aminopropyl)amino]heptan-2-one (replacement of 8-nitrogen of N^8-acetylspermidine with a carbon), caused an increase in cellular N^8-acetylspermidine. The increase in N^8-acetylspermidine resulted in increased rates of cell division and cell density. These effects were replicated when N^8-acetylspermidine was added to cell culture medium at a concentration of 1 mM. However, polyamines N^1-acetylspermidine, spermidine, spermine, and putrescine did not stimulate cell growth (Wang *et al.*, 1999). The enhancement of cellular growth is suggestive of the role N^8-acetylspermidine and acetylation may play in cellular regulation.

1.7 ENZYME KINETICS

Most enzyme –catalyzed reactions have a rate of reaction that increases linearly at low substrate concentrations and starts to level off at higher concentrations (see Fig. K-1). This is usually described as hyperbolic production of product and can be described by the following reaction:

$$\mathbf{E + S} \underset{k_2}{\overset{k_1}{\Leftrightarrow}} \mathbf{ES} \overset{k_3}{\Rightarrow} \mathbf{E + P} \qquad \textbf{(1)}$$

where **E** is the enzyme concentration, **S** is the substrate concentration, **ES** is the enzyme substrate complex concentration, and **P** is the product concentration. When the enzyme E combines with substrate S, it forms the enzyme substrate complex ES, with a rate constant of k_1. The ES complex, once formed, has two possible destinies. It can dissociate and form E + S at a rate constant of k_2, or it can proceed to the formation of product P at a rate constant of k_3. In 1913, Michaelis and Menten provided the first definitive mathematical description of enzyme kinetics (Michaelis & Menten, 1913). Using the above reaction and rate constants **(1)** an equation can be derived and is described as the Michaelis-Menten equation:

$$\nu = \frac{\mathbf{V_{max}}\,[\mathbf{S}]}{\mathbf{K_m} + [\mathbf{S}]} \qquad \textbf{(2)}$$

where ν is the rate of product formation and **S** is the substrate concentration. $\mathbf{V_{max}}$ is the maximum velocity of product formation and occurs at very large substrate concentration [S]. V_{max} is dependent on the enzyme concentrations; therefore it is not considered a fundamental property of the enzyme (Cornish-Bowden, 1979). $\mathbf{K_m}$, known as the Michaelis constant, is defined as the substrate concentration at which the reaction

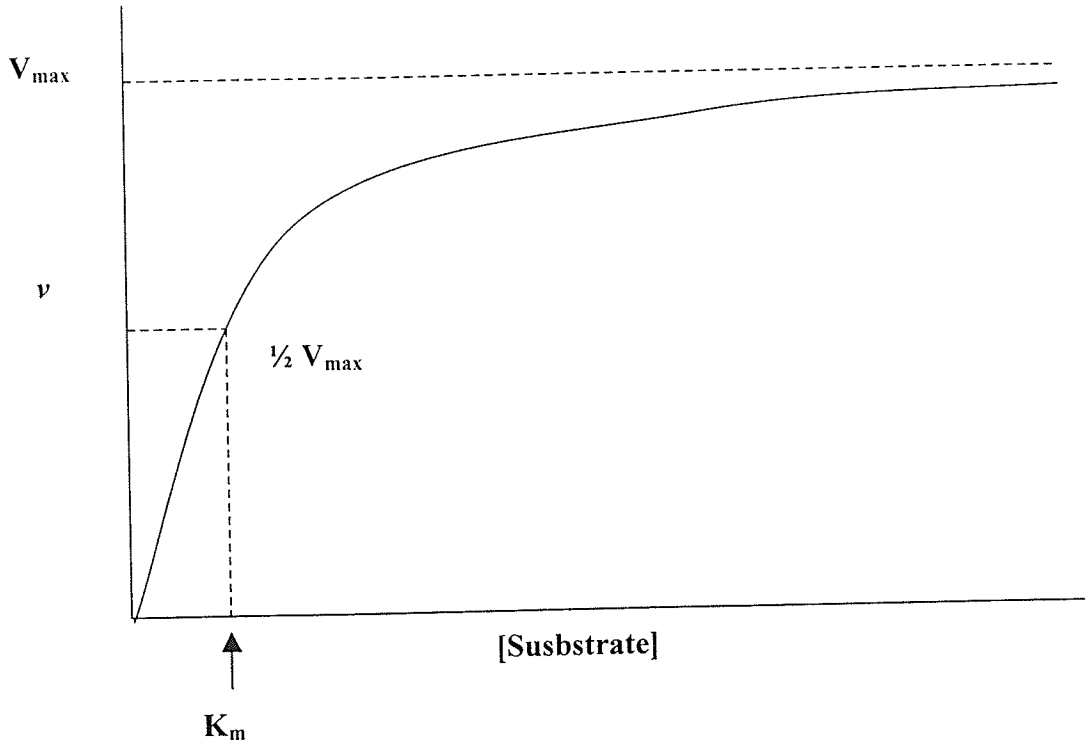


Figure K-1. Representative plot of a rectangular hyperbola obtained with the Michaelis-Menten realationship of an enzyme-catalyzed reaction. K_m, the Michaelis constant, is the substrate concentration at which the reaction proceeds at ½ V_{max}. V_{max} is the maximum rate of an enzyme catalyzed reaction.

proceeds at half maximum velocity (1/2 V_{max}) (Fig K-1). The Michaelis constant K_m is equal to the dissociation constant of the enzyme substrate complex [ES] only when $k_2 >> k_3$ **(1)**. When this criterion is met, the expression of $1/K_m$ is used as measure of the enzyme's affinity for the substrate (Hofstee, 1952). Thus, an enzyme-catalyzed reaction with a high K_m indicates low affinity for the substrate and requires a higher substrate concentration to reach optimal catalytic activity. In contrast, an enzyme-catalyzed reaction with a low K_m indicates a high affinity for the substrate and requires low substrate concentrations for the reaction to proceed at optimal rate. The constant K_m is also dependent on factors such as pH and temperature of the experimental environment; so great care must be observed when comparing K_m values with other laboratories. In general, when designing an experiment to determine kinetic parameters, it is essential that one use a wide range of substrate concentrations in which the rates vary appreciably (Cornish-Bowden, 1979). If the enzyme being studied is thought to obey the Michaelis-Menten equation, one needs substrate concentrations that will define both K_m and V_{max}. As a general rule, the concentration range of the substrate should be between $0.1K_m$ and $10K_m$. In our SSAT experiments the concentrations chosen were based on the human K_m data. No K_m data existed for the mouse. The derivation of the Michaelis-Menten equation can be found in most introductory biochemistry or enzyme kinetic textbooks (Cornish-Bowden, 1979; Stryer, 1988).

1.7.1 Graphical methods of representing the Michaelis-Menten equation

For enzyme-catalyzed reactions, different graphical methods have been developed for the determination of K_m and V_{max} values and are described below.

The Michaelis-Menten plot (Fig K-1) describes a series of velocities v versus substrate concentrations [S] that produce a rectangular hyperbola. Before the advent of computers, this plot had disadvantages: it was difficult to fit the rectangular hyperbola accurately by eye; the difficulty of locating the asymptote for V_{max} could lead to error in determining V_{max}; and therefore could lead to variable results when estimating K_m. With the introduction of computers and of non-linear regression programs, the Michaelis-Menten plot became useful for estimating K_m and V_{max}. The Michaelis-Menten plot also allows for the observation of enzyme inhibition caused by increases in substrate concentrations. Failure to observe expected increases in v as [S] increases may indicate substrate inhibition or product inhibition.

Lineweaver & Burke, 1934, proposed the following the equation by taking reciprocals of both sides of equation **(2)** to give:

$$\mathbf{1/v = K_m/V_{max} \times (1/[S]) + 1/V_{max}} \qquad \mathbf{(3)}$$

By plotting $1/v$ versus $1/[S]$ a straight line should result with a slope K_m/V_{max}, with a y-intercept equal to $1/V_{max}$, and an –x-intercept that will equal $-1/K_m$. The Lineweaver-Burk plot has probably been the most widely used plot in enzyme kinetics but is commonly used inappropriately for the determination of K_m and V_{max} (Cornish-Bowden, 1979). The major advantage of using this transformation of the Michaelis-Menten

equation is that the K_m and V_{max} can be determined from intersection points on the graph. The major disadvantage of the double reciprocal plot is that low values of substrate concentration inordinately influence the determination Km and Vmax. At low substrate concentrations, the nature of the double reciprocal plot causes small errors in v to lead to large errors in $1/v$, which lead to errors in the determination of K_m and V_{max}. To increase the precision of the K_m and V_{max} estimates, appropriate data point weighting must be used such as described by Wilkinson, which minimizes errors in the data fit (Wilkinson, 1961). If the data fit is extremely good, the double reciprocal plot will provide the same estimates as other linear graphical methods of plotting the Michaelis-Menten equation.

Three other transformations of the Michaelis-Menten equation are the Eadie-Hofstee plot (Eadie, 1942; Hofstee, 1952), Hanes plot (Hanes, 1932), and Eisenthal and Cornish-Bowden plot (Eisenthal & Cornish-Bowden, 1974). These plots are also used in enzymatic data analysis. They also have advantages and disadvantages when data are fitted. In our data analysis, these plots were not used and therefore are not discussed further.

1.7.2 Kinetics of Enzyme Inhibition

Compounds that bind to enzymes and alter their substrate affinity or rate of an enzyme-catalyzed reactions are known as inhibitors. Inhibitors come in two forms, reversible and irreversible. Reversible inhibitors bind an enzyme and inhibit substrate to product formation, resulting in various equilibria between enzyme, substrate and inhibitor. Reversible inhibitors bind and unbind to the enzyme, but may not be

transformed by the enzyme (Lehninger, 1982). Irreversible inhibitors bind the enzyme in such a way (e.g. covalently) that they inactivate catalytic activity permanently. Since our studies involved only reversible inhibition, no further reference to irreversible inhibitors will be made. Reversible inhibition, depending on the type of interaction, can be classified into one of the following categories: competitive, non-competitive, uncompetitive, or mixed. Each of these categories possesses its own kinetic characteristics.

1.7.2.1 Competitive Inhibition

Cornish-Bowden, suggested that competitive inhibition is the commonest type of inhibition observed (Cornish-Bowden, 1979). In general, competitive inhibition occurs when a substrate and inhibitor are in direct competition for the binding site on the enzyme, and may be described by the scheme below:

$$\begin{array}{l} \mathbf{E + S \Leftrightarrow ES \Rightarrow E + P} \\ + \\ \mathbf{I} \\ \Updownarrow \\ \mathbf{EI} \end{array} \qquad (4)$$

Where **I** is the inhibitor, **EI** is the enzyme inhibitor complex and **S** substrate being studied; all other terms are as described previously. In this type of inhibition, the increase in substrate concentration will overcome the inhibition, and thus V_{max} will remain the same. However, a greater substrate concentration will be required to reach saturation in the presence of an inhibitor. The inhibitor and substrate compete for the

same binding site on the enzyme, resulting in the increase of K_m with an apparent decrease in the affinity of the enzyme for the substrate. Competitive inhibition is described by the following equation:

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (5)$$

Where $\mathbf{K_i}$ is the dissociation constant of the enzyme inhibitor complex; **I** is the inhibitor concentration, and the other constants are as described previously. Competitive inhibition data can be presented using the Lineweaver-Burk plot. The plot will show an increase in K_m, reflecting the presence of inhibitor, without change in V_{max}.

In order to determine K_i, Dixon, proposed a plot of $1/v$ versus I (Fig K-2)(Dixon, 1953). Using two or more different substrate concentrations, the plot will give lines with different slopes intersecting with each other at a single point to the left of the y-axis. Where the lines intersect at the single point, the negative x-value is equal to $-K_i$. The associated problems observed with the Lineweaver-Burk plot are also present with the Dixon plot. The reciprocal transformation of v to $1/v$ in the Dixon plot may result in poor estimates of K_i unless experimental error is very small (Cornish-Bowden, 1974).

Another problem associated with the Dixon plot is that it yields similar plots for both competitive and mixed inhibition (see below), and does not provide a measure of $\mathbf{K_i'}$, which describes the dissociation constant of the ESI complex (Cornish-Bowden, 1974)(Fig K-2). To distinguish between competitive and mixed inhibtion, a complementary plot was proposed by Cornish-Bowden (Cornish-Bowden, 1974) and is described by the following transformation of equation **(5)**:

$$\frac{[S]}{v} = \frac{K_m}{V_{max}}\left[1 + \frac{[I]}{K_i}\right] + \frac{[S]}{V_{max}} \qquad (6)$$

A competitive inhibition plot of S/v versus I (Fig K-2), yields parallel lines representing different substrate concentrations. By constructing Dixon and Cornish-Bowden plots, it is possible then to distinguish between different types of inhibitions and to determine the inhibition constants.

1.7.2.2 Non-competitive Inhibition

Non-competitive inhibition is a special type of inhibition in which the inhibitor can bind either the enzyme or the enzyme substrate complex without changing the affinity of the enzyme for the substrate. However, the maximum kinetic rate, V_{max}, decreases. Non-competitive inhibition is decribed by the following scheme:

$$\begin{array}{ccccc} \mathbf{E} + \mathbf{S} & \Leftrightarrow & \mathbf{ES} & \Rightarrow & \mathbf{E} + \mathbf{P} \\ + & & + & & \\ \mathbf{I} & & \mathbf{I} & & \\ \Updownarrow \mathbf{K_i} & & \Updownarrow \mathbf{K_i'} & & \\ \mathbf{EI} + \mathbf{S} & \Leftrightarrow & \mathbf{ESI} & \Rightarrow & \mathbf{E} + \mathbf{P} \end{array} \qquad (7)$$

where **ESI** is the enzyme substrate inhibitor complex, and its dissociation constant is denoted by $\mathbf{K_i'}$. All other terms are as previously described. In this scheme, product formation can occur due to breakdown of ES, ESI or both. In this type of inhibition, the apparent maximum rate of reaction (V_{max}) is decreased without affecting the enzyme affinity for the substrate (K_m). This effect is exactly the opposite of competitive inhibition. The equation describing non-competitive inhibition is as follows:

$$v = \frac{V_{max}[S]}{([S] + K_m)\left[1 + \frac{[I]}{K_i}\right]} \quad (8)$$

The Lineweaver-Burk plot of non-competitive inhibition gives lines of each different inhibitor concentration that intersect at the same point on the negative x-axis, and each line has a different y-axis intersect. From this plot it is not possible to determine K_i. The Dixon plot can be used to determine K_i, and the plot will be visually similar to the Lineweaver-Burk plot, in that the lines intersect at the same point on the negative x-axis giving a value - K_i. Similarly, the Cornish-Bowden plots both intersect at the same point in the x-axis, making the inhibitor constant K_i and K_i' equal (Fig K-2).

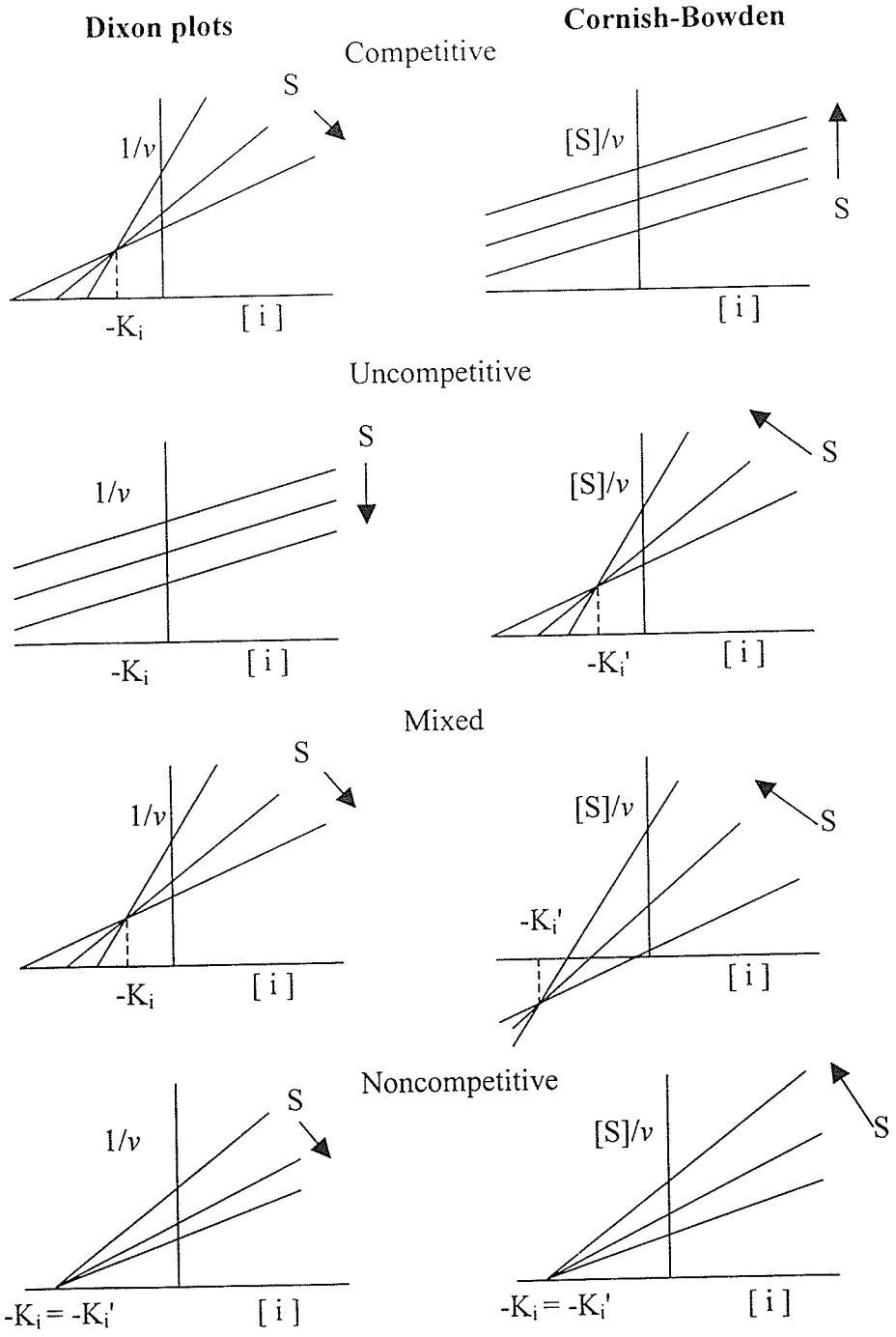


Figure K-2. Dixon and Cornish-Bowden plots for the determination of inhibition types and inhibitory constants Ki and K_i' (Cornish-Bowden, 1974).

1.7.2.3 Uncompetitive Inhibition

In uncompetitive inhibition the inhibitor does not bind the free enzyme. There is no binding site available. However, when substrate binds the enzyme, the inhibitor can combine with the enzyme complex and may be described by the following scheme:

$$\begin{array}{ccccc} E + S & \Leftrightarrow & ES & \Rightarrow & E + P \\ & & + & & \\ & & I & & \\ & & \Updownarrow K_i' & & \\ & & ESI & & \end{array} \qquad (9)$$

This form of inhibition is characterized by a decrease in V_{max} and K_m in the presence of an inhibitor by a factor of $(1 + [I]/ K_i')$. According to Cornish-Bowden, uncompetitive inhibition occurs almost exclusively as a type of product inhibition that takes place in reactions with several substrates and products (Cornish-Bowden, 1979). Uncompetitive inhibition can be described by the following rate equation:

$$v = \frac{V_{max}[S]}{K_m + [S]\left[1 + \frac{[I]}{K_i'}\right]} \qquad (10)$$

where K_i' is the dissociation constant of the enzyme-substrate-inhibitor complex, ESI. By convention, in this scheme no E and I interaction occurs; therefore K_i cannot be determined. A Lineweaver-Burk plot of $1/v$ versus $1/S$ for a series of inhibitor concentrations yields a number of parallel lines with the same slope. The parallel lines of the Lineweaver-Burk plot in uncompetitive inhibition suggest a decrease in affinity (K_m) of the enzyme for the substrate when theoretically there should be none, because the

enzyme does not interact with the inhibitor until the substrate is bound. Similarly, the Dixon, plot yields parallel lines for each substrate concentration (Fig K-2). The problem with the Dixon plot is that the inhibition constant K_i' cannot be determined from the parallel lines. To determine K_i', the Cornish-Bowden plot of $[S]/v$ versus I can be used. The lines for different substrate concentrations have different slopes that intersect at a point to the left of the y-axis and above the negative x-axis. The point of intersection represent - K_i'.

1.7.2.4 Mixed Inhibition

The mixed inhibition kinetic behavior may be described as a combination of both competitive and uncompetitive inhibition. In this situation, the inhibitor can bind the free enzyme or the enzyme substrate complex; however the substrate cannot bind the enzyme inhibitor complex. The following scheme visually describes mixed inhibition:

$$\begin{array}{ccccc} \mathbf{EI} & & \mathbf{ESI} & & \\ \Updownarrow K_i & & \Updownarrow K_i' & & \\ \mathbf{E+S} & \Leftrightarrow & \mathbf{ES} & \Rightarrow & \mathbf{E+P} \\ + & & + & & \\ \mathbf{I} & & \mathbf{I} & & \end{array} \qquad (11)$$

In this scheme, two inhibition constants may be determined, K_i for the enzyme inhibitor complex, and K_i' for the enzyme substrate inhibitor complex. Mixed inhibition can be described by the following rate equation:

$$v = \frac{V_{max}[S]}{Km\left[1 + \frac{[I]}{K_i}\right] + [S]\left[1 + \frac{[I]}{K_i'}\right]} \qquad (12)$$

For mixed inhibition, a Lineweaver-Burk plot yields lines with different negative x-intercepts and y-intercepts. The kinetic parameters of K_m and V_{max} vary depending on the K_i and K_i'. To determine the inhibition constants, the Dixon plot will yield K_i and the Cornish-Bowden plot the K_i' (Fig K-2).

1.7.3 Cheng-Prusoff Analysis

In the present studies, the Cheng and Prusoff method for determining the enzyme inhibition constant K_i was used. The Cheng-Prusoff equation relates the K_i for an inhibitor to its concentration required to reduce the reaction rate of a fixed substrate concentration to 50% (IC_{50}) of that in the absence of inhibitor. To determine IC_{50}, the reaction rates of the fixed substrate concentration are converted to percent control and plotted against log of inhibitor concentrations that gives a straight line (Fig K-3). From the slope and intercept of the straight line, the IC_{50} is determined by the y = 50% point. If the type of inhibition that occurs is known, the following formulae may be used to determine K_i:

for competitive inhibition $$\mathbf{Ki} = \frac{IC_{50}}{1 + \frac{S}{K_m}} \qquad (13)$$

and for uncompetitive and non-competitive inhibition $$\mathbf{IC_{50} = K_i} \qquad (14)$$

A major advantage of the Cheng-Prusoff relationship is that only a single substrate concentration with the various inhibitor concentrations is required to determine the K_i, making assays much smaller. In contrast, the Dixon or Cornish-Bowden plots require 2 or more substrate concentrations before determination of inhibition constants is possible. However, Dixon and Cornish-Bowden plots must be used if the type of inhibition is unknown and before the equations 13 and 14 can be used. A major disadvantage with the determination of IC_{50} is that it is dependent on substrate concentration used in the assay; therefore comparisons with other laboratories are impossible unless identical assay conditions are used (Cheng & Prusoff, 1973).

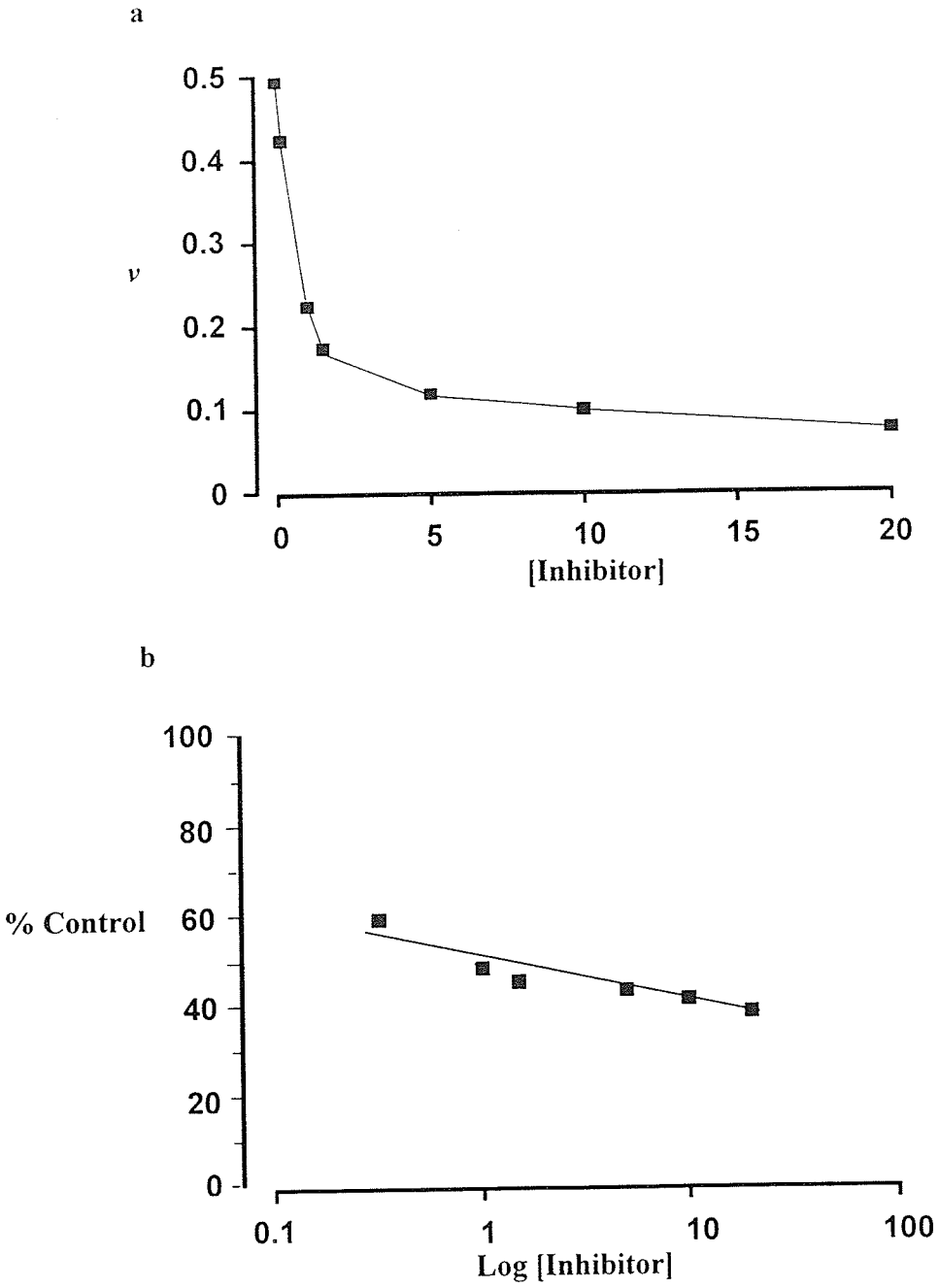


Figure K-3: Example of data conversion to determine IC_{50}. a. Reaction rate versus inhibitor concentration. b. Reaction rate as % control versus log inhibitor concentration.

1.7.4 Assignment of Mechanism in Multi-substrate Enzyme Systems

In the current studies, enzyme reactions have two substrate reaction mechanisms, and as such have been classified as Ping Pong Bi Bi for the NAT1 and NAT2 enzymes (Weber & Cohen, 1967), and ordered Bi Bi for SSAT (Della Ragione & Pegg, 1983).

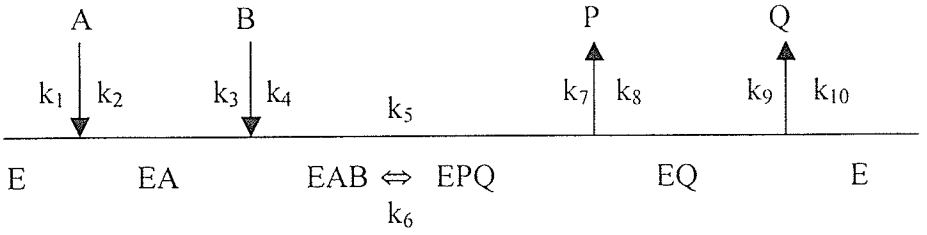
Cleland developed a classification system for two or more substrates in enzyme catalyzed reactions according to the number of substrates, products and the reaction mechanism. The number of kinetically important reactant substrates were designated A, B, C, and D in the order in which they added to the enzyme, and products were designated P, Q, R, and S in the order that they leave the enzyme. Syllables Uni, Bi, Ter, and Quad designated the number of kinetically important substrates and products of the reaction (Cleland, 1963). For example

$A \Leftrightarrow P$	Uni Uni
$A \Leftrightarrow P + Q$	Uni Bi
$A + B \Leftrightarrow P$	Bi Uni
$A + B \Leftrightarrow P + Q$	Bi Bi
$A + B + C \Leftrightarrow P + Q$	Ter Bi

This sequence of the reaction mechanism, that is the addition of substrates or release of products, was designated in descriptive terms. The term *sequential* describes the mechanism where all substrates must add to the enzyme before any products are released. Mechanism where the substrates add in an obligatory order and leave similarly are termed *ordered*. If substrates do not add in an obligatory order and depart in the same manner, then they are termed *random*. Cleland defined the mechanism of *Ping Pong* " when one or more products are released before all substrates have added, the enzyme will

exist in two or more stable forms between which it oscillates during the reaction" (Cleland, 1963).

A simple diagrammatical sequence of the reaction mechanism was proposed by (Cleland, 1963). The system used the designated letters described above for substrates and products, with an horizontal line representing the enzyme and arrows; those pointing downwards indicating addition of substrates, and those pointing upwards denoting formation of product. At any of these steps, the reaction may be reversible. For example, the ordered Bi Bi mechanism is described by the following scheme:



From these graphical representations, rate equations could then be derived. To limit the complexity of multi-substrate reactions and therefore their kinetics, a simple approach is to hold all substrate concentrations constant, except the substrate being analyzed. The kinetics can then be described by the Michaelis-Menten equation.

1.8 OBJECTIVES OF THE DISSERTATION

The focus of the present dissertation was to determine the enzyme that catalyzes the N-acetylation of amantadine. An initial report from our laboratory indicated that that acetylation of amantadine was not correlated with NAT2 acetylator phenotype (Sitar *et al.*, 1991). This initial observation led to the work presented in this dissertation. The

objective of the dissertation was to test the following hypotheses; 1. The N-acetyltransferases, NAT1 and NAT2 do not catalyze the acetylation of amantadine. 2. The enzyme SSAT, that catalyzes the acetylation of spermidine and spermine, is responsible for the N-acetylation of amantadine, when it is induced or overexpressed.

2. METHODS

2.1 PART 1

2.2 NAT *IN-VITRO* METHODS

2.2.1 Animals

Selected tissues as described below were obtained from the following animals Male Sprague-Dawley rats (250-300 g) (Canada-Charles River breeding stock, University of Manitoba, Winnipeg, Manitoba), New Zealand White female rabbits (3.5 kg), and male cross breed pig (6 months old), that were obtained from the University of Manitoba. Experimental procedures involving the use of animals were approved by the University of Manitoba Faculty of Medicine Protocol Management and Review Committee.

2.2.2 Preparation of NAT enzymes

The rats and rabbits were anaesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally for the rat, and into an ear vein for the rabbit. A laparotomy was performed through which the rat and rabbit were sacrificed by sectioning of the aorta, and the livers were extracted. Pig lung tissue was salvaged from a post-surgical procedure performed at another laboratory. Throughout the rest of the procedures the tissue and homogenates were maintained on ice to prolong the viability of the NAT enzymes. The liver and lung tissue was placed in ice cold normal saline (0.9 % w/v) (4^0C) washed, blotted, weighed and placed in 3 volumes (1 volume equals the weight of

the liver tissue) of ice cold TEDK buffer which was composed of the following: 10 mM triethanolamine-HCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DDT), 50 mM KCl, pH 7 (pH adjusted with KOH) (Grant *et al.*, 1990) which also included 10 μM leupeptin, 0.1 mM phenylmethylsulfonyl flouride, and 0.05 mM butylated hydroxytoluene (Cribb *et al.*, 1991). 1 ml of TEDK buffer was then placed on the stage of the tissue chopper (Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, UK); strips of liver cut with scissors were placed on the stage and minced. Once minced, the liver was placed back into the TEDK buffer and homogenized using a Polytron Homogenizer (Brinkmann Instruments, Westburg, NY) at power setting of 6 for approximately 2 min, taking care not to allow frothing of the homogenate. The homogenate was centrifuged at 9000 x g for 20 min at 4^0C (Sorvall RC-5B automatic refrigerated super-speed centrifuge, with SM24 centrifuge rotor, Du Pont Instruments). The supernatant was removed and the pellet discarded. The supernatant was centrifuged at 105,000 x g for 60 min at 4^0C (Beckman L8-80M automatic refrigerated ultracentrifuge, with T60 centrifuge rotor). TEDK buffer was added to the supernatant to bring it to the original volume of the crude homogenate. The supernatant was used as the source of cytosolic N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2). When the supernatant was not used immediately, it was stored in microcentrifuge tubes (1.5ml) at -80^0C until needed. Previous reports have indicated that freezing-thawing and storage of NATs at –20^o C for up to 3 weeks had negligible effect on activity (Meisler & Reinke, 1979; McQueen & Weber, 1980; Mandelbaum-Shavit & Blondheim, 1981) and storage in liquid nitrogen for up to 6 weeks had no effect on NAT activity (Cribb *et al.*,

1991). Grant *et al.*, reported that freeze-thawing of liver cytosol decreased NAT1 activity (Grant *et al.*, 1991). We used the frozen supernatant within 2 days of preparation.

The pig lung tissue was handled in the same manner as for liver tissue and modified in the following steps. A lobe of a lung (78 g) was finely minced by hand using a razor blade in a cold room (4°C). Tough fiber tissue was removed. Half of the tissue was placed in TEDK buffer and homogenized at speed setting of 4 for 5 min with a Polytron homogenizer. The second half of the tissue was placed in Cross-Taggart buffer that contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM, 1.4 mM KH_2PO_4, 1.2 mM $MgCl_2H_2O$, 11 mM glucose, 1mM $CaCl_2$, 15 mM sodium phosphate buffer pH 7.4 (pH adjusted with NaOH), and homogenized. Homogenization proceeded as described previously.

Lysates of human recombinant NAT1 and NAT2 wild-type enzymes expressed in *Escherichia coli* (*E. coli*) were generated at the Division of Clinical Pharmacology and Toxicology, Research Institute (The Hospital for Sick Children, Toronto, Canada) as previously described (Dupret & Grant, 1992). These lysates were used as described below.

2.2.3 N-Acetyltransferase assay

The assay conditions have been previously described (Grant *et al.*, 1989; Grant *et al.*, 1990; Cribb *et al.*, 1991; Derewlany *et al.*, 1994) and are as follows. Liver cytosolic supernatant was appropriately diluted using TEDK buffer containing 1mg/ml bovine serum albumin, and 40 µl are placed in 1.5ml microcentrifuge tubes to start reaction after adding the following: 20 µl acetyl-coezyme A (acetyl-CoA)(450 mM dissolved in

distilled deionized water), and 20 µl of acetyl-CoA regenerating system composed of acetyl-DL-carnitine HCl 5.4 mg/ml and carnitine acetyltransferase 1 U/ml dissolved in assay buffer that contained: 225 mM triethanolamine-HCl, 4.5mM EDTA, 4.5mM DDT dissolved in distilled deionized water (DDW), pH 7 at 37^0C (pH adjusted with NaOH) (Cribb *et al.*, 1991; Derewlany *et al.*, 1994), and 10 µl of substrate are added. The acetyl-CoA regenerating system maintains acetyl-CoA concentrations at a physiological level of 100 µM during the incubations (Andres *et al.*, 1985; Cribb *et al.*, 1991). The substrate solutions containing SMZ, PABA, N-acetyl-*p*-aminobenzoic acid (AcPABA), N-acetyl-sulfamethazine (AcSMZ), and amantadine were prepared fresh for each experiment. Dimethylsulfoxide (DMSO) 25% was used to prepare the stock solution of each substrate. The substrates were first dissolved in 100% DMSO and water was added to make a 25% concentration of DMSO. The concentration ranges of the substrates used in the final incubation volume of 90 µl were as follows, SMZ 27 to 1000 µM, and PABA 5 to 50 µM. These substrates were incubated with or without amantadine 1000 µM. Incubations occur for an appropriate amount of time such that no more then 20% of substrate is converted to the acetylated product. The enzyme is tested for linearity of velocity versus time and protein concentration at a fixed concentration of either SMZ or PABA. The microcentrifuge tubes were placed on a rack and were incubated at 37^0C with shaking (60 oscillations/min) in a Fisher incubator (model 125, Fisher Laboratory Products, Pittsburgh, PA). The experiments were conducted in triplicate. One set of microcentrifuge tubes contained substrate without amantadine and the second set contained substrate with amantadine. Incubations were carried out simultaneously to preclude variation in the rate due to temperature fluctuations. Incubation times were the

following: rat SMZ = 30 min, PABA = 15min, rabbit SMZ and PABA = 10 min. In each experiment 40 µl of the following protein concentrations were used: rat SMZ = 5.5 mg/ml, PABA = 0.88 mg/ml; rabbit SMZ and PABA = 0.84 mg/ml (albumin subtracted from protein concentration values). The reaction was stopped with 15% perchloric acid. The samples were centrifuged at 12,000 x g for 3 min to pellet the protein (Fisher Scientific Micro Centrifuge model 235c). The supernatant was used to quantify the acetylated substrates by high performance liquid chromatography (HPLC).

2.2.4 Human recombinant NAT1 and NAT2 assay

We performed identical acetylation studies with PABA and SMZ in the absence and presence of amantadine HCl using the human recombinant NAT1 and NAT2 enzyme from lysates of *E. coli* as described above in N-Acetyltransferase assay section. The lysates containing the recombinant NAT1 enzymes (1 ml) were diluted 100 x using TEDK buffer and the protein concentration used ranged from 0.015 to 0.020 mg/ml. For the lysates containing the recombinant NAT2 (1 ml) the dilution was 5 x using TEDK buffer and the protein concentration used ranged from 0.36 to 1.17 mg/ml. The incubation time for the assay containing NAT1 and NAT2 were 10 and 15 min, respectively.

2.2.5 Amantadine acetylation in vitro assay

Amantadine was incubated with *E. coli* lysates containing human recombinant NAT1, or NAT2 enzymes, and liver 105,000 x g supernatant from rat, and rabbit as a

source of NAT1 and NAT2 enzymes in the absence of competing PABA and SMZ. The incubation procedures were as described above in the N-acetyltransferase assay section, with the following modifications: the concentration of amantadine ranged from 10 to 1000 μM, and the incubation times were extended to 30 and 60 min to increase the probability of detecting acetylamantadine. The volumes of all the substrates added for incubation were multiplied 10 x, to give sufficient volume for quantification of acetylamantadine.

Crude homogenates of pig lung and 9000 x g supernatant from both buffers were used in the incubation without regard for protein concentration with 1000 μM amantadine (final concentration in incubation medium) for 30 min. All other procedures that were as described above. The quantification of acetylamantadine from the supernatant was performed by gas liquid chromatography as described below in part two of the methods.

2.2.6 Analytical

HPLC analysis for AcSMZ and AcPABA were performed using a Beckman pump (Beckman instruments, Mississauga, Canada) Shimadzu SIL-9A autoinjector (Shimadzu Scientific, Kyoto, Japan), Waters model 441 Absorbance detector (Waters Associates, Milford, Mass.), Hewlett Packard 3380A Integrator, and Whatman 4.6 x 216 mm EQC 10U 125A C_{18} Column. The mobile phase has been previously described (Cribb *et al.*, 1991; Derewlany *et al.*, 1994) and the proportions modified in the following manner: distilled deionized water (DDW)/acetonitrile/acetic acid/triethylamine in the following ratios: for SMZ and AcSMZ 880:120:10:0.5 ml, and for PABA and AcPABA 905:95:10:0.5 ml. Both substrates and products were detected at a wavelength of 254

nm. Retention times were 12.4 and 15.9 min for SMZ and AcSMZ respectively, and 8.1 and 16.5 min for PABA and AcPABA respectively, at a flow rate of 1 ml/min. The unknown acetylated products were quantified against calibration curves of known acetylated product concentrations that ranged from 0.78-100 μM for both AcPABA and AcSMZ. The curves were linear over the range of selected concentrations used, $r^2 >$ 0.99, C.V. < 1%. The limit of quantitation for both metabolites was 0.78 μmol/l. For analysis of acetylation kinetics 5 or 6 concentration points were chosen and performed in triplicate.

2.2.6.1 Protein Analysis

Protein analysis of the supernatant was performed by the Biuret method (Gornall *et al.*, 1949). 0.4ml of Biuret reagent (0.15% $CuSO_4 \cdot 5H_2O$, 0.6% $NaKC_4H_9O_6 \cdot 4H_2O$, 3% NaOH (w/v)) and 0.1ml of the protein solution wered mixed and allowed to stand for 30 min. Samples were read on a spectrophotometer at 550 nm (Milton Roy Spectronic 3000 Array, Milton Roy, Rochester, NY). Results were compared to a standard curve constructed using known amounts of bovine serum albumin (1 to 10 mg/ml).

2.2.7 Data analysis

Kinetic parameters were initially estimated by Lineweaver-Burk plots in a graphical manner, to detect experimental errors, such as data points that were not linear or inhibition by excess substrate concentrations (Lineweaver & Burke, 1934). The

Michaelis-Menten equation kinetic parameters K_m and V_{max} were estimated by the method of Wilkinson, 1961, on EXCEL spreadsheets (Microsoft Corp., Seattle). Means of experiments (n=4) were compared by two-tailed paired Student's t-test for significance. Data are expressed as means ± SE. Specific differences between means with a $P \leq 0.05$ were considered significant.

3. METHODS

3.1. PART 2

3.1.1 Animals

CD2F1 transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase were generated at the A.I. Virtanen Institute for Molecular Sciences (University of Kuopio, Kuopio, Finland). The transgene construct for these mice was from a genomic sequence isolated 129 SVJ mouse genomic library as previously described (Marko Pietilä et al., 1997). The transgenic mice were propagated and maintained at the Grace Cancer Drug Center (Roswell Park Cancer Institute, Buffalo, New York). CD2F1 non-transgenic mice were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Experimental procedures involving the use of animals were approved by the University of Manitoba Faculty of Medicine Protocol Management and Review Committee

3.2 In *vivo* Experiments

Both transgenic and non-transgenic CD2F1 mice were injected s.c. with a dose of

3 mg/kg amantadine HCl (0.5 mg/ml). The stock amantadine for injection was prepared by dissolving amantadine HCl in normal saline for injection. The solution was then filtered into a sterile vial using a 0.22 μ GV Millex filter (Millipore Canada, Mississauga, Ontario) and stored at 4°C. The mice were placed in separate metabolic cages. The total urine was collected and washed from the sides of the metabolic cages with DDW at 3, 6, 9, and 24 hr after drug injection. The urine was frozen at –20° C until analyzed for acetylamantadine. Six hr after the last urine collection, the mice were injected s.c. with a dose of 80 mg/kg MGBG (Pegg *et al.*, 1985), dissolved in normal saline for injection just before each experiment, 18 hr later, animals were injected with amantadine HCl (3 mg/kg). The total excreted urine was collected as described above.

Acetylamantadine was quantified by gas-liquid chromatography as previously described (Bras *et al.*, 1998), and modified to improve sensitivity. The modified procedures used solid phase extraction as follows: Supelclean™ ENVI™-18 SPE tubes, 3 ml (Supelco, Bellafonte, PA.) were primed with 2 ml of methanol, and 2 ml of DDW, followed by 2 ml of 0.2 M sodium phosphate buffer pH 7.4. The mouse urine samples of 1.0 ml were mixed with 50 μl acetanilid (5 mg/l, dissolved in DDW), as an internal standard and 1.0 ml of buffer. The samples were loaded on the columns and allowed to permeate. The columns were washed with 2 ml 0.2 M sodium phosphate buffer followed by two washes of 2.5 ml DDW. Excess water was removed under low vacuum pressure. The column was eluted with 2 ml ethyl acetate to extract acetylamantadine and internal standard, and evaporated under a stream of nitrogen to dryness in a fume hood at room temperature. The residue was reconstituted with toluene (35 μl), allowed to stand for 30 min, and mixed for 2 min. Samples (1 μl) from the reconstituted residue were injected

into an Hewlett Packard model 5890 series II gas chromatograph using helium as the carrier gas and an Hewlett Packard high performance (crossed-linked methyl silicone gum) 25 m x 0.2 mm x 0.3 μm film thickness capillary column. The detector temperature was 250°C, injection port 250°C, and oven temperature was programmed so that the initial temperature was 150°C for 2 min, then increased by 10°C/min to 200°C and held for 1 min, increased by 70°C/min up to 240°C and held for 15 min. A nitrogen-specific detector was used.

Standard curves for acetylamantadine were derived from blank urine (diluted 1:10) with DDW or plasma (for subcellular metabolism studies) to which known amounts of acetylamantadine were added. The concentration range of the calibration curves for both urine and plasma was from 6.25 – 800 ng/ml. Plasma and urine assays for the quantitation of acetylamantadine were performed in duplicate. Duplicates differing by more than 10% were reanalyzed. Urine samples outside of the calibration range were diluted and reanalyzed. In urine, r^2 was 0.99 and C.V. = 10% (n = 6). In plasma, r^2 was 0.98 and C.V. = 16% (n = 3) over an 11 month period.

3.3 Enzyme Preparation

Transgenic and non-transgenic male CD2F1 mice weighing 25 to 38 g were anaesthetized with pentobarbital sodium (200 mg/kg) injected intraperitoneally. A laparotomy was performed through which the mice were sacrificed by sectioning of the aorta, and the liver was removed immediately and placed in ice-cold Tris buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5 (pH adjusted using NaOH)), 25 mM

KCl, 5 mM $MgCl_2$ and modified to include 1 mM EDTA, and 2.5 mM DTT to improve enzyme stability when homogenizing the liver (Matsui & Pegg, 1980; Wallace & Evans, 1998). The liver was blotted, weighed, and finely minced with a tissue chopper (Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, UK.) at 4°C. The minced tissue was placed in two volumes of the Tris buffer and homogenized using a Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland) for 2 min at a power setting of 6. The homogenate was centrifuged at 100,000 x g for 1 h at 4°C (Beckman L8-80M automatic refrigerated ultracentrifuge, with T80 centrifuge Rotor) and the supernatant served as the source of spermidine/spermine N^1-acetyltransferase (SSAT). Supernatant protein was determined using the Biuret reagent method (Gornall *et al.*, 1949), as follows: 30 µl of supernatant was mixed with 120 µl of DDW and 600 µl of Biuret reagent, providing a five times dilution of the supernatant, and compared to a standard curve constructed using crystalline bovine serum albumin with a concentration range between 1 and 10 mg/ml. The color was allowed to develop for 30 min and then read on a spectrophotometer at a wavelength of 540 nm (Pharmacia Biotech Ultraspec 4000, Pharmacia Biotech (Biochrom) Ltd., Milton Road, Cambridge, UK.). The dilution of the supernatant was necessary to prevent Tris homogenizing buffer from interfering with the Biuret assay (Price, 1996). To confirm that no interference was occurring, an experiment was performed where the bovine serum albumin was mixed with either Tris homogenizing buffer or water to give the same protein concentration (10 mg/ml). The Tris buffer bovine serum albumin mixture was diluted 5 x as described above and compared with albumin mixed with water after mixing with Biuret reagent and allowed to develop for 30

min. The results were identical for both (data not shown), indicating that this procedure provided results without interference from the Tris buffer.

3.4 Spermidine Acetylation Assay Procedures

The spermidine acetylation assays were completed by measuring the incorporation of radioactivity from [acetyl-1-^{14}C]-acetyl CoA (Matsui & Pegg, 1980), and modified as follows: tubes were prepared in triplicate by the addition of 20 µl spermidine dissolved in Tris-HCl buffer such that the concentrations in a final incubation volume of 100 µl were between 50 to 1000 µM for spermidine, and 100 mM Tris-HCl, pH 7.8 at 37°C (pH adjusted with NaOH). A solution (20 µl) containing 40 nCi of [acetyl-1-^{14}C]-acetyl CoA dissolved in DDW was added to the tubes. The supernatant was adjusted with Tris buffer to give a protein concentration in the incubation medium of 1 mg/ml in a final incubation volume of 100 µl. Assay blanks were prepared in the absence of spermidine (Sessa & Perin, 1991). The assay was initiated with the addition of 100,000 x g supernatant (60 µl) to the tubes and incubated for 10 min in a 37°C water bath with shaking (80 oscillations/min) in a Haake SWB 20 incubator (Fisons Instruments, Haake Mess-Technik GmbH, Karlsrutte, Germany) (Wallace & Evans, 1998). The reaction was terminated by the addition of 20 µl of ice cold aqueous 1M hydroxylamine HCl; tubes were briefly mixed with a vortex-type mixer, and placed on ice. The reaction mixture was then placed in a boiling water bath for 3 min. The tubes were centrifuged at 12,000 x g for 3 min to pellet the protein (Fisher Scientific Micro Centrifuge model 235c). An aliquot of the resultant incubation supernatant (50 µl) was

applied and allowed to permeate 2.5 cm cellulose phosphate paper discs (Whatman, P-81, Whatman International Ltd., Maidstone, UK) that were previously wetted with DDW and placed on a vacuum box. Negative pressure was applied and the discs were washed 5 times with DDW, followed by three washes with 1.0 ml 95% v/v ethanol. The dried discs were placed into scintillation vials containing 4 ml of Ready Safe scintillation fluid (Beckman Coulter Inc., Fullerton, CA) and counted in a Beckman model LS6000TA scintillation counter (Beckman Instruments Inc., Fullerton, CA). To determine SSAT activity, the nonspecific radioactivity of the blanks was subtracted from the total radioactivity of the samples containing the added spermidine.

3.5 Inhibition Studies

Inhibition of spermidine acetylation by SSAT was determined by the addition of amantadine, 1 to 10,000 μM, to fixed concentrations of spermidine, 50 to 80 μM. The NAT1 selective substrate PABA with a concentration range of 200, 500 and 1000 μM or the NAT2 selective substrate SMZ with range of 200, 500 and 700 μM were added to tubes containing spermidine (200 μM) and the acetylation reagents as described above. The PABA and SMZ were dissolved in 4% DMSO Tris-HCl buffer solution. The PABA and SMZ are first dissolved in 100% DMSO and then diluted using Tris-HCl buffer to the desired concentration of substrates; the final concentration of DMSO depended on the volume of buffer used for dilution. To ascertain that DMSO did not interfere with SSAT, two controls were run simultaneously, containing 200 μM spermidine dissolved in Tris-

HCl buffer with and without DMSO. Spermidine acetylation was quantified as described above. All assays were performed in triplicate.

3.6 Amantadine Acetylation *in vitro* assay

Amantadine was incubated with transgenic mouse liver 100,000 x g supernatant as the source of SSAT. An acetyl-CoA regenerating system was used as a source of acetate (Andres *et al.*, 1985). The assay was performed in 1.5 ml microcentrifuge tubes. The assay buffer contained 225 mM Tris-HCl, 4.5 mM EDTA, and 4.5 mM DTT (pH 7.5 at 37°C). In brief, 100 µl of acetyl-CoA (1 mM in DDW) and 100 µl of acetyl-CoA regenerating system (5.4 mg/ml acetyl-DL-carnitine HCl and 1 U/ml carnitine acetyltransferase dissolved in assay buffer) were added to the tubes. Then 200 µl of amantadine (50, 100, 200 µM) or a mixture of amantadine and SMZ (2500 µM) were dissolved in Tris-HCl buffer (pH 7.8) and added in manner such that the final incubation concentration of the buffer was 100 mM in the final volume of 1000 µl. To start the reaction, 600 µl of supernatant were added and incubated for 10 min at 37°C. The reaction was stopped by the addition of 200 µl of ice cold aqueous 1M hydroxylamine HCl and placed on ice. The protein was removed as previously described in the spermidine acetylation procedures section. The resultant incubation supernatant was frozen at –20°C until analyzed for acetylamantadine using gas liquid chromatography.

3.7 Malme-3M cell incubation with amantadine

SSAT in human malignant melanoma cultured cells (Malme-3M) has been shown to be greatly induced (100-200 fold) by a polyamine analogue N^{1},N^{11}-diethylnorspermine (Libby *et al.*, 1991). The cell incubations took place and were performed by personell at the Grace Cancer Drug Center (Roswell Park Cancer Institute, Buffalo, New York) and the analysis for acetylamantadine took place in our laboratory. The experiment was performed in the following manner. Malme-3M cells (2 x 10^{6} cells) were added to 30 ml of media placed in 150 x 25 mm dish; this was repeated for a total of 8 dishes. The cells were counted with a Z1 Coulter Counter. The cells were then plated overnight. The next day 4 dishes were incubated with the inducer, 10 μM N^{1},N^{11}-diethylnorspermine and 4 dishes without inducer. After incubating for 24 hours, the media was aspirated from the dishes and refreshed with new media. Then two dishes of the induced group were treated with 10 μM and the other two with 100 μM amantadine. The un-induced dishes are treated in the same manner. After incubating for 24 hours with amantadine present, 1.5 ml of media was removed and stored in a 1.8 ml Corning Cryostat vial and placed on ice. Once all samples were collected, they were placed in a $-70^{\circ}C$ freezer. The remainder of the media was aspirated from the plates and these were then washed with phosphate buffered saline (PBS). The cells were then trypsinized in trypsin/EDTA (GibcoBRL, 10 x Solution, diluted 1:10) for 3 minutes, and inactivated with a wash (RPMI with serum). One ml was taken for cell count. The cells were collected and pelleted in 50 ml Falcon tubes. The pellet was washed with 10 ml PBS and transferred to a 15 ml Falcon tube.

Cotton swabs eliminated any residual liquid. The samples were kept on ice at all times until the final sample is collected and then placed in a $-70^{\circ}C$ freezer.

3.8 Data Analysis

Data are expressed as mean ± SE of at least three experiments. Apparent K_m and V_{max} values were determined by nonlinear regression fit to the Michaelis-Menten equation with the computer program (Fig P. version 6.0a, Biososft, Ferguson, MO). IC_{50} values for inhibition of spermidine acetylation were determined using regressive probit analysis (Cheng & Prusoff, 1973). The complementary graphical methods of Dixon (1953) and Cornish-Bowden (1974) were used to assess the type of inhibition caused by amantadine. Urinary acetylamantadine excretion between transgenic mice with or without induction by MGBG was evaluated by the two-tailed Student's *t* test. Regression analysis was used to evaluate inhibitory activity of PABA and SMZ against spermidine acetylation. Differences between means with values $P \leq 0.05$ were considered significant. Analysis of variance (ANOVA) was used to detect significance among multiple groups.

4. Chemicals and reagents

Sucrose, potassium chloride, dithiothreitol (DTT), magnesium chloride ($MgCl_2.6H_2O$), perchloric acid, glacial acetic acid, anhydrous dimethylsulfoxide, acetonitrile, and hydroxylamine HCl were acquired from Fisher Scientific, (Fisher Chemical, Fair Lawn, New Jersey). Spermidine, Tris-HCl, EDTA, acetyl-coenzyme A (sodium salt), acetyl-DL-carnitine HCl, carnitine acetyltransferase (from pigeon breast muscle), sulfamethazine (SMZ), *p*-aminobenzoic acid (PABA), N-acetyl-*p*-aminobenzoic acid (AcPABA), triethanolamine-HCl, leupeptin, phenylmethylsulfonyl flouride, butylated hydroxytoluene, triethylamine, bovine serum albumin and methylglyoxal bis-(guanylhydrazone) (MGBG) were obtained from Sigma Chemical Company (St. Louis, MO.) and [acetyl-1-^{14}C]-acetyl CoA (58.9 mCi/mmol) was purchased from New England Nuclear (Boston, MA.). Amantadine HCl was provided by Dupont Canada Inc. (Mississauga, Ontario, Canada). Human albumin crystallized was purchased from Miles Scientific (Naperville, Illinois). N-acetyl-sulfamethazine (AcSMZ) was synthesized according to a previously published method (Svensson *et al.*, 1991).

5. RESULTS

5.1 *In vitro* Studies of Rat NAT2 and NAT1 Acetylation.

NAT2. Using the liver subcellular fraction from Sprague-Dawley rats as the source of the NAT2 enzymes, the apparent enzyme kinetic constants (Fig R-1) for acetylation of PABA, a selective substrate for NAT2 enzyme in the rat were, K_m = 10.8 ± 0.5 μM and the V_{max} = 0.93 ± 0.10 nmol/min/mg protein (mean ± SE). The acetylation of PABA in the presence of 1000 μM amantadine did not alter the apparent kinetic constants, which were K_m = 11.2 ± 1.1μM and V_{max} = 0.93 ± 0.05 nmol/min/mg protein (mean ± SE). This results indicates that amantadine had no affinity for the NAT2, therefore causing no inhibition of PABA acetylation that continued at the same rate.

NAT1. Using the liver subcellular fraction from Sprague-Dawley rats as the source of the NAT1 enzymes, the apparent enzyme kinetic constants (Fig R-2) for acetylation of SMZ, a selective substrate in the rat for NAT1 enzyme were, K_m = 580 ± 49 μM and the V_{max} = 0.30 ± 0.02 nmol/min/mg protein (mean ± SE). The acetylation of SMZ in the presence of 1000 μM amantadine did not alter the apparent kinetic constants, which were K_m = 579 ± 45 μM and V_{max} = 0.30 ± 0.02 nmol/min/mg protein (mean ± SE). The high K_m observed with the acetylation of SMZ, using Sprague-Dawley rat liver as the source of the NAT1 enzyme, indicates that SMZ has a low affinity for the enzyme. At the high SMZ concentrations used, the high K_m suggests also that SMZ is not a preferred substrate and that the enzyme acetylating SMZ is not NAT1. Nevertheless, the presence of amantadine did not alter SMZ kinetic constants for the enzyme.

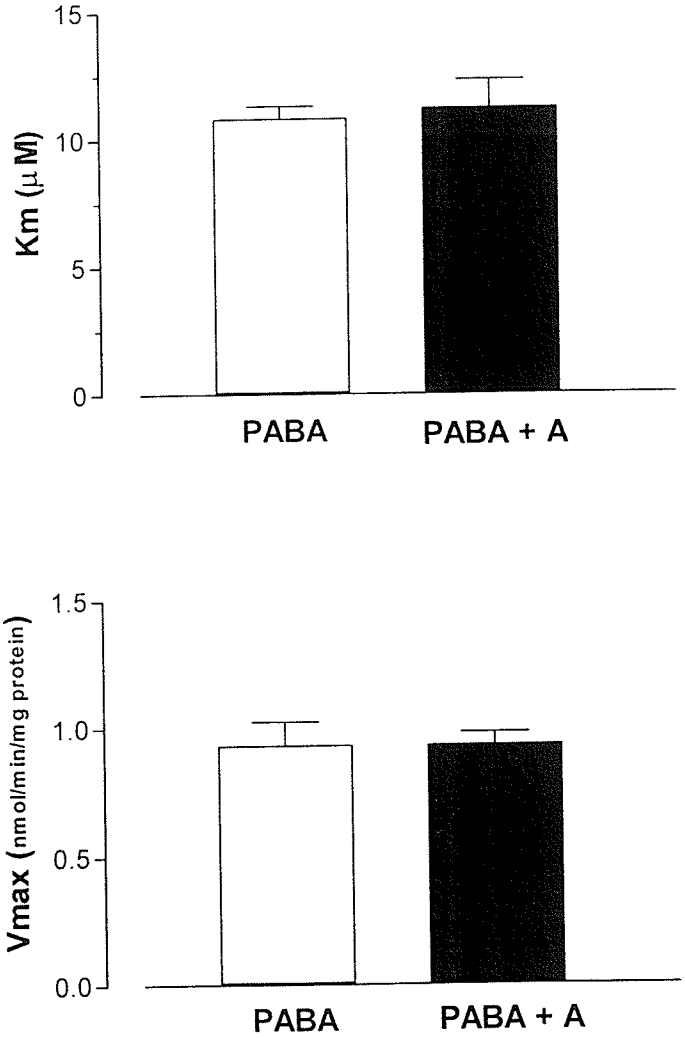


Figure R-1: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for *p*-aminobenzoic acid acetylation, the preferred substrate for NAT2, derived from rat liver 100,000 x g supernatant in the presence or absence of 1000 μM amantadine. Values are represented as mean ± SE of 4 separate experiments with different rats.

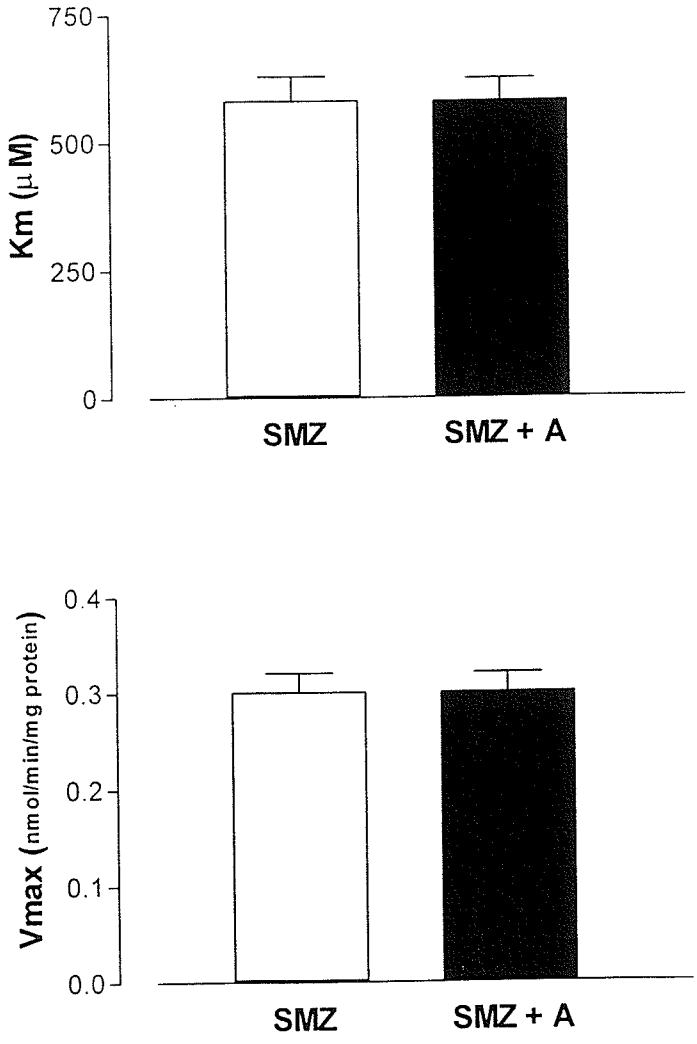


Figure R-2: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for sulfamethazine acetylation, the preferred substrate for NAT1, derived from rat liver 100,000 x g supernatant in the presence or absence of 1000 μM amantadine. Values are represented as mean ± SE of 4 separate experiments.

5.2 *In vitro* Studies of New Zealand White Rabbit Liver NAT1 and NAT2 Acetylation.

NAT1. Using the 100,000 x g liver supernatant from New Zealand white rabbit as a source of NAT1 enzymes, the apparent enzyme kinetic constants (Fig R-3) for acetylation of PABA, the selective substrate in the rabbit for NAT1 enzyme, were K_m = 94 ± 7 μM and the V_{max} = 5.8 ± 0.2 nmol/min/mg protein (mean ± SE). The addition of 1000 μM amantadine to the incubation medium did not alter the apparent kinetic constants of PABA acetylation, which were K_m = 91 ± 4 μM and the V_{max} = 5.8 ± 0.3 nmol/min/mg protein (mean ± SE). With the rabbit species, we also observed that the presence of amantadine did not inhibit the acetylation of PABA. These results suggest that amantadine has no affinity for rabbit NAT1. Therefore, amantadine is not a substrate for rabbit liver NAT1.

NAT2. Using the same 100,000 x g liver supernatant from New Zealand white rabbit as the source of the NAT2 enzymes, the apparent enzyme kinetic constants (Fig R-4) for acetylation of SMZ were, K_m = 119 ± 9 μM and the V_{max} = 7.3 ± 0.3 nmol/min/mg protein (mean ± SE). Incubation in the presence of 1000 μM amantadine did not alter the apparent enzyme kinetics of SMZ acetylation, and were K_m = 119 ± 12 μM and the V_{max} = 7.1 ± 0.5 nmol/min/mg protein (mean ± SE). The observation that amantadine did not inhibit the acetylation of SMZ in rat, was also consistent with the data obtained from the rabbit. This result suggests, again, that amantadine in this second species, has no affinity for the NAT2 enzyme.

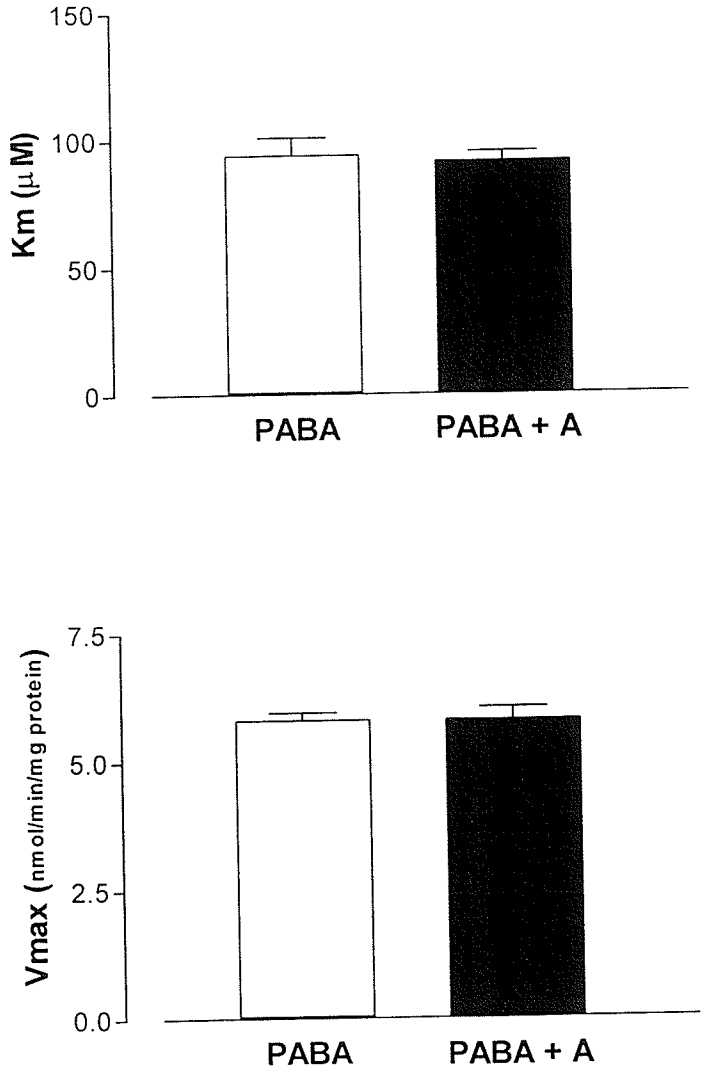


Figure R-3: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for *p*-aminobenzoic acid acetylation , the preferred substrate for NAT1, derived from rabbit liver 100,000 x g supernatant in the presence or absence of 1000 μM amantadine. Values are represented as mean ± SE of 4 separate determinations.

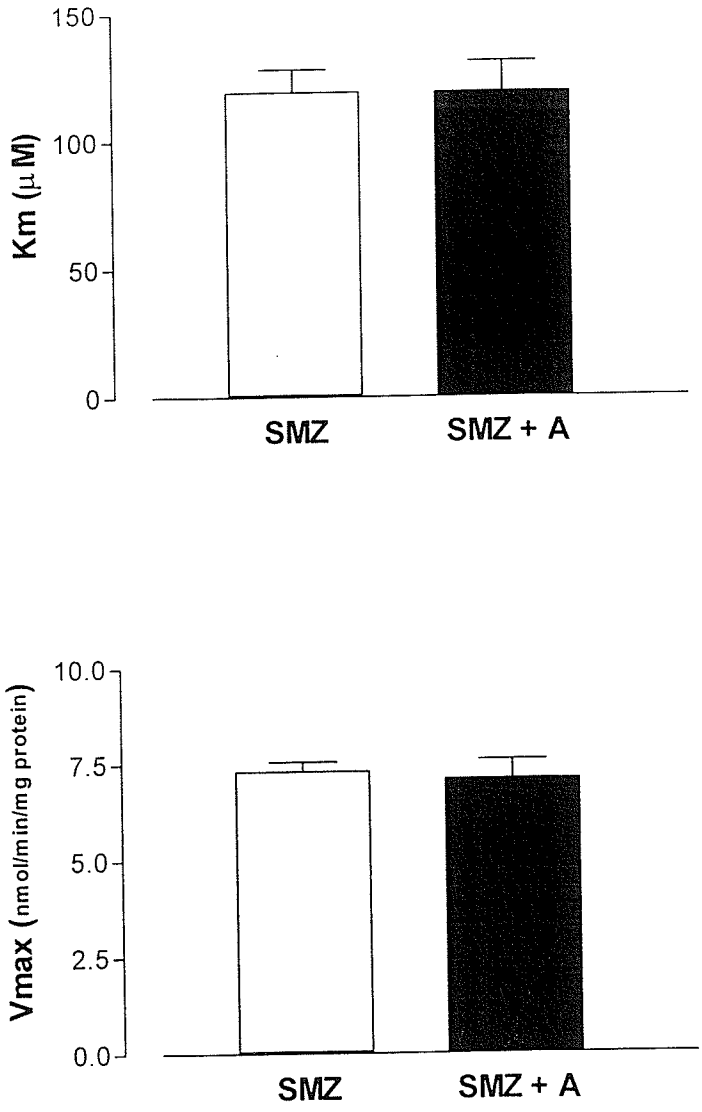


Figure R-4: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for sulfamethazine acetylation , the preferred substrate for NAT2, derived from rabbit liver 100,000 x g supernatant in the presence or absence of 1000 μM amantadine. Values are represented as mean ± SE of 4 separate determinations.

5.3 *In vitro* Studies Using Wild Type Human Recombinant NAT1 and NAT2.

NAT1. The wild type human recombinant NAT1 expressed in *E. coli* was our third enzyme species. The human recombinant NAT1 allowed us to determine if there was any species variation with respect to amantadine's ability to inhibit PABA acetylation. Using the *E. coli* lysates as our source of NAT1 enzymes, the apparent enzyme kinetic constants (Fig R-5) for acetylation of PABA were, K_m = 21.8 ± 2.4 μM and the V_{max} = 269 ± 32 nmol/min/mg protein (mean ± SE). PABA incubation in the presence of 1000 μM amantadine did not alter the apparent enzyme kinetics of PABA acetylation, and were K_m = 21.3 ± 1.3 μM and the V_{max} = 250 ± 24 nmol/min/mg protein (mean ± SE). Amantadine's inability to inhibit PABA acetylation, even a concentration of 1000 μM, suggests that it has no affinity for the human recombinant NAT1. Therefore, we found no species variation with respect to amantadine's inability to inhibit PABA acetylation. These results suggested that amantadine may not serve as a substrate for the human NAT1 enzyme.

NAT2. The wild type human recombinant NAT2 expressed in *E. coli* was our third enzyme species, the apparent enzyme kinetic constants (Fig. R-6) for acetylation of SMZ were, K_m = 108 ± 12 μM and the V_{max} = 6.2 ± 1.4 nmol/min/mg protein (mean ± SE). SMZ incubation in the presence of 1000 μM amantadine did not alter the apparent enzyme kinetics of SMZ acetylation, with the K_m = 110 ± 5 μM and the V_{max} = 6.1 ± 1.5 nmol/min/mg protein (mean ± SE) indicating that amantadine had no affinity for human recombinant NAT2 enzyme. Therefore, in all three species, no variation was found with respect to amantadine's ability to inhibit acetylation by NAT1 or NAT2.

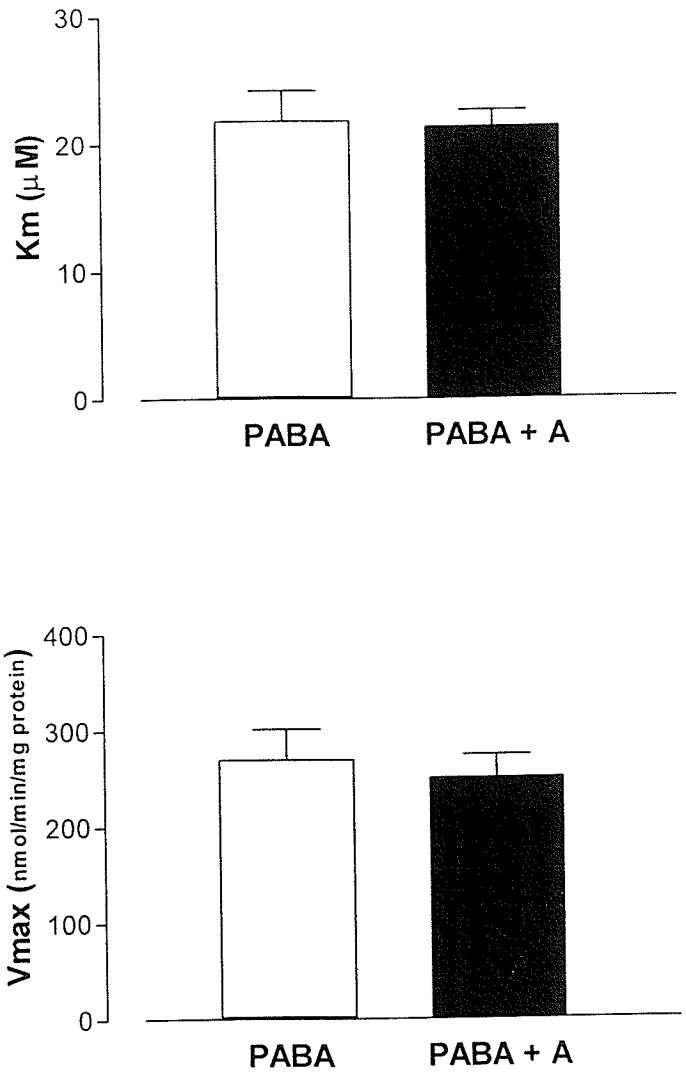


Figure R-5: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for *p*-aminobenzoic acid acetylation, the selective substrate for NAT1, from human recombinant NAT1 from *E. coli* lysates, in the presence or absence of 1000 μM amantadine. Values are reported as mean ± SE of 4 separate determinations.

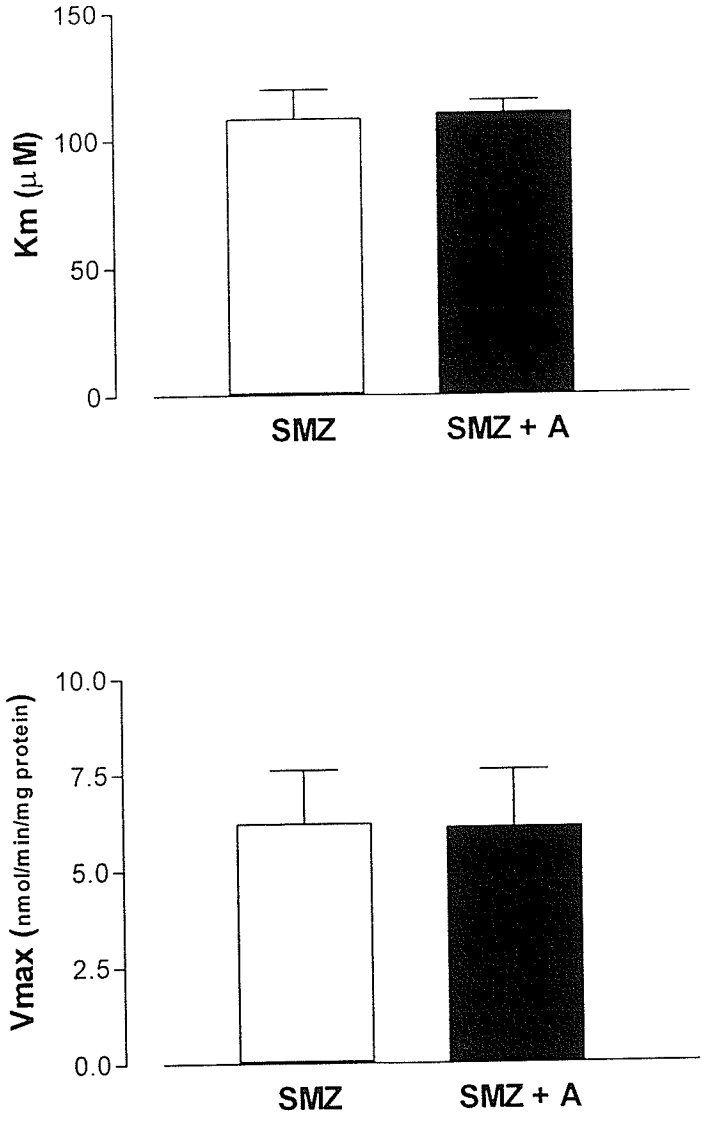


Figure R-6: Measured apparent K_m (upper panel) and V_{max} (lower panel) values for sulfamethazine acetylation, the selective substrate for NAT2, from human recombinant NAT2 from *E. coli* lysates, in the presence or absence of 1000 μM amantadine. Values are reported as mean ± SE of 4 separate determinations.

5.4 *In vitro* Incubations of Amantadine with NAT1 and NAT2

Amantadine incubations were performed with rat and rabbit 105,000 x g liver supernatant, and wild type human recombinant NAT1 and NAT2 enzymes that contained an acetyl-CoA regenerating system as the acetyl donor. These incubations took place in the absence of PABA and SMZ. No acetylamantadine was detectable from these incubations, even with the extended periods of incubation of 30 and 60 min, and concentrations of 10 to 1000 μM amantadine. These results suggested that NAT1 and NAT2 were unable to acetylate amantadine.

5.5 *In vitro* Amantadine Incubation with Pig Lung Tissue.

The NAT1 and NAT2 enzyme experiments demonstrated that they were unlikely to catalyze amantadine acetylation. Therefore, an as yet uncharacterized acetyltransferase enzyme may be acetylating amantadine. The acetyltransferase enzyme chosen for the next experiment was the inducible spermidine/spermine N^1-acetyltransferase (SSAT). The tissue chosen was pig lung. Two different buffer systems (TEDK and Cross-Taggart) were used to homogenize the lung tissue. The two buffer systems were used to increase the likelihood that the SSAT enzyme might function optimally in one of the buffers. Using both crude and 9000 x g lung homogenates in the two buffers, we incubated these tissue preparations with the acetyl-CoA regenerating system as the acetyl donor and 1000 μM amantadine. The crude and 9000 x g homogenates were used in case they contained a cofactor needed by the SSAT enzyme.

The incubation periods were 30 and 60 min. No acetylamantadine was detected in the supernatant in these extended periods of incubation and crude homogenate preparations. At this time we did not realize that in order for there to be sufficient concentrations of the SSAT enzyme for in vitro experiments to take place, induction of the enzyme was required.

5.6 EXPERIMENTS USING TRANSGENIC MICE OVEREXPRESSING SSAT

5.6.1 *In vivo* Studies of Amantadine Acetylation.

Urine samples from amantadine-treated CD2F1 transgenic mice overexpressing SSAT consistently demonstrated metabolism of the parent compound to acetylamantadine in all timed collection periods (Fig R-7). The acetylamantadine excreted in the urine as a cumulative percent of administered dose at 24 hr ranged between 2-6% with a mean of 4.5 ± 1.0 %. Subsequently the CD2F1 transgenic mice were injected s.c. with a known inducer of SSAT, methylglyoxal bis-(guanylhydrazone) (MGBG), and served as their own controls. At 24 hr, the acetylamantadine excreted as a cumulative percent of administered dose was 4.5 ± 1.0 %, not different from the initial result without MGBG treatment. The urine samples from CD2F1 non-transgenic control mice and those treated with MGBG contained no acetylamantadine after a dose of amantadine.

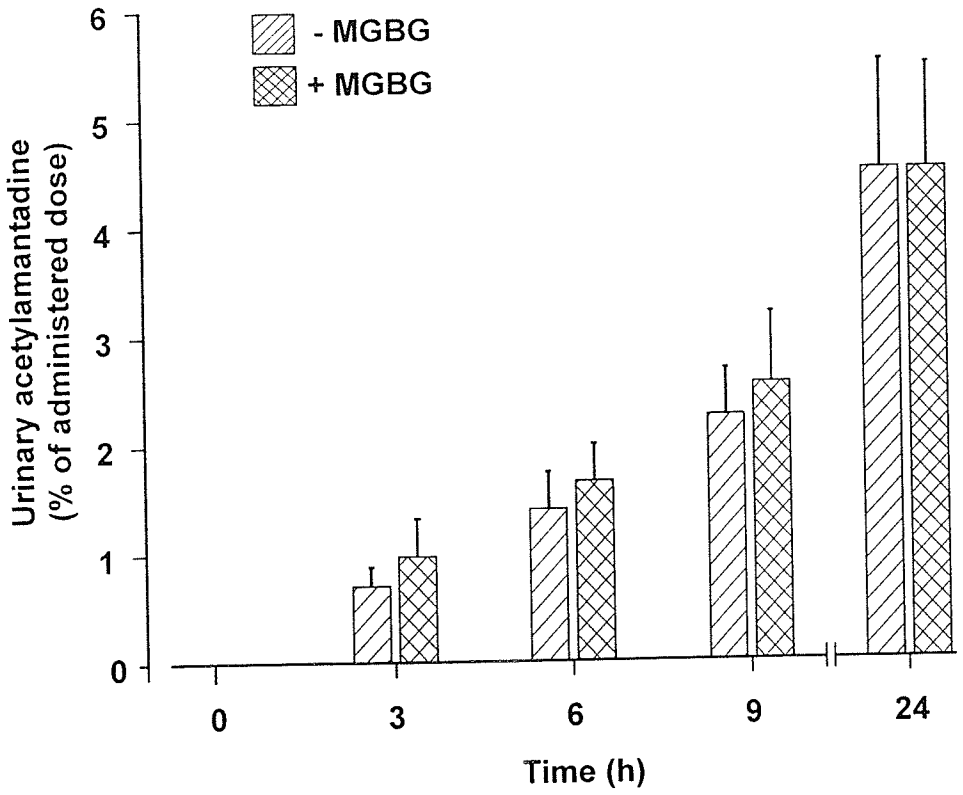


Figure R-7: Urinary excretion of acetylamantadine by transgenic mice overexpressing SSAT and injected with amantadine HCl (3mg/kg) either before (-) or after (+) treatment with MGBG, an inducer of SSAT. Control non-transgenic mice undergoing the same procedures did not excrete acetylamantadine in their urine. The values reported are mean ± S.E. of 4 separate experiments. The timed collections for each treatment group were compared by two tailed Student's t-test.

5.6.2 *In vitro* Studies of Spermidine Acetylation

A representative non-linear velocity versus substrate concentration plot for spermidine acetylation by SSAT is shown in Fig R-8. The kinetic parameters derived from these plots indicate an apparent K_m of 267 ± 46 μM and a V_{max} of 0.009 ± 0.002 nmol/min/mg protein (n = 10, different mice). Using the non-transgenic mouse liver supernatant as a source of SSAT, we were not able to detect spermidine acetylation.

5.6.3 Amantadine Inhibition Studies

We first evaluated the ability of amantadine to inhibit spermidine acetylation by including it with spermidine incubations. The addition of a therapeutic concentration of amantadine (2.5 μM) impeded the acetylation of spermidine, indicating it could serve as a substrate for the SSAT enzyme (Fig R-8). Subsequently, inhibition studies using fixed concentrations of spermidine (50 μM) and various concentrations of amantadine ranging from 1 to 10,000 μM were completed. A representative inhibition profile (Fig R-9) showed complete inhibition of the SSAT enzyme at 10,000 μM amantadine. Amantadine inhibition profiles were used to determine IC_{50} values for spermidine acetylation. Representative regressive probit plots of these data are shown in Fig R-10. From the IC_{50} values, the inhibitor dissociation constant (K_i) was calculated. Dixon and Cornish-Bowden analyses supported the interpretation that amantadine inhibition of spermidine

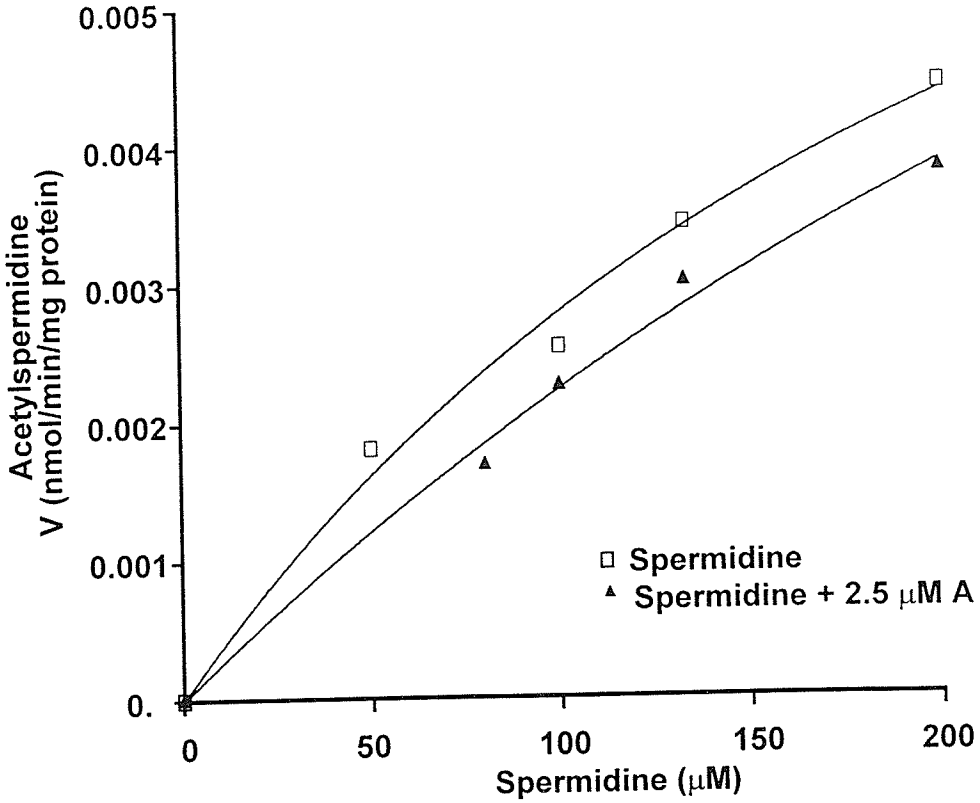


Figure R-8: A representative plot of V vs. S demonstrating spermidine acetylation by transgenic mouse liver 100,000 x g supernatant containing SSAT with apparent K_m = 263 μM and V_{max} = 0.010 nmol/min/mg protein (r^2 = 0.99) □. The addition of a therapeutic concentration (2.5 μM) of amantadine (A) causes inhibition of spermidine acetylation and an increase in the apparent K_m to 542 μM and V_{max} to 0.014 nmol/min/mg protein (r^2 = 0.99), and suggests that amantadine may be a substrate for SSAT.

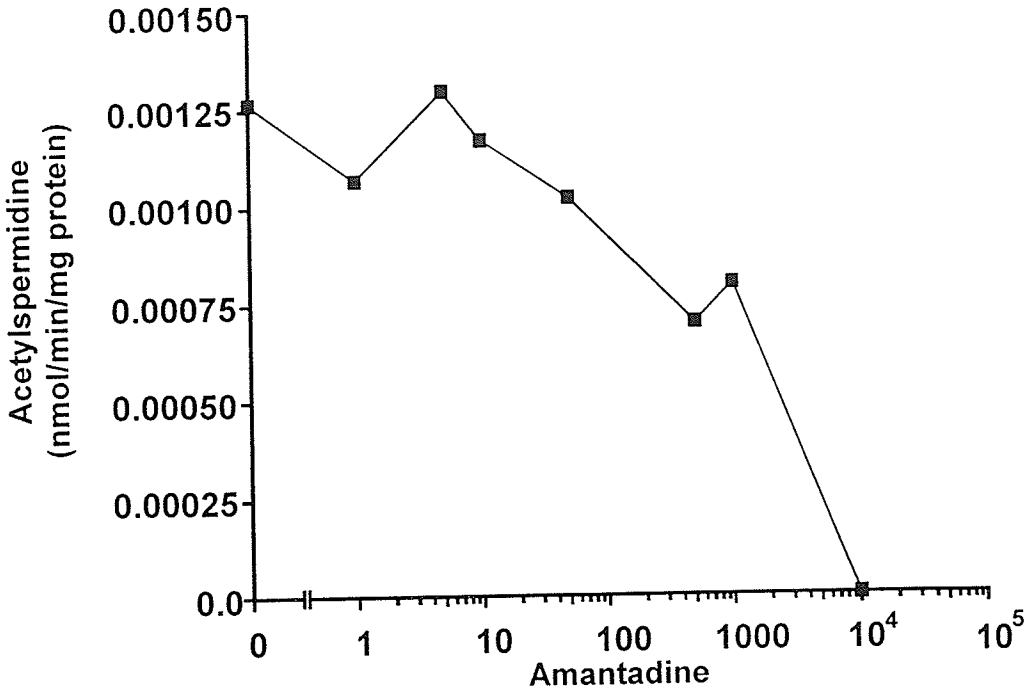


Figure R-9: A representative plot for the inhibition of acetylspermidine production by increasing amantadine concentrations in the presence of 50 μM spermidine. Spermidine acetylation is completely inhibited by 10,000 μM amantadine.

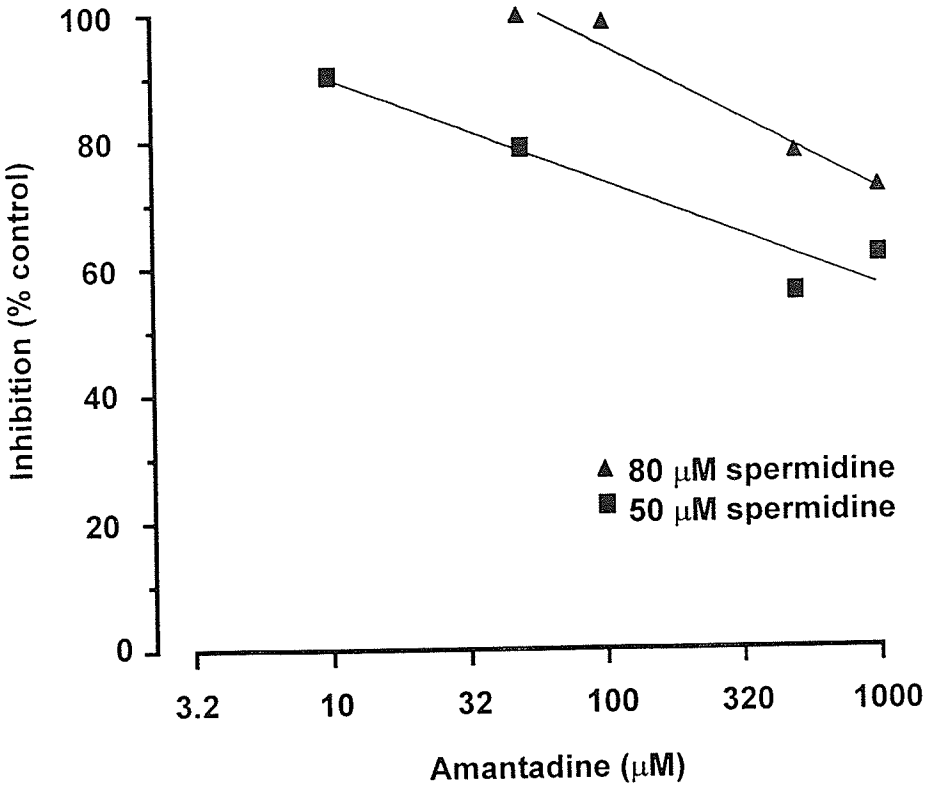
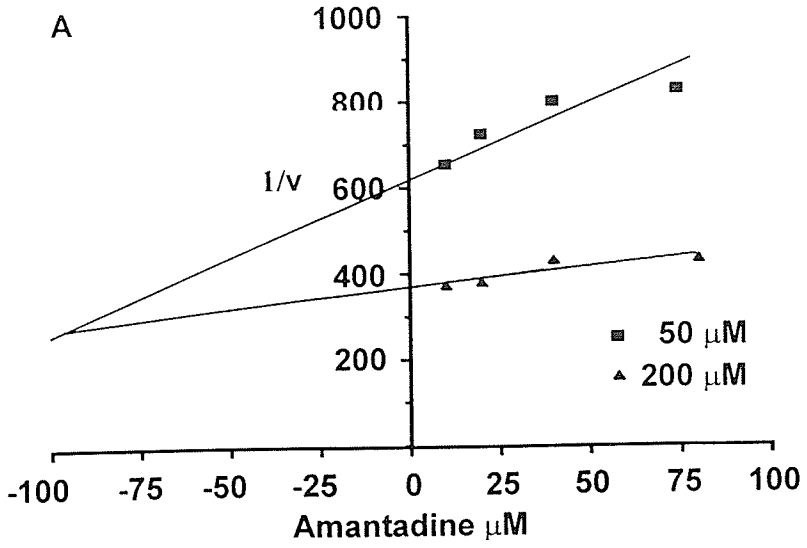


Figure R-10: Representative regressive probit plots of two spermidine concentrations (50 μM and 80 μM) with increasing amantadine (inhibitor) concentrations to determine IC_{50} values.



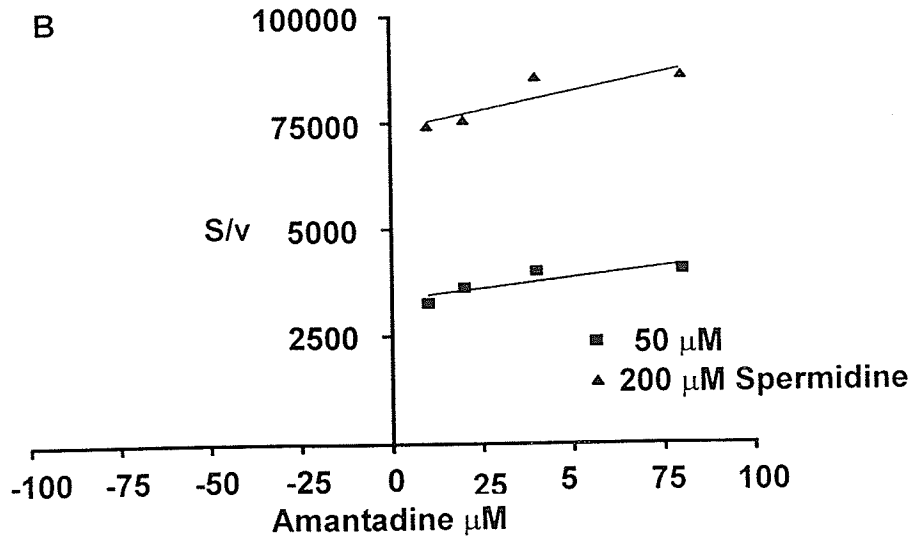


Figure R-11: A. Representative Dixon plot of 1/v versus [I], showing the inhibition of acetylspermidine production by increasing amantadine [I] concentrations in the presence of 50 μM and 200 μM spermidine. The intersection of the two lines to left of y-axis and above the negative x-axis indicate the inhibition could be competitive or mixed inhibition. B. Representative Cornish-Bowden plot S/v versus [I]of the same data as in A. The two parallel lines in the plot indicate competitive inhibition.

acetylation was consistent with competitive inhibition (Fig R-11A and R-11B), and the use of IC_{50} values to calculate the K_i (Cheng & Prusoff, 1973). The apparent IC_{50} and K_i values were 935 ± 191 μM and 738 ± 157 μM respectively (n = 13).

Inhibition studies were undertaken to determine if PABA and SMZ, selective substrates in humans for NAT1 and NAT2 respectively, would inhibit SSAT in the mouse. Fig R-12A demonstrates the ability of SMZ (n = 4) to inhibit SSAT activity in a concentration related manner and suggests the potential for it to serve as substrate for SSAT. Assuming SMZ was competitively inhibiting spermidine acetylation, we calculated an apparent K_i of 3511 μM. When PABA was used, no inhibition was observed (Fig. R-12B) suggesting that PABA may not be a substrate for SSAT.

5.6.4 Amantadine Acetylation studies

Transgenic mouse liver supernatant, containing overexpressed SSAT and incubated with three concentrations of amantadine in the presence of an acetyl CoA regenerating system as the acetyl donor, produced modest amounts of acetylamantadine (Fig. R-13) that did not increase with substrate concentration over the narrow range studied. Co-incubation of amantadine with SMZ inhibited the production of acetylamantadine.

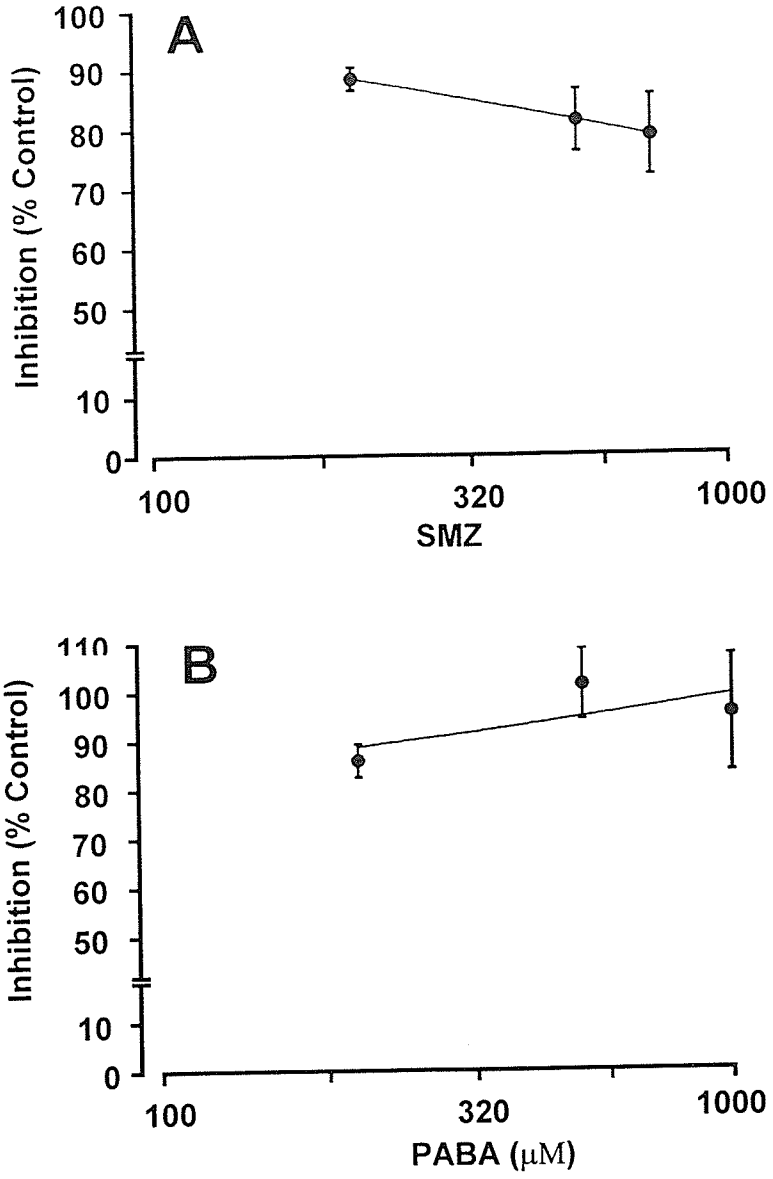


Figure R-12: A. Using a fixed concentration of spermidine (200 μM), its acetylation by SSAT is inhibited by SMZ (200, 500, 700 μM), an NAT2 selective substrate in humans. SMZ has a calculated K_i = 3511 μM. Each data point is representative of the mean ± SE of 4 separate experiments, r^2 = 0.99. B. Using fixed concentrations of PABA (200, 500, 1000 μM), an NAT1 selective substrate in humans did not inhibit spermidine acetylation. Each data point is representative of the mean ± SE of 5 experiments, r^2 = 0.23.

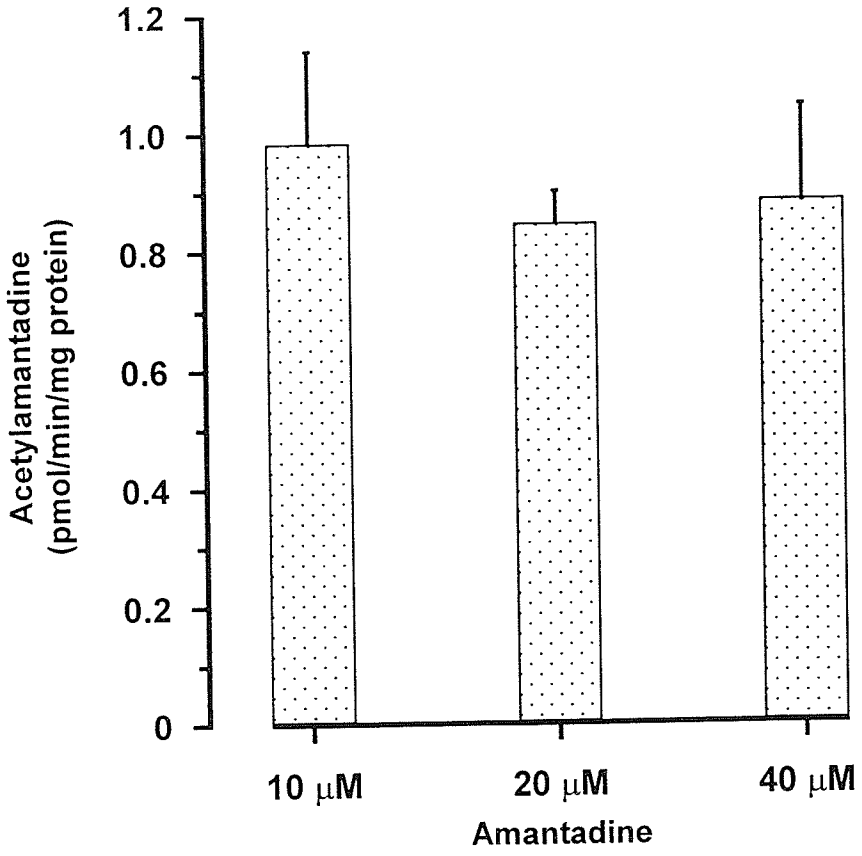


Figure R-13: Acetylamantadine production after a 10 min incubation of amantadine with 100,000 x g liver supernatant as a source of SSAT from transgenic mice, and an acetyl CoA regenerating system. Values are represented as mean ± SE of 3 separate experiments. Groups were compared by repeated measures ANOVA. No differences among treatment groups were found.

5.6.5 Amantadine Incubation with MALME-3M Cells

SSAT in cultured human malignant melanoma cells (Malme-3M) were induced by a polyamine analogue N^{I},N^{II}-diethylnorspermine. Two different concentrations of amantadine (10 and 100 μM) were added to the incubation medium. After incubating the cells in the presence amantadine for 24 hr, an aliquot of the incubation medium was analyzed for the presence of acetylamantadine. No acetylamantadine was detected in incubation medium for both amantadine concentrations used. This result suggestes two possibilities; First, the amount of acetylamantadine produced by the cells was too dilute in the volume of the medium for our limits of detection or there was an insufficient number of cells in the medium to produce the amount of acetylamantadine needed for dectection. Therefore, in our methods we needed to increase the volume of cellular growth medium used in the solid phase extraction procedure to increase the likelihood of acetylamantadine detection. The second possibility is that the MALME-3M cells may not uptake amantadine to be metabolized. To characterize this possibility, MALME-3M cells could be incubated with radiolabelled amantadine present in the medium. The presence of radiolabled amantadine within cells would confirm that amantadine is taken up by the MALME-3M cells.

6. DISCUSSION

The most important finding of this study was the demonstration that amantadine was acetylated by SSAT, and that it may be a previously unrecognized drug acetylating enzyme. In addition, this study also demonstrated that for SSAT to be a drug acetylating enzyme, it must be induced from basal levels. In this study we demonstrated that amantadine is selective for SSAT, and therefore could be used to distinguish acetylation that is proceeding by NAT1 or NAT2 from SSAT. Furthermore, a small but potentially relevant interaction between SSAT and the NAT2 selective substrate SMZ was observed.

Amantadine was initially reported not to be subject to metabolism in humans (Bleidner *et al.*, 1965). Acetylamantadine was first detected in the urine of three healthy young male volunteers who ingested a 200 mg dose of amantadine (Koppel & Tenczer, 1985). Since that report no further research was conducted to localize the enzyme site responsible for the N-acetylation of amantadine until the studies from our laboratory were done.

Our initial hypothesis was that amantadine, a primary amine, was not metabolized by the NAT1 and/or NAT2 enzymes. In order to determine if the polymorphic NAT1 or NAT2 participated in the N-acetylation of amantadine, *in vitro* experiments were carried out. We used three sources for the NAT1 and NAT2 enzymes, rat liver, rabbit liver, and human recombinant wild type NAT1 and NAT2 expressed in *E. coli*. The kinetic parameters K_m and V_{max} for NAT1 and NAT2 with the selective substrate PABA and SMZ respectively, were similar to previous reports (Andres & Weber, 1986; Dupret & Grant, 1992; Hickman *et al.*, 1995; Tannen & Weber, 1979). The addition of amantadine

to the selective substrate in the same incubation medium did not inhibit their acetylation (Bras *et al.*, 1998). Incubations of amantadine without the selective substrates demonstrated that it was not acetylated by NAT1 or NAT2 (Bras *et al.*, 1998). These findings demonstrated that the NAT1 and NAT2 enzymes were not the catalytic enzymes responsible for the N-acetylation of amantadine.

To determine the responsible enzyme, we proposed our second hypothesis that the enzyme catalyzing the acetylation of amantadine was SSAT.

Our *in vivo* data showed that only transgenic male mice overexpressing SSAT excreted acetylamantadine in their urine. These mice contain at least 20 copies of the SSAT transgene, and have a level of SSAT activity in the liver that is 4 fold higher than seen in nontransgenic mouse liver (Pietila *et al.*, 1997), strongly suggesting that SSAT is the enzyme catalyzing the acetylation of amantadine and possibly other primary amine-containing drugs and xenobiotics. Futhermore, our laboratory previously reported that Sprague-Dawley rats receiving the same therapeutic dose of amantadine did not excrete acetylamantadine in their urine (Goralski *et al.*, 1999). These results are similar to our study showing that the non-transgenic mice of the same strain as the transgenic mice also failed to excrete acetylamantadine in their urine. These observations strongly suggest the need for increased levels of SSAT to be present before the acetylation of amantadine can occur. The percentage of the administered dose excreted by the transgenic mouse in their urine as acetylamantadine was similar to that shown in previous reports in humans (Bras *et al.*, 1998; Koppel & Tenczer, 1985).

To determine if further induction could be achieved, MGBG that has been reported to be an inducer of SSAT in rat liver, increasing its activity by 7 to 700 fold, was

used (Karvonen & Poso, 1984; Pegg *et al.*, 1985; Persson & Pegg, 1984). Under the experimental conditions in this study, the percentage of acetylamatandine excreted in the urine of transgenic mice did not increase. In addition, the same treatment with the non-transgenic mice of the same strains did not result in any detectable acetylamantadine in their urine. The failure to see an increase in the percentage of acetylamantadine in transgenic mice and no acetylamantadine in the non-trangenic mouse urine suggests that MGBG may not be active as an SSAT inducer in the mouse model. There are two possible explanations for these observations. First, mice excrete three quarters of the MGBG in 24 hr (Oliverio *et al.*, 1963), suggesting that the levels of MGBG at 18 hr may be insufficient to maintain induction of SSAT, the time at which we injected the amantadine. Alternatively, although induction in rats occurred by 18 hr, it is possible that insufficient time had elapsed for the induction to have taken place the in the mouse (Pegg *et al.*, 1985). Secondly, the mouse may handle MGBG differently then the rat, suggesting a species difference. In addition Porter *et al.*, suggested that the effects of MGBG may be reversed more rapidly or to a greater extent in the presence versus the absence of spermidine (Porter *et al.*, 1981).

Our *in vitro* data using the transgenic mouse liver supernatant as the source of SSAT implicates it as the enzyme that acetylates amantadine, giving support to our *in vivo* observations. Our data demonstrated that when amantadine was incubated with the transgenic mouse liver derived supernatant in the absence of spermidine, modest amounts of acetylamantadine were produced. This observation is consistent with low amounts of acetylation that occur in humans when they ingest amantadine (Bras *et al.*, 1998; Koppel & Tenczer, 1985). These observations are further supported by our in vitro data that

showed that neither NAT1 nor NAT2 could explain amantadine acetylation (Bras *et al.*, 1998). Our interpretation of these result suggests that amantadine may be a specific substrate for SSAT, eventhough its capacity and affinity for the enzyme are low. This interpretation is consistent with the small amount of acetylated amantadine observed in the urine after a dose of the drug, and is reflected in the apparent high K_m and low V_{max} values seen in our *in vitro* studies. In contrast, the non-transgenic mice have inherently low levels of SSAT in their livers; *in vitro* experiments were performed and no acetylation of spermidine was detected. In addition, amantadine was not acetylated in our *in vitro* experiments using pig lung supernatant as the source of SSAT. In support of these findings, previous studies using non-induced and non-transgenic rat-derived liver supernatant revealed no detectable levels of acetylputrescine or acetylspermidine were produced (Blankenship & Walle, 1977; Seiler & al-Therib, 1974).

Drugs that contain the substituted diaminopropane structure that resembles part of spermidine, have been acetylated by SSAT. For *in vitro* experiments using human SSAT expressed in *E. coli*, Parry *et al.*, demonstrated that the antitumor and immunosuppressive agent 1-deoxyspergualin, a metabolite of amifostine, the radioprotective and chemoprotective agent S-2-(3-aminopropylamino)ethanethiol (WR1065), and the spermine synthase inhibitor N-(*n*-butyl)-1,3-diaminopropane were acetylated by SSAT (Parry *et al.*, 1995). However, besides these three drugs, no other studies have been reported that examined drugs that are not diaminopropane substituted as potential substrates for SSAT.

The other two potential substrates for SSAT that we examined in our in vitro experiments were the prototypical substrates of NAT1 and NAT2, SMZ and PABA,

respectively. Interestingly, the NAT2 but not the NAT1 selective substrate, interacted with SSAT in a concentration dependent manner and inhibited spermidine acetylation. In addition, when we incubated amantadine and SMZ together, no acetylamantadine was detected in the incubation supernatant. This finding suggests that SMZ inhibited amantadine acetylation, and for this to occur, it had to interact with SSAT. Since SMZ interacted with transgenic mouse SSAT with an apparent K_m of 267 ± 46 μM toward spermidine, the expectation is that the affinity of the human enzyme for SMZ would be greater since human SSAT has an apparent K_m of 55 μM for spermidine (Libby *et al.*, 1991). Furthermore, the concentration of the spermidine used in these experiments was close to the apparent K_m (200 μM). Possibly at these concentrations the weak inhibitory effect of SMZ was reversed more readily than if the concentration was lower. Therefore, for SMZ inhibition to occur and overcome the substrate concentration, significantly higher concentrations of inhibitor were required, resulting in the high calculated K_i. Although, the concentration of SMZ used exceeded therapeutic concentrations by several fold, these observation suggest that NAT2 selective substrates may interact with SSAT when it is induced by drugs, experimental and pathological processes. It remains to be determined whether SSAT can acetylate SMZ.

SSAT, the rate-limiting enzyme in the catabolic pathway, is ubiquitously distributed in mammalian tissues and plays a regulatory role in maintaining spermidine and spermine homeostasis (Seiler, 1987). Normally, SSAT is present in very small amounts in mammalian cells. It has been estimated that less then 1000 molecules are present in a rat hepatocyte, compared to 60,000 molecules in an induced cell (Matsui & Pegg, 1981; Pegg *et al.*, 1982). Induction of mammalian cells can result from a number

of factors, including various drugs, growth factors, hormones, polyamines, polyamine analogues, and toxic substances and pathological processes (Casero & Pegg, 1993). The induction of mammalian tissues with these factors can result in increased levels of SSAT that in turn may serve as a potential acetylator of primary amine-containing compounds.

Induction or over-expression of SSAT is usually required for there to be sufficient SSAT enzyme present in cells or 100,000 x *g* supernatant before in vitro experiments can be successfully undertaken (Casero & Pegg, 1993; Fogel-Petrovic *et al.*, 1997; Matsui & Pegg, 1980; Pietila *et al.*, 1997). In our study, we incubated amantadine with induced and non-induced malignant melanoma cells and acetylamantadine was not detected in the growth medium in either case. The volume of the medium used for the solid phase extraction may contain inadequate levels of acetylamantadine for our capability to detect it. Therefore, further methodological development is required to determine if acetylamantadine is produced by these cells. The development would include increasing the volume of the medium used for the solid phase extraction, determine if the cells uptake amantadine and use the cytosolic fraction from these cells to determine if SSAT could acetylate amantadine. It is also possible that this cell model is inappropriate to determine if amantadine is acetylated by malignant cells.

However, in pathological states cellular polyamine levels are increased along with increased levels of N^1-acetylspermidine and polyamines excreted in the urine (Morgan, 1998; Russell, 1971; Suh *et al.*, 1997). The increased levels of N^1-acetylspermidine observed in the urine of cancer patients suggests increased SSAT activity. Interestingly, in pathological states, it has been demonstrated that NAT acetylation activity also increases. (Dilman *et al.*, 1976), demonstrated that the growth of transplantable tumors

involves an intensification of sulfadimidine acetylation and treatment of the tumors caused a decrease in the rate of acetylation. However, when tumor suppression failed, the rate of acetylation did not diminish (Dilman *et al.*, 1976). In support of this finding, the level of N-acetylation of sulfadimidine in cancer patients is higher in both fast and slow acetylator phenotypes, compared to corresponding controls (Bulovskaya *et al.*, 1978; Chekharina Ye *et al.*, 1978). Effective treatment of malignant lymphoma caused a decrease in sulfadimidine acetylation (Chekharina Ye *et al.*, 1978). It has been assumed that the increased acetylation activity could be attributed to NAT. However, it is generally accepted that NAT1 and NAT2 are constitutive enzymes, and no induction is necessary to enhance enzyme concentrations before 100,000 x g supernatant is used for in vitro experiments. In addition, induction of NAT2 has only been demonstrated to occur in mouse kidney under the influence of glucocorticoid or androgen (Estrada-Rodgers *et al.*, 1998).

Our data suggest that the increase in acetylation observed in pathological states may be attributable to the increased SSAT activity. The levels of SSAT activity in malignant tissues are greatly increased compared to normal tissues. Cancers with increased levels levels of N^1-acetylspermidine the metabolite of SSAT, include gastric carcinoma, ovarian cancer, acute myelocytic leukemia, lymphoma, breast cancer, liver cancer, renal cancer, colorectal cancer, prostate cancer and others (Kingsnorth & Wallace, 1985; Pine *et al.*, 1989; Sessa & Perin, 1991; Suh *et al.*, 1997; Takenoshita *et al.*, 1984). The increased expression and activity of SSAT seen in cancer cells prevents polyamines from reaching levels that would be toxic to the cell (Bettuzzi *et al.*, 2000). The above observations strongly support the interpretation that the increase in acetylation

of NAT2 substrates observed in malignancy probably is due to increased levels of SSAT enzyme. However, this hypothesis remains to be tested.

Interestingly, basal levels of SSAT have been shown to increase in the presence of ethanol (Casero & Pegg, 1993; Perin & Sessa, 1993). Olsen & Morland, observed that when volunteers ingested alcohol concurrently with the NAT2 substrate sulfadimidine, enhanced acetylation of the substrate occurred in both fast and slow acetylators compared to controls. The same phenomenon was also demonstrated with procainamide, and these authors suggested that another pathway uninfluenced by acetylator phenotype or ethanol could possibly be responsible for the enhanced acetylation that they observed (Olsen & Morland, 1978;Olsen & Morland, 1982). INH half-life was reduced in two test subjects when alcohol was ingested concurrently (Lester, 1964), and in male rats, ethanol increased urinary excretion of N-acetylisoniazid (Thomas & Solomonraj, 1977). These observations that SSAT induction by ethanol may explain increased acetylation of NAT2 selective substrates presents a testable hypothesis. Amantadine could be used to differentiate between acetylation by SSAT and NAT, since it is not subject to acetylation by either NAT1 or NAT2. Furthermore, amantadine could be used to determine if there are increased levels of SSAT in the body that could indicate the presence of malignancy.

In conclusion, our initial question was to determine how amantadine acetylation occurred, and this led us to the finding that SSAT may be a previously unrecognized drug acetylating enzyme. Our data suggest that amantadine is a novel drug substrate that can be used to evaluate and detect SSAT activity. Our results also suggest the potential for SSAT to contribute to the acetylation of NAT2 selective substrates when it is induced. Further investigation of drugs and xenobitotics that undergo acetylation are warranted to

determine if they are substrates for SSAT. Exposure to alcohol may affect drug acetylation by increasing SSAT's participation. Amantadine, being a specific substrate for SSAT, could be used to evaluate SSAT contribution to drug acetylation when there is concurrent alcohol consumption.

The identification of SSAT as the site of amantadine acetylation, raises the possibility that this drug may be used as a novel agent in identifying patients with cancer. As discussed above, tumor cells have a much higher SSAT activity. Our findings support the interpretation that amantadine may be metabolized by the increased activity of the enzyme. The excreted metabolite could be used to indicate if further investigation for cancer is warranted. Therefore, a clinical trial is the next step to determine if amantadine is acetylated in patients with cancer and if the metabolite production can be related to specific types of cancer. This finding, if positive, would be a novel approach to cancer diagnosis. This approach would be minimally invasive and cost effective compared to other cancer diagnostic procedures in current use.

In future fundamental studies, human recombinant SSAT may be used in a purified form to conclusively prove that this is the site of amantadine acetylation. Further studies using the recombinant enzyme will determine if SMZ inhibits spermidine acetylation and is acetylated by this enzyme in the absence of spermidine. It will also be essential to determine if other NAT1 and NAT2 substrates (arylamines, hydrazines, and primary amines) inhibit or are acetylated by SSAT using the human recombinant enzyme. This finding would establish SSAT, when induced or overexpressed, as a contributer to acetylation of amino-containing drugs.

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