Characterization of acetylator genotype-dependent and -independent hamster hepatic n-acetyltransferases and their role in the metabolism of arylamine and n-hydroxyarylamine carcinogens

Alma C. Trinidad
Atlanta University

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CHARACTERIZATION OF ACETYLATOR GENOTYPE-DEPENDENT
AND -INDEPENDENT HAMSTER HEPATIC N-ACETYLTRANSFERASES
AND THEIR ROLE IN THE METABOLISM OF ARYLAMINE AND N-HYDROXYARYLAMINE CARCINOGENS

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SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
ALMA C. TRINIDAD

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA
JULY, 1989
Doctor of Philosophy Dissertation

of

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Advisor: Dr. David W. Hein
Dissertation dated July, 1989

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To my mother (Ines) and my sister (Sandra),
Los Santos, and Los Protectores for their love,
support, guidance, and understanding.
CHARACTERIZATION OF ACETYLATOR GENOTYPE-DEPENDENT AND -INDEPENDENT HAMSTER HEPATIC N-ACETYLTRANSFERASES AND THEIR ROLE IN THE METABOLISM OF ARYLAMINE AND N-HYDROXYARYLAMINE CARCINOGENS

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ACKNOWLEDGEMENTS

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<tr>
<td>AAF</td>
<td>2-Acetylaminofluorene</td>
</tr>
<tr>
<td>ABP</td>
<td>4-Aminobiphenyl</td>
</tr>
<tr>
<td>AcCoA</td>
<td>Acetyl Coenzyme A</td>
</tr>
<tr>
<td>ADT</td>
<td>Arylhydroxamic acid deacetylase</td>
</tr>
<tr>
<td>AF</td>
<td>2-Aminofluorene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMAB</td>
<td>Dimethylaminobenzaldehyde</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>x g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>KD</td>
<td>Kilodalton</td>
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</table>

x
Logarithm
Molar
Monomorphic acetyltransferase
Millicurie
Milligram
Milliliter
Minute
Millimeter
Millimole
Millimolar
Normal
N-Acetyltransferase
Nanometer
Nanomole
N,0-Acetyltransferase
N-Hydroxy-N-acetylaminobiphenyl
N-Hydroxy-N-acetylaminofluorene
N-Hydroxy-4-aminobiphenyl
N-Hydroxy-2-aminofluorene
N-Hydroxy-3,2'-dimethyl-4-aminobiphenyl
O-Acetyltransferase
p-Aminobenzoic acid
Polyacrylamide gel electrophoresis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAPS</td>
<td>3'-Phosphoadenosine 5'-phosphosulfate</td>
</tr>
<tr>
<td>PAT</td>
<td>Polymorphic acetyltransferase</td>
</tr>
<tr>
<td>pmole</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RR</td>
<td>Homozygous rapid acetylator genotype</td>
</tr>
<tr>
<td>Rr</td>
<td>Heterozygous acetylator genotype</td>
</tr>
<tr>
<td>rr</td>
<td>Homozygous slow acetylator genotype</td>
</tr>
<tr>
<td>S-AcCoA synthetase</td>
<td>Acetyl coenzyme A synthetase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sp. act.</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>ug</td>
<td>Microgram</td>
</tr>
<tr>
<td>ul</td>
<td>Microliter</td>
</tr>
<tr>
<td>uM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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CHAPTER I

INTRODUCTION

The involvement of aromatic amines (arylamines) in certain types of human cancer was established in the late nineteenth century (Rehn, 1895). Aromatic amines were first used as precursors for the chemical synthesis of dyes and subsequently as antioxidants in the manufacture of rubber products and lubricating oils (Miller and Miller, 1983). Aromatic amines were recognized as human carcinogenic agents when workers exposed to these substances were found to exhibit a high incidence of urinary bladder cancer (Parkes and Evans, 1984). Benzidine, beta-naphthylamine, and 4-aminobiphenyl are examples of arylamine compounds widely used in the manufacture of plastic and dyes that have been shown to be carcinogenic (Case et al., 1954; Case and Pearson, 1954). In addition, arylamine compounds are used in industries that manufacture or use antioxidants, polymers, and pesticides (Schulte and Eard, 1988). It is now recognized that occupational exposure is one of the critical risk factors involved in the development of neoplasia from environmental chemicals.

Studies in drug metabolism and disposition indicate that arylamines usually induce most tumors in excretory tissues (e.g., liver, bladder, and intestine) rather than at sites of administration. It was also found that arylamine metabolites are more carcinogenic than their parent compounds. These findings
demonstrate that arylamines require metabolic activation by the host, into reactive electrophilic species that readily bind to the nucleophilic sites of macromolecules (e.g., DNA, RNA, and proteins) and initiate carcinogenesis in target tissues (Miller and Miller, 1981a, b; Lower, 1982; Dipple et al., 1985). Metabolic activation and deactivation are recognized as important determining factors that influence individual susceptibility to arylamine-induced carcinogenesis.

Many different enzymatic reactions are involved in the metabolic activation/deactivation of arylamines (as shown in Fig. 1). Among these reactions, N-acetylation, N-deacetylation, N-hydroxylation, and conjugation with glucuronides and sulfates are the main contributors to the activation of arylamines to toxic (carcinogenic) substances. Conversely, ring hydroxylation produces less toxic substances (Weber and Hein, 1985; Hein, 1988a). The metabolic pathway implicated in arylamine activation involves the biotransformation of arylamines to their corresponding arylamides via cytosolic N-acetyltransferase(s) (NAT) and back to the corresponding arylamines by microsomal deacetylase(s). Arylamines and arylamides can be further activated by N-hydroxylation to corresponding N-hydroxyarylamines and N-hydroxyarylhydroxamic acids (arylhydroxamic acids) respectively. These proximate carcinogens can be further metabolized to ultimate carcinogens via UDP-glucuronyltransferases, sulfotransferases, deacetylases, and/or acetyltransferases (Hein, 1988a).
Fig. 1. Metabolic Pathways Leading to DNA-Adducts from Arylamines and Arylamides (Beland and Kadmubăr, 1985).
Deacetylated DNA-adducts

Acetylated DNA-adducts
Acetylation is a major metabolic pathway in the biotransformation of amines and hydrazines. The acetylation reaction is catalyzed by NAT(s) (EC 2.3.1.5), an enzyme responsible for the enzymatic transfer of an acetyl group, usually from acetyl coenzyme A (AcCoA), to molecules that contain a primary amine, hydroxyl, or hydrazine group (Weber, 1973).

Individual differences in acetylation capacity have long been associated with drug-induced abnormalities such as peripheral neuritis, hepatitis, and lupus erythematosus (Weber and Hein, 1985). More recently, N-acetylation has been recognized to play a modulatory role in the activation/deactivation of carcinogenic aromatic amines (Weber and Hein, 1985; Hein, 1988a). Thus, acetylation reactions contribute to the cytotoxicity, mutagenicity, and carcinogenicity of aromatic amines.

The capacity for N-acetylation is inherited as a single autosomal Mendelian trait of two alleles at a single gene locus (Weber and Hein, 1985). The genotypes of such inheritance can be identified as homozygous rapid (RR), heterozygous (Rr), and homozygous slow (rr) acetylators, according to the rate at which they acetylate amines and hydrazines. This gene-dose relationship is referred to as the acetylation polymorphism. Human epidemiological studies have demonstrated that slow acetylator individuals are predisposed to amines-induced bladder cancer. An amines bladder carcinogenic model proposes that slow acetylators having low levels of hepatic NAT activity do not N-acetylate
arylamines very well, leading to transport of reactive N-hydroxyarylamines to the urinary bladder lumen where they can be converted into electrophilic molecules that bind to DNA (Poirier et al., 1963; Lower and Bryan, 1973; Lower et al., 1979; Poupko et al., 1979; Beland et al., 1983). In contrast, rapid acetylator individuals are more susceptible to colorectal cancer because they express higher levels of O-acetyltransferase (OAT) activity in colonic mucosa which converts N-hydroxyarylamines into reactive electrophiles that bind to DNA (Flammang et al., 1985; Flammang and Kadrubar, 1986). These findings indicate a relationship between genetic variability in acetylation capacity and differences in the metabolic activation/deactivation pathway of arylamine carcinogens (Weber and Hein, 1985; Hein, 1988a).

Because of the biological significance of genetic variability in acetylation status and arylamine-induced carcinogenesis, it is important to determine the genetic and biochemical factors that influence the metabolic activation/deactivation pathway of arylamine carcinogens.
Acetylation Polymorphism

Human hereditary differences in acetylation of drugs was first termed the "isoniazid acetylation polymorphism." Individual differences in isoniazid (INH)-induced toxicity of the nervous system were observed in patients receiving the drug for the treatment of tuberculosis. This pharmacogenetic trait, however, is now known as "acetylation polymorphism" because it relates to the difference in the capacity of individuals to metabolize numerous drugs and environmental chemicals that are acetylated in the body tissues (Fig. 2) (Weber, 1987). Slow acetylator individuals tend to accumulate high concentrations of INH and other hydrazine and arylamine drugs which are acetylated prior to excretion. Consequently, slow acetylators are often more susceptible to drug-induced toxicities than rapid acetylators (Weber and Hein, 1985). The significance of the acetylation polymorphism in drug-induced toxicity is best illustrated by the mortality of rapid and slow acetylator rabbits. When exposed to the same doses of INH for a specified period of time, rapid acetylators survived while slow acetylators did not (Hein and Weber, 1984).
Fig. 2. Structures of Representative Drugs and Environmental Chemicals that are Acetylated (Weber, 1987).
<table>
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<th>Arylamine Drugs</th>
<th>Arylamine Carcinogens</th>
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<td>Isoniazid</td>
<td>Acetylhydrazine</td>
<td>4,4'-Methylene-bis-(2-Chloroaniline)</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>Sulfamethazine</td>
<td>Benzidine</td>
</tr>
<tr>
<td>Phenelzine</td>
<td>Aminoglutethimide</td>
<td>2-Aminofluorene</td>
</tr>
<tr>
<td>Acetylhydrazine</td>
<td>p-Aminobenzoic Acid</td>
<td>β-Naphthylamine</td>
</tr>
<tr>
<td></td>
<td>p-Aminosalicylic Acid</td>
<td></td>
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Arylamine Drugs

- Acetylhydrazine
- Sulfamethazine
- Aminoglutethimide
- Acetylhydrazine
- p-Aminobenzoic Acid
- p-Aminosalicylic Acid
- Procainamide
- Dapsone
- Promizole
Acetylation

Acetylation is a major pathway of drug metabolism involved in the biotransformation of aromatic amines and hydrazines in the body (Williams, 1959). Usually, acetylation deactivates these chemicals into inert by-products that are excreted. In some instances, acetylation activates the chemicals into highly reactive substances which can initiate carcinogenesis (Weber, 1987).

The discovery by Lipman (1945) of coenzyme A derivatives, and their central role in the acetylation of drugs, led the way to the study of NAT enzymes. NAT is responsible for catalyzing the N-acetylation of arylamines and hydrazines. Recent studies on NAT have revealed the existence of various acetyltransferases in several mammalian species, including man (Weber and Hein, 1985).

Species and Hereditary Variation

The influence of hereditary factors in drug acetylation was first identified by Hughes et al. (1955), when studies on the disposition of INH in humans indicated that some individuals, treated with ordinary therapeutic doses of INH, had a toxic response to this drug. In addition, Hughes and co-workers found that individuals who developed a toxic response to INH also tended to excrete relatively small amounts of acetylated INH in their urine. These observations suggested that the extent of acetylation was an individual characteristic and that toxicity resulted in susceptible persons because they failed to sufficiently acetylate the drug.
Later, twin studies (Bonicke and Lisboa, 1957) and family studies (Evans et al., 1960) demonstrated that acetylation differences between individuals are almost exclusively inheritable. Individuals could be phenotypically identified as either rapid or slow acetylators. Sunahara et al. (1963) provided evidence that subjects could be identified into 3 subpopulations (phenotypes) corresponding to homozygous rapid, heterozygous, and homozygous slow acetylator genotypes.

Genetic analyses conducted in rabbits (Weber et al., 1976), mice (Tannen and Weber 1980), and hamsters (Hein et al., 1985a) have documented that acetylator differences are controlled by autosomal Mendelian inheritance of two alleles at a single gene locus. The genetically determined acetylator status of an individual has important pharmacokinetic (Weber and Hein, 1979) and toxicological significance (Drayer and Reidenberg, 1977). The frequency of a variety of drug-induced pathological conditions has been shown to be dependent on acetylator status (Bernstein and Lorincze, 1981; Weber, 1987). Susceptibility to arylamine-induced cancers has also been associated with acetylation polymorphism (Hein, 1988a).

Acetylator Status and Drug Response

Neurotoxicity. INH-induced neurotoxicity was the first drug toxicity disorder to be associated with the human acetylator status. Slow acetylators appeared to be more susceptible to
INH-induced neurotoxicity than rapid acetylators. Devadatta and co-workers (1960) reported that INH-induced peripheral neuropathy, associated with chronic ingestion of INH, occurred more frequently in slow acetylators than in rapid acetylators. These studies also demonstrated that the occurrence of this toxicity was related to the total dose of INH ingested.

No human epidemiological studies of the relationship between acetylator status and acute neurotoxicity have been reported. Several animal studies, however, suggest that hydrazine-induced neurotoxicity may be influenced by acetylator status (O'Brien et al., 1964; Hein and Weber, 1984). The relationship between drug-induced neurotoxicity and acetylator status was demonstrated by studies conducted in rapid and slow acetylator rabbits. Both rabbit populations received a similar daily injection of INH over the same period of time. All slow acetylator rabbits died of neurotoxicity, while rapid acetylator rabbits survived (Hein and Weber, 1984).

Drug-Induced Systemic Lupus Erythematosus. Numerous drugs are capable of producing systemic lupus erythematosus (SLE). The most important of these are aromatic amines such as procainamide (an antiarrhythmic agent) and the hydrazines such as hydralazine (an antihypertensive agent). The signs and symptoms found in patients with drug-induce SLE are similar to those found in idiopathic lupus erythematosus. The precise mechanism by which arylamine and hydrazine drugs produce this disorder is not known. However, evidence from chemical studies have shown that an N-hydroxylated metabolite rather than the parent compound may trigger this disorder.
Hereditary differences in acetylation capacity appears to be related to the susceptibility of individuals in developing drug-induced SLE (Weber, 1987; Hein and Weber, 1989). Several studies (Litwin et al., 1981; Mansilla-Tinoco et al., 1982) have demonstrated that lupus patients produce antinuclear auto-antibodies. It was found that patients in which SLE was drug-induced were predominantly slow rather than rapid acetylators. This suggests that acetylator status is not only a factor in arylamine-induced carcinogenesis but also with several pathological disorders.

Isoniazid-Hepatitis. It was initially proposed that INH was more hepatotoxic for rapid than slow acetylators because rapid acetylators are expected to form monoacetylhydrazine more rapidly than slow acetylators. Monoacetylhydrazine could then be converted to potent reactive electrophiles that bind covalently to hepatic macromolecules causing hepatic necrosis (Mitchell et al., 1975). This hypothesis has been challenged by more complete INH pharmacokinetic studies (Timbrell et al., 1977) that examined the percentages of metabolites formed and excreted after INH ingestion. The data confirmed that higher amounts of acetyl-INH are excreted in rapid acetylators than in slow acetylators. As expected, the amount of monoacetylhydrazine formed was higher in rapid than in slow acetylators. However, monoacetylhydrazine can be excreted unchanged or can be further metabolized in at least 2 ways (Fig. 3): it can be acetylated to diacetylhydrazine, a nontoxic metabolite; or it can
Fig. 3. The Metabolic Activation of Isoniazid in Liver.
Isoniazid \( \downarrow \) NAT
  \[ \text{Acetyl-isoniazid} \]
  \[ \downarrow \]
  \[ \text{Isonicotinic Acid} \quad \text{Monoacetylhydrazine} \]
  \[ \downarrow \quad \downarrow \]
  \[ \text{Excretion} \quad \text{Conjugation with Glycine} \]
  \[ \downarrow \quad \downarrow \]
  \[ \text{Excretion} \quad \text{NAT} \quad \text{P-450} \]
  \[ \downarrow \quad \downarrow \]
  \[ \text{Diacetylhydrazine} \quad (?) \]
  \[ \downarrow \quad \downarrow \]
  \[ \text{Excretion} \quad \text{Liver Damage} \]
undergo N-hydroxylation through the cytochrome P-450 system to a highly electrophilic intermediate. The last pathway is reported to be responsible for hepatotoxicity; whereas, the first is a detoxification pathway (Mitchell et al., 1976; Timbrell et al., 1980). In vivo studies indicated that INH inhibits the acetylation of monoacetylhydrazine. These observations have suggested that after normal therapeutic doses of INH, higher amounts of free INH remain for longer periods of time in slow acetylators than in rapid acetylators. Consequently, slow acetylators would have a greater inhibition of the acetylation reaction (detoxification pathway) resulting in a greater fraction of monoacetylhydrazine being available for the microsomal pathway which leads to hepatotoxicity. These results indicate that slow acetylators are more susceptible to drug-induced hepatotoxicity than rapid acetylators.

Acetylator Status and Cancer

Recently, N-acetylation has been identified as an important genetic factor with a modulatory role in the metabolic activation and deactivation of mutagenic and carcinogenic arylamines and of heterocyclic arylamines (Fig. 4) formed during the cooking of beef and other foods (Weber and Hein, 1985; Hein, 1988a; Sugimura, 1988; Weber et al., 1989). Epidemiological studies have shown associations between acetylator status and cancers of the urinary bladder (Matnoski and Elliot, 1981; Cartwright, 1983), and colon (Lang, et al., 1986; Ilett et al., 1987).
Fig. 4. Structures of Some Mutagenic Heterocyclic Arylamines Isolated from Cooked Foods (Sugimura, 1988). Compounds with asterisks have been subjected to long-term carcinogenesis experiments and proved to be carcinogenic. IQ, 2-amino-3-methylimidazo [4,5-f] quinoline; MeIQ, 2-amino-3,4-dimethylimidazo [4,5-f] quinoline; MeIQx, 2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline; 7,8-DiMeIQX, 2-amino-3,7,8-trimethylimidazo [4,5-f] quinoxaline; 4,8-DiMeIQX; 2-amino-3,4,8-trimethylimidazo [4,5-f] quinoxaline; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido [4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido [4,3-b]indole; Glu-P-1, 2-amino-6-methyldipyrido [1,2-a: 3'2'-d]imidazole; A α C, 2-amino-9H-pyrido [2,3-b]indole; MeA α C, 2-amino-3-methyl-9H-pyrido [2,3-b] indole.
Industrial exposure to arylamine chemicals has been recognized as a causal agent responsible for the induction of bladder cancer (Parkes and Evans, 1984). Rehn (1895) was the first to conduct epidemiological investigations of industrial bladder cancer. He reported the unusual occurrence of urinary bladder cancer among fuchsin dye workers. In the 1940's, numerous basic clinical and epidemiological evidence in humans and animals, indicated that certain aromatic amines constituted a grave bladder cancer hazard. Studies by Case and co-workers (Case et al., 1954; Case and Pearson, 1954) clearly showed an association between bladder cancer deaths and exposures to certain arylamines in dye-manufacturing companies.

The fact that carcinogenic aromatic amines are acetylated by the same enzymes(s) which acetylates INH and other drugs (Glowinski et al., 1978) suggested a potential role for the acetylation polymorphism in individual susceptibility to bladder cancer (Weber, 1978; Lower et al., 1979). Studies on several populations have indicated that slow acetylators are more susceptible to arylamine-induced bladder cancer than rapid acetylators (Cartwright et al., 1982; Evans et al., 1983; Ladero et al., 1985; Mommsen et al., 1985; Mommsen and Aagard, 1986). Evans et al. (1983) suggested that association of slow acetylator status with bladder cancer might signify that either rapid acetylators are protected because they are more capable of rendering aromatic amines non-carcinogenic by N-acetylation, or that slow acetylators survive longer with bladder cancer than rapid acetylators. Whether or not acetylator status has
an appreciable effect on the survival of patients with occupational bladder cancer requires further investigation.

Although colorectal cancer is the second leading cause of cancer death in the United States (Lang et al., 1986), the etiology of this disease is poorly understood. Dietary habits and their effects on intestinal microflora, bile acids, and fecal bulk are considered to be the major predisposing factors for this disease (Lang et al., 1986). In addition, several aromatic amines identified as pyrolysis products formed during cooking of food have been shown to induce intestinal and other tumors in experimental animals (Takayama et al., 1984a, b).

In recent studies, an association between acetylator status and colorectal cancer has been documented. Rapid acetylator individuals may be more susceptible to colorectal cancer than slow acetylators due to higher levels of N-hydroxyarylamine OAT activity present in the colon of rapid acetylators (Lang et al., 1986; Ilett et al., 1987). OAT catalyzes the metabolic activation of N-hydroxyarylamines to electrophilic intermediates that bind readily to DNA and initiate colon cancer.

**Metabolic Activation of Arylamine Carcinogens**

Arylamine chemicals require metabolic activation into reactive electrophilic species which bind covalently to DNA, forming derivatized bases which are thought to induce the initiation of carcinogenesis (Miller and Miller, 1981b). The metabolic pathways indicated in arylamine-induced carcinogenesis are catalyzed by
various host enzymes. Hypotheses exist which suggest that individual susceptibility to drug-induced toxicities is determined by inheritable differences in these enzymes.

Several reviews (Miller and Miller, 1981a, b; Lower, 1982; Kadlubar and Beland, 1985; Hein, 1988a) of arylamine-induced carcinogenesis have suggested that arylamine chemicals are N-acetylated by cytosolic NAT to arylamides, and deacetylated by microsomal deacetylases to the parent arylamines. Arylamines and arylamides undergo N-oxidation to N-hydroxyarylamines and N-hydroxy-N-acyetylarylamines, respectively, by cytochrome P-450 and flavin-containing monooxygenases. Further activation to their ultimate electrophilic forms occurs via enzymatic conjugation reactions with acetate, sulfate, or phosphate groups.

Arylamines and N-hydroxyarylamines can be transported as N-glucuronide conjugates to target tissues, such as colon and bladder. Bacterial glucuronidases in the colon or the mildly acidic urine conditions in the bladder can regenerate the arylamine and N-hydroxyarylamine forms. The slightly acidic conditions in the bladder can protonate the N-hydroxy group of N-hydroxyarylamines, forming electrophiles that bind to bladder DNA. In contrast, N-hydroxyarylamines in the colon undergo O-acetylation to the acetoxy ester, which immediately converts into an electrophile that binds to colon DNA.
Distribution and Physicochemical Properties of N-Acetyltransferase (NAT)

NAT is found in several tissues of a number of species. NAT activity has been detected in several organs including liver, small intestine, brain, kidney, lung, spleen, testis, thymus, ovary, salivary glands, and some peripheral blood cells (Weber, 1973). Mammalian liver NAT has a broad substrate specificity. An amino group attached directly to unsaturated rings or by way of a carbonyl group is an important structural component of reactive compounds.

Purification studies on avian liver have demonstrated that pigeon NAT is a monomer with a pi of 4.8 and a molecular mass of 32,900 D (Andres et al., 1983); whereas, chicken NAT has a molecular mass of 34,000 D (Deguchi et al., 1988). Liver NAT from homozygous rapid acetylator rabbits is a monomer with a pi of 5.2 and a molecular mass of 33,000 D (Andres et al., 1987; Kabishev and Patrushev, 1987). The hepatic NAT from rapid (C57BL/6J) and slow (A/J) acetylator mice has a molecular mass of 31,500 D (Mattano et al., 1988).

Partial amino acid sequences have been determined for the hepatic NAT of rapid rabbit acetylator (Andres et al., 1987) and the chicken (Deguchi et al., 1988). An oligonucleotide probe has been synthesized and used to screen a lambda gt10 cDNA library enabling the isolation of a cDNA clone encoding chicken liver NAT (Ohsako et al., 1988). The sequence of this cDNA consists of 1,320 nucleotides including a 861-nucleotide region coding for 287 amino acids. Comparisons of NAT amino acid sequences in chicken and rabbit livers revealed approximately 60-80% homology (Ohsako et al., 1988).
Multiple Forms of Acyltransferases and Deacetylases

Different enzymatic acetyl transfer reactions (as shown in Fig. 5) are involved in the metabolic activation of arylamine carcinogens and their N-hydroxyarylamine and N-hydroxy-N-acetyarylamine (arylhydroxamic acid) derivatives (Hein, 1988a). Recent studies have suggested that many of these enzymatic pathways are mediated by one or more enzymes with diverse catalytic activity (Smith and Hanna, 1986; Mattano et al., 1988; Kato et al., 1988).

Acetyl Coenzyme A-dependent (AcCoA) Arylamine N-Acetyltransferase (NAT). Enzymatic transfer of the acetyl group from AcCoA to acceptor arylamines was first characterized in pigeon liver preparations by Bessman and Lipmann (1953). Their data suggested the formation of an acetylated enzyme intermediate via various co-factors, including AcCoA. Subsequent studies demonstrated that N-acetylation is a two-step process in which the enzyme oscillates between a free and an acetylated form (Weber and Hein, 1985). The reaction is usually written as follows:

\[
\text{AcCoA} \xrightarrow{\text{NAT}} \text{CoA} \xrightarrow{\text{Ac-NAT}} \text{Acceptor Amine} \xrightarrow{\text{Acetyl Amine}} \text{NAT}
\]

The overall reaction can be written

\[
\text{AcCoA} \xleftarrow{\text{NAT}} \text{NAT} \xleftrightarrow{\text{Ac-NAT}} \text{CoA} \xleftarrow{\text{Ac-NAT}} \text{Ac-NAT}
\]

\[
\text{Ac-NAT} \xleftrightarrow{\text{Amine}} \text{Acetyl-amine} \xrightarrow{\text{NAT}} \text{NAT}
\]
Fig. 5. Different Enzymatic Acetyl Transfer Reactions (Hein, 1988a).
Acetyl Coenzyme A

Cytosolic Acetyltransferase Enzymes

Acetyl Coenzyme A

Coenzyme A

Acetylated Enzymes

Arylhydroxamic Acid

N-Hydroxyarylamine

Deacetylated Enzymes

N-Hydroxyarylamine

N-Acetoxyarylamine

Nonenzymatic DNA-Adducts

Deacetylase (Microsomal)

OAT

N-Acetoxyarylamine

Nonenzymatic DNA-Adducts
Studies of pigeon liver (Jacobson, 1961) and rabbit liver (Andres et al., 1987) NATs have demonstrated that transfer of the acetyl group from the acetylated enzyme to the acceptor amine is the rate-limiting step for the N-acetylation of strongly basic amines (Weber and Hein, 1985).

**Arylhydroxamic Acid-Dependent Arylamine N,N-Transacetylase.**

N,N-Transacetylase catalyzes the transfer of the acetyl group from N-hydroxy-N-acetylarylamine to form an arylamide (Booth, 1966). The transacetylase activity has been found in many organs (Weber, 1973).

Studies on substrate specificities have indicated that arylamine carcinogens, such as AF and ABP, serve as acetyl acceptor molecules; while, arylhydroxamic acids such as N-OH-AAF and N-OH-AABP serve as acetyl donor molecules. In contrast, AcCoA does not serve as an acetyl donor molecule for this reaction in rat liver (Booth, 1966). Based on these results, Booth concluded that N,N-transacetylase activity was different from the AcCoA-dependent NAT. Recent studies of rabbit (Glowinski et al., 1980) and hamster (Hein et al., 1986a; Kato and Yamazoe, 1988) liver cytosols, however, have provided evidence that support the existence of an enzyme capable of catalyzing both N,N-transacetylase and AcCoA-dependent NAT activities. Thus, further research is necessary to determine if these activities are catalyzed by different enzyme(s) in certain species.

**Arylhydroxamic Acid N,O-Acyltransferase (N,O-AT).** N,O-AT is responsible for the intramolecular N,O-acyltransfer of arylhydroxamic acids to form electrophilic intermediates (King, 1974; King
The interaction of arylhydroxamic acids and N,O-AT is outlined in Fig. 6. The arylhydroxamic acid binds to the active site of the enzyme and acetylates it. The acetylated enzyme may then acetylate the oxygen atom of the arylhydroxylamine derived from the arylhydroxamic acid donor. This intramolecular N- to O-acyltransfer yields a highly reactive acetoxy arylamine intermediate (Fig. 6, step b) which undergoes heterolytic cleavage to form an arylnitrenium ion. This ion reacts with tissue nucleophiles (Fig. 6, step c) such as nucleic acids and proteins, including the nucleophilic sites (Fig. 6, step d) on N,O-AT (King, 1974; King and Allaben, 1978; Hanna et al., 1982). Inhibition studies by Smith and Hanna (1988) and Wick et al. (1988) have shown that bioactivation of arylhydroxamic acids by N,O-AT is accompanied by irreversible inactivation of arylhydroxamic acid N,N-transacetylase activity (Fig. 6, step a).

Evidence supporting the formation of an acetoxy intermediate is indirect, since the acetoxyarylamine products formed via arylhydroxamic acid N,O-AT are too unstable to allow their isolation (Kadlubar and Beland, 1985). Product formation, however, is deduced from the structure of adducts formed when various nucleophiles are used as trapping agents.

The arylhydroxamic acid N,O-AT is expressed in several species including rat, hamster, rabbit, and mouse. The derivatives formed by arylhydroxamic acid N,O-AT can induce tumors in a wide spectrum
Fig. 6. Interaction of N-Arylhydroxamic Acids and N,O-Acyltransferase (Smith and Hanna, 1988).
Reaction with biological nucleophiles
d irreversible inactivation of N₂O-AT

\[
\begin{align*}
\text{Ar-N-H} + \text{Ar'}\text{NHCCH}_3 & \rightarrow \text{Ar-N-H} + \text{Ar'}\text{NH}_2 \\
\text{Ar-N-H} + \text{CH}_3\text-C-\text{enzyme} & \rightarrow \text{Ar-N-H} + \text{CH}_3\text-C-\text{enzyme} \\
\text{Ar-N-CH}_3 + \text{N}_2\text{O-AT} & \rightarrow \text{Ar-N-H} + \text{CH}_3\text-C-\text{enzyme} \\
\end{align*}
\]
of tissues such as kidney, mammary gland, and the gastrointestinal tract (King, 1974; Weber, 1987). Rat liver N-O-AT has been shown to be responsible for the activation of N-OH-AAF to a mutagen in the Salmonella typhimurium mutagenesis assay system (King, 1974).

Arylhydroxamic acid N,O-AT has been purified to electrophoretic homogeneity in rat (Allaben and King, 1984), mouse (Mattano et al., 1988), rabbit (Glowinski et al., 1980), and hamster (Saito et al., 1986) liver. It co-purifies with AcCoA-dependent NAT, providing further evidence for the existence of a common protein capable of catalyzing several acetyltransferase activities.

Arylhydroxamic Acid-Dependent N-Hydroxyarylamine N,O-Transacetylase. Bartsch et al. (1972, 1973) were the first to describe the intermolecular transfer of the N-acetyl groups of certain carcinogenic arylhydroxamic acids (e.g., N-OH-AABP and N-OH-AAF) to the oxygen atom of N-hydroxyarylamines (e.g., N-OH-ABP and N-OH-AF). This reaction gives rise to N-acetoxyarylamine metabolites, highly unstable derivatives, which are immediately hydrolyzed into strongly electrophilic intermediates capable of binding to the nucleophilic sites of cellular macromolecules.

Bartsch and co-workers (1972) demonstrated that AcCoA did not serve as an acetyl donor for the N,O-transacetylase activity, whereas N-OH-AABP and N-OH-AAF are the most efficient acetyl donors. These observations suggested that N,O-transacetylase activity differed from the AcCoA-dependent NAT activity. Arylhydroxamic acid N,O-transacetylase activity has many
similarities with arylhydroxamic acid N,N-transacetylase activity described by Booth (1966).

The N,O-transacetylase activity has been identified in various species (e.g., rat, rabbit, and hamster) and it is expressed in several tissues. Bartsch and co-workers (1972) observed 6-fold variability between rabbit livers for N,O-transacetylase and suggested that the variation might be related to the NAT hereditary polymorphism.

Acetyl Coenzyme A-Dependent N-Hydroxyarylamine O-Acetyltransferase (OAT). It was postulated that a major pathway leading to the formation of arylamine-DNA adducts involves the N-acetylation of the N-hydroxyarylamine to an N-hydroxy-N-acetylarylamine followed by the formation of an N-acetoxyarylamine via an intramolecular N,O-acyltransfer (King, 1974). The N-acetoxy derivatives then bind covalently with DNA, forming derivatized bases which are thought to initiate the neoplastic process. However, studies have demonstrated that the formation of arylamine-DNA adducts usually involves the N-oxidation of primary arylamines to N-hydroxy derivatives, followed by direct O-acetylation of the oxygen atom by AcCoA-dependent N-hydroxyarylamine OAT (Beland and Kadlebar, 1985; Flammang and Kadlebar, 1986).

AcCoA-dependent N-hydroxyarylamine OAT activity has co-purified with NAT activity in hamster liver (Saito et al., 1986) and mouse liver (Mattano et al., 1989). In addition, genetic co-regulation has been shown in hamster liver between OAT and NAT activities (Hein et al., 1987a) and has been suggested to occur in human liver.
(Flammang et al., 1987), colon (Flammang et al., 1988) and bladder (Kirlin et al., 1989).

**Arylamide Deacetylase.** The acetyl group of arylacetamides (e.g., AAF and AABP) is removed by microsomal deacetylases. This activity has been reported in the hepatic and extrahepatic tissues of several mammalian species (Hein, 1988a).

Initial studies with guinea pig, a species with low N-hydroxylation activity (Gutmann and Bell, 1977), provided evidence that deacetylation can lead to the formation of mutagenic products from AAF. In addition, Aune et al. (1985) indicated that the pathway which activates AAF to mutagenic products included deacetylation followed by monoxygenation catalyzed by cytochrome P-450.

No correlation between acetylator status and microsomal deacetylase has been detected in several species (reviewed in Hein, 1988a). However, a study by Hultin and Weber (1987) reported differences in microsomal AAF deacetylase activity between rapid and slow acetylator mice.

**Arylhydroxamic Acid Deacetylase.** Deacetylation of arylhydroxamic acids (e.g., N-OH-AAF) to N-hydroxyarylamines (e.g., N-OH-AF) has been proposed as one of the critical metabolic steps in the formation of hepatic DNA adducts and the initiation of liver tumors in mouse (Lai et al., 1988). Furthermore, microsomal deacetylase activity has been implicated in the metabolic activation of N-OH-AAF to electrophilic species mutagenic in bacterial tester systems (Schut et al., 1978).
Acetylation Polymorphism in the Inbred Hamster

Genetic animal models, which exhibit biochemical and physiological properties similar to the human trait, are used to assess the significance of hereditary factors affecting drug toxicity and carcinogenesis (Weber and Hein, 1985). Although investigating human traits in animal models is an indirect approach, animal studies facilitate the acquisition of information about the human condition under circumstances that may be impossible to simulate in humans due to ethical constraints or methodological difficulties.

Unlike rabbit and mouse, the hamster has high levels of acetyltransferase activities, as well as relatively high levels of deacetylase activities in liver. The ability to readily measure acetylase and deacetylase activities in hamster tissues makes it a unique model for investigating the role of acetylator genotype on the following: the expression of acetyltransferases and deacetylasers; drug-induced toxicities; and arylamine-induced carcinogenesis.

An acetylator gene-dose relationship towards hepatic NAT activity in slow and rapid acetylator Syrian inbred hamsters has been demonstrated (Hein et al., 1985a). NAT polymorphic expression has also been shown in blood lysates, intestine, kidney, lung, bladder, and colon (Hein et al., 1986b; 1987b, c; Ogolla et al., 1988). In each tissue, highest levels of activity are found in
homozygous rapid acetylators, lowest levels in homozygous slow acetylators, and intermediate levels in heterozygous acetylator progeny.

Upon partial purification by anion-exchange chromatography (Hein et al., 1985b; 1987a), two distinct acetyltransferase proteins have been identified in both rapid and slow acetylator hamster liver cytosol. One isozyme exhibited catalytic activity levels that are acetylator genotype-dependent (NAT activity levels differ according to acetylator genotype) and is referred to as the polymorphic acetyltransferase (PAT) isozyme. In contrast, the other isozyme exhibited catalytic levels that are acetylator genotype-independent (NAT activity levels are similar across acetylator genotype). This isozyme is referred to as the monomorphic acetyltransferase (MAT) isozyme.

Multiple forms of acetyltransferase activity have been identified (Hein et al., 1985b; Smith and Hanna, 1986). Hein and co-workers (1985b) identified the liver N-OH-AAF/AAB N,N-transacetylase activity in rapid and slow acetylator hamsters. The N,N-transacetylase activity exhibited an acetylator genotype-independent expression. Also, multiple forms of acetyltransferase activity have been demonstrated by Smith and Hanna (1986), who were able to separate hepatic N-OH-AAF N,O-AT and PABA NAT activities by affinity chromatography. It was further shown that sulfamethazine NAT activity and N-OH-AAF N,O-AT activity are both inactivated by N-hydroxyphenacetin. In addition, Smith and
Hanna (1986) found that neither of these activities is protected by cysteine, a low molecular weight nucleophile, but it does protect PABA NAT activity. Consequently, it was proposed that sulfamethazine NAT and N-OH-AAF N,O-AT activities are associated with a single enzyme protein; while, PABA NAT activity is not associated with N-OH-AAF N,O-AT activity.

Rationale for this Study

Inherited differences in acetyltransferase activities are thought to play an important role in the metabolic activation and/or deactivation of aromatic amine carcinogens. Two genetically variant forms of N-acetyltransferases (PAT and MAT) have been identified in hamster liver. However, the relationship between the PAT and MAT isozymes, as well as their role in the metabolic activation of aromatic amines is not well understood.

Genetic and biochemical characterization of the isozymes is needed in order to elucidate the biochemical factors affecting the mechanism(s) responsible for the activation of carcinogenic aromatic amines. It is expected that determination of the factors involved in the activation of arylamine and N-hydroxyarylamine carcinogens will enhance the understanding of the biochemical and molecular mechanisms associated with the biotransformation of arylamines, as well as N-hydroxyarylamines.

This study was designed to purify the acetyltransferase activity responsible for the N-acetylation polymorphism in the inbred hamster. The main objective of this investigation was to determine
the role and contribution of the PAT and MAT isozymes in the metabolism of aromatic amine carcinogens.

Research Aims

The specific aims of this investigation are to:

1. Partially purify the PAT and MAT isozymes from homozygous rapid and homozygous slow acetylator hamster livers.
2. Determine the expression of multiple forms of acetyltransferase isozymes.
3. Perform kinetic characterization studies on the isozymes.
4. Purify the PAT and MAT isozymes from homozygous rapid acetylator hamsters to apparent homogeneity.
5. Determine the relationship between acetylator status and O-acetyltransferase activity.
CHAPTER III

MATERIALS AND METHODS

Chemicals

Reagent grade PABA potassium salt, DMAB, dithioerythritol, DTT, INH, ABP, S-AcCoA synthetase (EC 6.2.1.1), EDTA, PMSF, and ATP were purchased from Sigma Chemical Co., St. Louis, MO; AcCoA lithium salt was obtained from Pharmacia LKB, Inc. Piscataway, NJ; [3H]acetate (sp. act. 4.5 Ci/m mole) was obtained from Dupont, New England Nuclear Co., Boston, MA; AF and [ring 3H] N-OH-AF (112 mCi/m mole); were purchased from Chemsyn Laboratories, Lexena, KS; [ring 3H] N-OH-ABP (141 mCi/m mole); and [ring 3H] N-OH-DMABP (166 mCi/m mole) were donated by Drs. Fred Kadlubar and Thomas Flammang at the National Center for Toxicological Research, Jefferson, AR.

Animals

Bio. 87.20 homozygous rapid acetylator Syrian inbred hamsters were obtained from Bio. Breeders, Watertown, MA. Bio. 82.73/H homozygous slow acetylator Syrian inbred hamsters were originally derived from the Bio-Research Institute, Cambridge, MA but have since been maintained in the animal care facilities of The Morehouse School of Medicine for several generations. The two inbred strains have been described elsewhere (Altman and Katz, 1979) and characterized with respect to acetylator genotype (Hein et al., 1985a, b).
Preparation of Tissue Cytosol

Hamsters were sacrificed by decapitation after a preliminary carbon dioxide anesthesia. The livers were removed rapidly, washed, minced, and homogenized (25% w/v) in ice-cold 20 mM potassium phosphate buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, and 50 μM PMSF, using a motor driven homogenizer (Biospec Products, Bartlesville, OK). The homogenate was subjected to sequential centrifugation at 10,000 x g for 20 min followed by 105,000 x g for 60 min. The final supernatant (cytosol) was assayed for protein and enzymatic activity, or further purified as described below. All enzyme isolation and purification steps were performed at 4°C.

Anion-Exchange FPLC of Hamster Liver Cytosol

Separation of hamster liver cytosol NAT activities was accomplished by an ion-exchange method using a Fast Protein Liquid Chromatography (FPLC) system. Fifteen mg of Bio. 87.20 or Bio. 82.73/H liver cytosol protein was applied to a Mono Q HR 5/5 anion-exchange column (Pharmacia LKB, Inc., Piscataway, NJ) that had been equilibrated with filtered and degassed 20 mM potassium phosphate buffer (pH 7.4), containing 1 mM DTT and 1 mM EDTA (start buffer). The column was eluted with filtered and degassed 20 mM potassium phosphate buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, and 1 M KCl (elution buffer), using a Pharmacia FPLC system (model GP-250) at a flow rate of 1.0 ml/min and a linear gradient (60 ml)
from 0-400 mM KCl with an isocratic step at 120 mM KCl. This step gradient was designed to optimize the separation of the two NAT isozymes. All buffers and fractions collected were kept ice-cold. One ml fractions were collected and tested for protein and enzymatic activity as described in the following determinations.

**PABA NAT Activity Determinations**

AcCoA-dependent PABA NAT activities were determined in liver cytosol and chromatographic fractions as previously described (Hein et al., 1982b). The procedure measures the AcCoA-dependent disappearance of arylamine substrate as reflected by decreasing Schiff's base formation with DMAB. To 50 ul of suitably diluted enzyme, 20 ul of 1 mM arylamine substrate was added. The reaction was started with the addition of 20 ul of 4.5 mM AcCoA except in crude cytosol where 20 ul of 10 mM AcCoA was added. Initial concentrations in the reaction mixture were 0.22 mM PABA and 1.00 mM AcCoA except in crude cytosol where 2.22 mM AcCoA was used. The reaction was terminated by the addition of 50 ul of trichloroacetic acid (10% w/v) at specified time points to give linear time-activity plots. After centrifugation in a Brinkmann Eppendorf centrifuge 5414, color development was achieved with the addition of 1 ml of DMAB solution (equal volumes of DMAB in ethanol (1% w/v) and 1 M sodium acetate HCl buffer, pH 1.4). After final mixing and recentrifugation, the tubes were allowed to stand for at least 30 min at room temperature. Absorbance was measured at 460 nm against
an ethanol blank in a Perkin Elmer Lambda 3A spectrophotometer. The amount of acetylated product formed was determined by subtracting the experimental from the control absorbance readings. Controls received 20 ul of water instead of 20 ul AcCoA. All assays were performed in duplicate at 37°C. The assay was calibrated by constructing a standard curve of known PABA concentrations versus absorbance.

INH NAT Activity Determinations

AcCoA-dependent INH NAT activities were determined in crude cytosol and in chromatographic fractions by a modification of the method of Weber (1971) as previously described (Hein et al., 1982a). The assay measures the AcCoA-dependent formation of acetyl-INH. To 50 ul of suitably diluted enzyme, 20 ul of 50 mM INH was added. The reaction was started with the addition of 20 ul of 4.5 mM AcCoA except in crude cytosol where 20 ul of 10 mM AcCoA was added. Initial concentrations in the reaction mixture were 11.1 mM INH and 1.00 mM AcCoA except in crude cytosol where 2.22 mM AcCoA was used. The reaction was stopped at specified time points by the addition of 50 ul trichloroacetic acid (10% w/v) to give linear-activity plots. After centrifugation in a Brinkmann Eppendorf centrifuge 5414, 1 ml of 0.8 M potassium borate buffer, pH 9.0, was added. The mixture was vortexed and then recentrifuged. Absorbance was measured at 303 nm versus a water blank in a Perkin Elmer Lambda 3A spectrophotometer. The amount of acetylated product
formed was determined by subtracting the control from the experimental absorbance reading. Controls received 20 ul of water instead of 20 ul AcCoA. All assays were performed in duplicate at 37°C.

AF and ABP NAT Activity Determinations

AcCoA-dependent AF and ABP NAT activities in crude cytosol and in chromatographic fractions were determined by a modification of the method of Andres et al. (1985) as previously described (Hein et al., 1987a). The procedure measures AcCoA-dependent formation of radiolabeled arylamide product. To 60 ul of suitably diluted enzyme preincubated at 37°C, 20 ul of 450 mM Tris buffer (pH 8.0), containing 4.5 mM EDTA, 22.5 mM MgCl₂, 4.5 mM dithioerythritol, 5 mM AcCoA, 22.5 mM ATP, 1.5 U/ml S-AcCoA synthetase (EC 6.2.1.1), and 2.25 mM [³H]acetate (sp. act. 4.5 Ci/mmole), was added. The reaction was started with the addition of 20 ul of 2.5 mM arylamine substrate (in 25% DMSO). Initial concentrations in the reaction mixture were 0.5 mM for arylamine substrate and 1.0 mM for AcCoA. The reaction was terminated at multiple time points by the addition of 20 ul of a mixture of 5 N NaOH/100 mM sodium acetate to hydrolyze any remaining [³H]AcCoA to [³H]acetate. The recovery of [³H]arylamide was readily accomplished by a single extraction with chloroform. After the chloroform extract was washed twice with water, an aliquot of the organic layer was transferred to mini scintillation vials, evaporated to dryness, redissolved in 300 ul of
DMSO, and counted with a Beckman LS5801 scintillation counter after the addition of scintillation cocktail (Liquiscint; National Diagnostics, Menville, NJ). Controls received 20 uI of 25% DMSO rather than the arylamine substrate. All assays were performed in duplicate, and enzyme activity was calculated as previously described (Andres et al., 1985).

**AcCoA-Dependent N-Hydroxyarylamine OAT Activity Determinations**

This assay measures the extent to which ring-labeled N-hydroxyarylamines (e.g., N-OH-AF, N-OH-ABP, and N-OH-DMABP) are incorporated into DNA adducts as a consequence of the production of reactive N-acetoxyarylamines by AcCoA-dependent O-acetylation. The procedure was conducted as previously described (Flammang et al., 1985). The assays were performed in argon-saturated 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA, calf thymus DNA (2 mg/ml), and tissue cytosol protein (0.5 mg/ml) or chromatographic fractions as enzyme source. The initial [3H] N-hydroxyarylamine and AcCoA concentrations were 100 uM and 2 mM respectively. The control reactions did not contain AcCoA. Incubations were performed at 37°C for up to 15 min and terminated by adding 1 ml of ice cold water-saturated N-butanol. One ml of potassium phosphate buffer (pH 7.4), containing 1 mM DTT and 1 mM EDTA, was added to the samples. The samples were frozen for 1 h to separate the aqueous from the organic layer. The N-butanol was removed and the aqueous layer was washed twice with 1 ml of N-butanol, followed by two washes with 1 ml of Tris-washed phenol.
The final aqueous solution was made 100 mM NaCl by adding 100 μl of 1 M NaCl. The samples were placed on ice and the DNA was precipitated by layering with 5 ml of ice-cold ethanol. The precipitated DNA was isolated and redissolved with 1 ml of 5 mM Tris, 10 mM MgCl₂ buffer pH 7.0. An aliquot of this solution was transferred to scintillation vials and counted in a Beckman LS5801 counter to measure the extent of covalent binding to DNA. The amount of DNA in the aliquot was determined by a colorimetric assay with diphenylamine as previously described (Shatkin, 1969). Results are expressed as nmoles bound/15 min/mg DNA/mg protein.

**Purification of PAT and MAT Isozymes**

The protocol for the purification of PAT and MAT isozymes from homozygous rapid acetylator hamsters is summarized in Fig. 7. A detailed discussion of each purification step is given below.

All purification steps were conducted at 4°C. The following buffers were used: buffer I (20 mM potassium phosphate, pH 7.4, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, and 10% glycerol), buffer II (same as buffer I without PMSF), and buffer III (same as buffer I except for the addition of 1 M KCl).
Fig. 7 Summary of Purification Procedure.
PURIFICATION OF POLYMORPHIC AND MONOMORPHIC N-ACETYLTRANSFERASES

Crude Cytosol (Bio. 87.20)

Sequential Centrifugation

Q-Sepharose Fast Flow Anion-Exchange Chromatography

Polymorphic NAT Isozyme

Sephacryl S-200 Chromatography

Anion-Exchange FPLC Chromatography

Monomorphic NAT Isozyme

Sephacryl S-200 Chromatography

Anion-Exchange FPLC Chromatography

SDS-PAGE
Step 1. **Q-Sepharose Fast Flow Anion-Exchange Chromatography.** Crude cytosol (2.7 g) from Bio.87.20 Syrian inbred hamster liver was filtered and applied to a Q-Sepharose anion-exchange column (2.6 x 70 cm) previously equilibrated with 2-3 bed volumes of buffer I until the protein concentration in the effluent was less than 50 ug/ml (approximately 10-15 h). The column was eluted at a flow rate of 2 ml/min by a linear salt gradient (900 ml, 0-400 mM KCl) in buffer III. This gradient separated the PAT and MAT isozymes. Ten ml fractions were collected and tested for enzymatic activity as described above. Fractions containing maximum PAT and MAT activities were pooled, separately. These two enzymatic fractions were then concentrated by ultrafiltration (Amicon Centriprep concentrators membrane cutoff 10 KD).

Step 2. **Sephacryl S-200 Chromatography.** The enzyme solution, usually 60-200 mg (PAT or MAT), was applied to a Sephacryl S-200 column (1.6 x 70 cm) previously equilibrated with buffer II. The enzyme was eluted at a flow rate of 1 ml/min. Seven ml fractions were collected. Fractions containing maximum NAT activities (PAT or MAT) were pooled.

Step 3. **Anion-Exchange FPLC Chromatography.** Pooled fractions (PAT or MAT) from the previous step (approximately 5-10 mg of protein) were applied to a Mono Q HR 5/5 anion-exchange column that had been equilibrated with filtered and degassed buffer II. The column was eluted at a flow rate of 1.0 ml/min with filtered and degassed buffer III using a Pharmacia FPLC system as previously
described. For further details, see Anion-Exchange FPLC of Hamster Liver Cytosol.

Effects of Temperature on Polymorphic NAT and OAT Activities

Heat inactivation studies were performed with partially purified Sephacryl S-200 PAT and MAT liver preparations, as well as in the homogenous PAT liver preparation. A sample from each preparation was placed in a test tube and incubated in a water bath at 55°C. Aliquots were then removed at different time intervals and assayed for PABA NAT, AF NAT, and N-OH-AF OAT activities, as previously described.

Protein Determinations

Total protein concentration was determined in 105,000 x g crude cytosol and chromatographic fractions by the method of Bradford (1976).

DNA Determinations

The concentration of the recovered DNA from acetyltransferase binding assays was determined by a colorimetric assay with diphenylamine (Shatkin, 1969).

Apparent Km and Vmax Determinations

Michaelis-Menten kinetic constants of NAT activity were determined in liver cytosols as well as with the PAT and MAT isozymes obtained from three or more homozygous rapid and slow acetylator hamsters. NAT assays were done in duplicate using
various concentrations of substrate in the presence of a fixed saturating concentration of AcCoA. PABA concentrations ranged from 50-500 uM in rapid acetylators and from 50-2250 uM in slow acetylators. INH concentrations ranged from 111-11,100 uM and AF and ABP concentrations ranged from 5-1000 uM in both acetylator genotypes. The kinetic constants for AcCoA were determined with AcCoA concentrations ranging from 100-2220 uM in both acetylator genotypes in the presence of 0.44 mM PABA in crude cytosol, while the PAT and MAT isozymes of both acetylator genotypes were assayed in the presence of 0.22 mM PABA.

Apparent Km and Vmax values were calculated for each liver cytosol and for the PAT and MAT isozymes by ENZFITTER non-linear regression data analysis (Leatherbarrow, 1987) and Eadie-Hofstee linear regression analysis (Dixon and Webb, 1979).

**Polyacrylamide Slab Gel Electrophoresis**

Polyacrylamide gel electrophoresis (PAGE) of proteins catalyzing NAT/OAT activity was carried out in an Ephortec (Saddle Brook, NJ) vertical slab system. Native gels were prepared by the method of Laemmli (1970), using a Tris-glycine buffer system, pH 8.8. Protein samples (1-5 ug) were analyzed using either 10 or 12.5% polyacrylamide resolving gels and 3% stacking gels. A maximum of 20 ul of protein samples were applied onto the stacking gel. Electrophoresis was performed for 4 h at a constant current of 50 milliamps/slab gel. The temperature was 14°C. Gels were sliced
vertically with a scalpel. One vertical gel strip was subjected to silver stain (Merrill et al., 1981) to detect the protein band(s). The remaining vertical gel strips were cut into segments of 1-2 mm. Each segment was homogenized in 20 mM potassium phosphate buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, and 50 μM PMSF. After centrifugation in a Brinkman Eppendorf centrifuge 5414, each segment was assayed for PABA NAT activity as described previously.

**SDS-Polyacrylamide Gel Electrophoresis and Molecular Weight Determination**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) using the Tris-glycine buffer system, pH 8.8, containing 0.1% SDS. The gels were prepared as described above (see Polyacrylamide Slab Gel Electrophoresis) with the addition of 0.125% SDS. Protein samples (100 ul) were mixed with 10 ul of sample buffer [30 mM Tris pH 6.8, 20% SDS, 99% glycerol, 1% bromophenol, 8 M urea and water (0.1 ml/0.2 ml/4 ml/1 ml/0.5 ml/4 ml)]. The samples were denatured by heating at 95°C for 3 min and reduced by adding 10 ul of β-mercaptoproethanol. One to 5 ug of the reduced and denatured protein was applied onto the stacking gel in a maximum of 20 ul and electrophoresed as described for Polyacrylamide Slab Gel Electrophoresis. Protein bands were detected by silver stain (Merrill et al., 1981). Pictures of the gels were taken for permanent records.
Approximate molecular weights were determined by comparison of protein migration to relative mobilities of Enprotech mid range molecular weight standards (Hyde Park, MA) on silver stained gels (Merrill et al., 1981). The markers used were a mixture of proteins (cytochrome C (12,400 D); lactoglobulin (18,400 D); carbonic anhydrase (29,000 D); ovalbumin (43,000 D); glutamate dehydrogenase (55,000 D); and phosphorylase B (95,500 D)). Additionally, in some studies Pharmacia low molecular weight standards (Piscataway, NJ) were used to determine the relative mass of PAT. The molecular weight markers included α-lactalbumin (14,400 D); trypsin inhibitor (20,100 D); carbonic anhydrase (30,000 D); ovalbumin (43,000 D); albumin (67,000 D); and phosphorylase B (94,000 D).

Data Analysis

Initial reaction rates were determined in individual hamster cytosolic preparations. The rates were averaged and tested for significant differences between rapid and slow acetylator genotypes with a two-tailed Student's t-test.

Apparent Km and Vmax kinetic constants, determined for individual hamster cytosolic and isozyme NAT activities, were averaged and tested for significant differences between homozygous rapid and slow acetylator genotypes with a two-tailed Student's t-test. Comparisons of Km and Vmax values between PAT and MAT isozymes, from both rapid and slow acetylators, were assessed by one-way analysis of variance to test for kinetic differences. The
levels of significant difference were statistically analyzed by the Newman-Keuls multiple range test.

Comparisons of rate constants and heated/control ratios, determined for the PAT and MAT isozymes, were assessed by one-way analysis of variance to test for rate differences. The levels of significant difference were statistically analyzed by the Newman-Keuls multiple range test.

Purified protein samples were desalted in deionized double distilled water using an Amicon Centriprep concentrator. The samples were taken to the Microchemical Facility at Emory University (Atlanta, GA) for protein sequencing, where the desalted protein was lyophilized and dissolved in 70% formic acid. After lyophilization, the protein was dissolved in a mixture of 25% trifluoroacetic acid/20% acetonitrile (1/1). The protein samples were lyophilized again and redissolved in 70% acetonitrile in diluted trifluoroacetic acid. Automatic Edman degradation of the protein was conducted in a Model 477A pulse-liquid sequence analyzer equipped with a 120A PTH-analyzer (Applied Biosystems).
CHAPTER IV

EXPERIMENTAL RESULTS

Liver NAT Activity in Inbred Hamsters of Known Acetylator Genotype

Initial rates of AcCoA-dependent NAT activity were determined in liver cytosols towards PABA, INH, AF, and ABP. Comparisons were made between homozygous rapid acetylators (Bio. 87.20) and homozygous slow acetylators (Bio. 82.73/H). A gene dose-response relationship was expressed in hamster liver cytosol towards PABA, AF, and ABP; whereas, acetylator genotype-independent expression of NAT activity was found in hamster liver cytosol towards INH (Table 1).

Liver OAT Activity in Inbred Hamsters of Known Acetylator Genotype

AcCoA-dependent N-hydroxyarylamine OAT activity was determined in cytosolic liver preparations from homozygous rapid acetylators and homozygous slow acetylators. The expression of acetylator genotype in the metabolic activation of N-hydroxyarylamines was substrate dependent. Table 2 shows the initial rates of AcCoA-dependent OAT activity towards N-OH-AF exhibited an acetylator gene-dose response in hamster liver cytosol. In contrast, the AcCoA-dependent metabolic activation of N-OH-ABP and N-OH-DMABP expressed activity levels that did not differ significantly across acetylator genotypes (Table 2).
Table 1. AcCoA-Dependent NAT Activity in Inbred Hamster Liver Cytosol.

<table>
<thead>
<tr>
<th>Acetylator Genotype</th>
<th>PABA</th>
<th>AF</th>
<th>ABP</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>164.9 ± 5.0*</td>
<td>51.9 ± 5.9*</td>
<td>41.3 ± 11.2*</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>rr</td>
<td>1.8 ± 0.6</td>
<td>8.4 ± 1.2</td>
<td>9.1 ± 2.0</td>
<td>15.5 ± 2.4</td>
</tr>
</tbody>
</table>

Results expressed as nmoles/min/mg protein.
Table values represent Mean ± S.D. for four or more animals.
*NAT activity in RR acetylator genotype is significantly higher (P < 0.0001) than rr acetylator genotype.
Table 2. AcCoA-Dependent Metabolic Activation of N-Hydroxyarylamines by Inbred Hamster Liver Cytosol.

<table>
<thead>
<tr>
<th>Acetylator Genotype</th>
<th>Substrate</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-OH-AF</td>
<td>N-OH-ABP</td>
<td>N-OH-DMABP</td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>5.8 ± 1.0*</td>
<td>8.2 ± 2.1</td>
<td>3.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>rr</td>
<td>1.2 ± 0.4</td>
<td>8.2 ± 2.1</td>
<td>3.1 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as nmols bound/15 min/mg DNA/mg protein. Table values represent Mean ± S.D. for four animals.

*Metabolic activation in RR acetylator genotype is significantly higher (P < 0.001) than rr acetylator genotype.
Results of Partially Purified Acetyltransferase Isozymes in Liver Cytosol

Liver cytosols from homozygous rapid and homozygous slow acetylator hamsters were partially purified by anion-exchange FPLC. Two forms of AcCoA-dependent acetyltransferase isozymes were identified in both rapid and slow acetylator hamsters. The isozymes have been designated as PAT (acetylator-genotype dependent) and MAT (acetylator-genotype independent) eluting at 60 mM and 120 mM KCl respectively (Fig. 8). The N-acetylation of PABA (Fig. 8) and AF (Fig. 9) and the metabolic activation of N-OH-AF (Fig. 10), N-OH-ABP (Fig. 11), and N-OH-DMABP (Fig. 12) by the PAT and MAT isozymes from rapid and slow acetylators were examined.

The PAT isozyme, derived from homozygous rapid acetylator hamster liver cytosol, exhibited significantly higher levels of AcCoA-dependent NAT and OAT activities, than the PAT isozyme derived from homozygous slow acetylator (Table 3). In contrast, the MAT isozymes showed similar levels of NAT and OAT activities in rapid and slow acetylator hamster liver cytosol (Table 3). By comparison the PAT and MAT isozymes, from both homozygous rapid and slow acetylators, exhibited similar NAT activity towards INH (Fig. 13).

Apparent Maximum Velocities of NAT Activity in Homozygous Rapid and Slow Acetylator Hamster Liver Cytosol

AcCoA-dependent NAT activity was determined in crude liver cytosol preparations from homozygous rapid acetylators and
Fig. 8. Anion-Exchange FPLC of PABA NAT Activity. Acetylation transferase activity in nmoles/min/ml is plotted on the left ordinate versus fraction number on the abscissa for AcCoA-dependent PABA NAT activity. The top panel represents liver cytosol from a homozygous rapid acetylator hamster (Bio. 87.20) and the lower panel represents liver cytosol from a homozygous slow acetylator hamster (Bio. 82.73/H). The right ordinate indicates % KCl of the elutant. For further details see Materials and Methods.
N-ACETYLTRANSFERASE ACTIVITY (nmoles/min/ml)
Fig. 9. Anion-Exchange FPLC of AF NAT Activity.

Acetyltransferase activity in nmoles/min/ml is plotted on the ordinate versus fraction number on the abscissa for AcCoA-dependent AF NAT activity. The top panel represents liver cytosol from a homozygous rapid acetylator hamster and the lower panel represents liver cytosol from a homozygous slow acetylator hamster. The salt gradient is illustrated in Fig. 8. For further details see Materials and Methods.
N-ACETYLTRANSFERASE ACTIVITY

Fraction Number

0 10 20 30

N-ACETYLTRANSFERASE ACTIVITY (nmols/min/ml)

0 10 20 30 40 50

AF
Fig. 10. Anion-Exchange FPLC of AcCoA-Dependent Metabolic Activation of N-OH-AF Via OAT Activity. Metabolic activation of N-OH-AF in nmoles bound/15 min/mg DNA/ml is plotted on the ordinate versus fraction number on the abscissa. The top panel represents liver cytosol from a homozygous rapid acetylator hamster and the lower panel represents liver cytosol from a homozygous slow acetylator hamster. The salt gradient is illustrated in Fig. 8. For further details see Materials and Methods.
Fig. 11. Anion-Exchange FPLC of AcCoA-Dependent Metabolic Activation of N-OH-ABP Via OAT Activity. Metabolic activation of N-OH-ABP in nmoles bound/15 min/mg DNA/ml is plotted on the ordinate versus fraction number on the abscissa. The top panel represents liver cytosol from a homozygous rapid acetylator hamster and the lower panel represents liver cytosol from a homozygous slow acetylator hamster. The salt gradient is illustrated in Fig. 8. For further details see Materials and Methods.
Fig. 12. Anion-Exchange FPLC of AcCoA-Dependent Metabolic Activation of N-OH-DMABP Via OAT Activity. Metabolic activation of N-OH-DMABP in nmoles bound/15 min/mg DNA/ml is plotted on the ordinate versus fraction number on the abscissa. The top panel represents liver cytosol from a homozygous rapid acetylator hamster and the lower panel represents liver cytosol from a homozygous slow acetylator hamster. The salt gradient is illustrated in Fig. 8. For further details see Materials and Methods.
<table>
<thead>
<tr>
<th>Reaction</th>
<th>ER Acetylator</th>
<th>rr Acetylator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol applied</td>
<td>PAT&lt;sup&gt;a&lt;/sup&gt; eluted</td>
</tr>
<tr>
<td>PABA</td>
<td>3122</td>
<td>1926</td>
</tr>
<tr>
<td>NAT&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF</td>
<td>264</td>
<td>95.6</td>
</tr>
<tr>
<td>NAT&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-OH-AF&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+AcCoA)</td>
<td>161</td>
</tr>
<tr>
<td>N-OH-DMABP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(+AcCoA)</td>
<td>65.3</td>
</tr>
<tr>
<td>N-OH-ABP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(+AcCoA)</td>
<td>134</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total activity eluted in PAT peak.
<sup>b</sup>Total activity eluted in MAT peak.
<sup>c</sup>AcCoA-dependent reaction; units are moles acetylated/min of NAT activity.
<sup>d</sup>AcCoA-dependent reaction; units are moles bound/15 min/mg DNA.
<sup>e</sup>AcCoA-dependent reaction; units are moles bound/15 min/mg DNA of OAT activity.
Fig. 13. Anion-Exchange FPLC of INH NAT Activity.
Acetyltransferase activity in nmoles/min/ml is plotted on the ordinate versus fraction number on the abscissa for AcCoA-dependent INH NAT activity. The top panel represents liver cytosol from a homozygous rapid acetylator hamster and the lower panel represents liver cytosol from a homozygous slow acetylator hamster. The salt gradient is illustrated in Fig. 8. For further details see Materials and Methods.
N-ACETYLMETHANOL TRANSFERASE ACTIVITY
(µmoles/min/ml)

FRACTION NUMBER
homozygous slow acetylators. As shown in Table 4, the apparent Vmax of NAT activity in homozygous rapid acetylator liver cytosol was significantly higher (p < 0.01) than the apparent Vmax of NAT activity of homozygous slow acetylator liver cytosol with varying substrate concentrations of AcCoA (Fig. 14), PABA (Fig. 15), AF (Fig. 16), and ABP (Fig. 17). In contrast, there was no significant difference (p > 0.05) in the apparent Vmax of liver cytosolic INH NAT activity between the acetylator genotypes (Table 4, Fig. 18).

Apparent Maximum Velocities of Partially Purified NAT Isozymes in Homozygous Rapid and Slow Acetylator Liver Cytosol

Homozygous rapid and slow acetylator hamster liver cytosol were partially purified by anion-exchange chromatography. The two forms of NAT isozymes, designated as PAT and MAT, were isolated in both rapid and slow acetylator liver cytosol. Both the PAT and the MAT isozymes catalyzed the AcCoA-dependent N-acetylation of PABA, INH, AF, and ABP. The apparent Vmax of the PAT isozyme in homozygous rapid acetylators exhibited a 95- to 110-fold higher PABA NAT activity with AcCoA (Fig. 14) and PABA (Fig. 15) as the variable substrates than the PAT isozyme of homozygous slow acetylators. Similarly, the apparent Vmax of NAT activity in the PAT isozyme of homozygous rapid acetylators was 7- to 10-fold higher (Table 4) than the PAT isozyme of homozygous slow acetylators with AF (Fig. 16) and ABP (Fig. 17). In contrast, the apparent Vmax of NAT activity catalyzed via the MAT isozyme did not exhibit a significant
### TABLE 4. Apparent Maximum Velocities of Hamster Liver NAT Activities.

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Acetylator Genotype</th>
<th>AcCoA</th>
<th>PABA</th>
<th>AF</th>
<th>ABP</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>192 ± 10*</td>
<td>208 ± 74*</td>
<td>60 ± 7*</td>
<td>64 ± 7*</td>
<td>22 ± 10</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>2 ± 0.5</td>
<td>11 ± 4</td>
<td>7 ± 3</td>
<td>10 ± 3</td>
<td>35 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>1760 ± 851*</td>
<td>1240 ± 241*</td>
<td>350 ± 25*</td>
<td>305 ± 82*</td>
<td>73 ± 5</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>16 ± 6</td>
<td>13 ± 5</td>
<td>53 ± 16</td>
<td>32 ± 6</td>
<td>53 ± 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>41 ± 9*</td>
<td>40 ± 22</td>
<td>18 ± 5</td>
<td>17 ± 5</td>
<td>165 ± 67</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>12 ± 6</td>
<td>95 ± 55</td>
<td>21 ± 4</td>
<td>18 ± 9</td>
<td>120 ± 56</td>
</tr>
</tbody>
</table>

Table values represent Mean ± S.D. for three or more animals.

*Apparent Vmax in RR acetylator genotype is significantly higher (P < 0.01) than rr acetylator.*
Fig. 14. Michaelis-Menten Kinetic Constants for Hamster Liver NAT Activity. PABA NAT activity in nmoles/min/mg is plotted on the ordinates versus micromolar concentration of AcCoA on the abscissae. The left panels represent rapid acetylators (RR) and the right panels represent homozygous slow acetylators (rr). The top panels represent determinations in unpurified cytosol, the center panels represent determinations with the polymorphic NAT isozyme (PAT) and the lower panel represent determinations with the monomorphic NAT isozyme (MAT). The initial PABA concentrations was 0.44 mM for each cytosol and 0.22 mM for each isozyme determination. The inset figures illustrate Eadie-Hofstee transformations of the data. In some cases, the enzyme rate in nmoles/min/mg is plotted as exponential. The kinetic constants derived from this data are shown in Tables 4 and 5.
AcCoA

N-ACETYLTRANSFERASE ACTIVITY
Fig. 15. Michaelis-Menten Kinetic Constants for Hamster Liver PABA NAT Activity. PABA NAT activity in nmoles/min/mg is plotted on the ordinates versus micromolar concentration of PABA on the abscissae. The initial concentration for AcCoA in each assay was 2.22 mM in cytosol and 1.0 mM for the two NAT isozymes. The inset figures represent Eadie-Hofstee transformations of the data. For further information, see the legend to Fig. 14.
N-ACETYLTRANSFERASE ACTIVITY

PABA

N-ACETYLTRANSFERASE ACTIVITY
Fig. 16. Michaelis-Menten Kinetic Constants for Hamster Liver AF NAT Activity. AF NAT activity in nmoles/min/mg is plotted on the ordinates versus micromolar concentration of AF on the abscissae. The initial concentration of AcCoA was 1.0 mM in all assays. The inset figures represent Eadie-Hofstee transformations of the data. For further information, see the legend to Fig. 14.
N-ACETYLTRANSFERASE ACTIVITY

AF

N-ACETYLTRANSFERASE ACTIVITY

[Graphs showing data with N-moles/20 min/mg protein]

Rate vs. substrate concentration

[NMoles/20 min/mg protein vs. mg protein]
Fig. 17. Michaelis-Menten Kinetic Constants for Hamster Liver ABP NAT Activity. ABP NAT activity in nmoles/min/mg is plotted on the ordinates versus micromolar concentration of ABP on the abscissae. The initial concentration of AcCoA was 1.0 mM in all assays. The inset figures represent Eadie-Hofstee transformations of the data. For further information, see the legend to Fig. 14.
Fig. 18. Michaelis–Menten Kinetic Constants for Hamster Liver INH NAT Activity. INH NAT activity in nmoles/min/mg is plotted on the ordinates versus micromolar concentration of INH on the abscissae. The initial concentrations of AcCoA were 2.22 mM in cytosol and 1.0 mM for the two NAT isozymes. The inset figures represent Eadie–Hofstee transformations of the data. For further information, see the legend to Fig. 14.
INH

N-ACETYLTRANSFERASE ACTIVITY

[Graphs showing enzymatic activity over time with rate calculations]
difference between acetylator genotypes for the N-acetylation of PABA, AF, ABP, or INH (Table 4, Figs. 15-18).

Comparisons of kinetic constants of the PAT and MAT isozymes, within the rapid acetylator genotype, revealed that the PAT isozyme showed an 18- to 30-fold higher apparent Vmax in NAT activity towards PABA (Fig. 15), AF (Fig. 16), and ABP (Fig. 17) than the MAT isozyme (Table 4). On the other hand, comparison of the kinetic constants of the PAT and MAT isozymes within the homozygous slow acetylator genotype indicated that the MAT isozyme expressed a 7-fold higher apparent Vmax in PABA NAT activity than the PAT isozyme (Fig. 15, Table 4). In contrast, the PAT isozyme showed a higher apparent Vmax towards AF (Fig. 16) and ABP (Fig. 17) NAT activities than the MAT isozyme in slow acetylators (Table 4). The MAT isozyme showed a 2-fold higher apparent Vmax for INH NAT activity than the PAT isozyme in both rapid and slow acetylators (Fig. 18, Table 4).

**Apparent Km Determinations of NAT Activity in Homozygous Rapid and Slow Acetylator Liver Cytosol**

Michaelis-Menten kinetic constants were derived for NAT activity in homozygous rapid and slow acetylator hamster liver cytosol as described in Materials and Methods. PABA NAT activity was measured as a function of varying AcCoA concentrations to derive an apparent Km for the AcCoA acetyl donor. The Km values were 931 and 256 µM for homozygous rapid and slow acetylators, respectively. Under these conditions, the apparent Km was 3.5-fold higher in the homozygous rapid acetylator genotype than the homozygous slow (Fig.
The apparent Km towards PABA, INH, AF, and ABP were determined in the presence of saturating levels of AcCoA. The kinetic constants differed between substrates and genotypes. The apparent Km for PABA was 8.5-fold lower in the homozygous rapid acetylator genotype than the homozygous slow acetylator genotype (Fig. 15, Table 5). In contrast, the apparent Km for INH, AF, and ABP were similar, yet slightly higher in the homozygous rapid acetylator genotype than the homozygous slow acetylator genotype (Figs. 16-18, Table 5).

Apparent Km Determinations of Partially Purified NAT Isozymes in Homozygous Rapid and Slow Acetylator Liver Cytosol

The apparent Km values of the PAT and MAT isozymes also differed between substrates and acetylator genotypes. The apparent Km of the PAT isozyme was significantly lower in the homozygous rapid acetylator genotype than in the homozygous slow acetylator genotype for PABA, whereas it was significantly higher for AF, ABP, and AcCoA (Figs. 14-17, Table 5). The apparent Km of the MAT isozyme was substantially lower (26-fold) in the homozygous rapid acetylator genotype (Km = 81 uM) than in the homozygous slow (Km = 2120 uM) for PABA, but it was significantly higher for AcCoA in the homozygous rapid acetylator (Km = 436 uM) than in the homozygous slow acetylator (Km = 142 uM) (Figs. 14-15, Table 5). The apparent Km of the MAT isozyme towards AF (Fig. 16), ABP (Fig 17), and INH (Fig. 18) did not differ significantly between the acetylator genotypes (Table 5).
### Table 5. Apparent Km of Hamster Liver NAT Activities.

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Acetylator Genotype</th>
<th>AcCoA</th>
<th>PABA</th>
<th>AF</th>
<th>ABP</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>931 ± 104*</td>
<td>189 ± 117*</td>
<td>155 ± 36*</td>
<td>142 ± 32*</td>
<td>100 ± 36</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>256 ± 70</td>
<td>1600 ± 897</td>
<td>82 ± 25</td>
<td>99 ± 11</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>PAT</td>
<td>RR</td>
<td>350 ± 49*</td>
<td>36 ± 10*</td>
<td>440 ± 10*</td>
<td>425 ± 77*</td>
<td>313 ± 75</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>45 ± 21</td>
<td>162 ± 29</td>
<td>44 ± 19</td>
<td>25 ± 3</td>
<td>589 ± 139</td>
</tr>
<tr>
<td>MAT</td>
<td>RR</td>
<td>436 ± 30*</td>
<td>81 ± 10*</td>
<td>11 ± 4</td>
<td>10 ± 6</td>
<td>547 ± 39</td>
</tr>
<tr>
<td></td>
<td>rr</td>
<td>142 ± 31</td>
<td>2120 ± 659</td>
<td>10 ± 6</td>
<td>15 ± 5</td>
<td>702 ± 18</td>
</tr>
</tbody>
</table>

Table values represent Mean ± S.D. for three or more animals.

*Apparent Km in RR acetylator genotype is significantly different (P < 0.05) than rr acetylator genotype.
Purification of PAT and MAT Isozymes

Q-Sepharose anion-exchange chromatography was used to separate the PAT and MAT isozymes from the liver cytosol of homozygous rapid acetylator hamsters. Subsequently, the PAT and MAT isozymes were further purified by sequential Sephacryl S-200 gel filtration and anion-exchange FPLC chromatography (Fig. 7). The data in Table 6 and Fig. 19 summarize a typical purification of hepatic PAT isozyme. PAT activities were determined at each step with the prototypic polymorphic substrate PABA (Table 6). The fold purification factor was typically between 500- and 2400-fold and the yield was about 2-10%.

Table 7 and Fig. 20 summarize the purification of the MAT isozyme. The specific activity for the MAT isozyme was determined at each purification step using PABA and INH as substrates. The specific activity with PABA as the acetyl-acceptor was 7796 units/mg of protein which represented only 4% of the catalysis rate for the purified PAT isozyme. The specific activity with INH as the acetyl-acceptor was 1764 units/mg of protein and represented a 150-fold purification with a 1.0% yield.

Protein Homogeneity and Molecular Weight Determinations

Native PAGE and SDS-PAGE were used to determine whether the PAT and MAT isozymes were purified to homogeneity. The PAT sample extracted from native PAGE exhibited high NAT activity (Fig. 21).
Table 6. Purification of Liver AcCoA-Dependent PAT from Homozygous Rapid Acetylator Hamsters.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (umoles/min)</th>
<th>Specific Activity (umoles/min/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 105,000 x g</td>
<td>491</td>
<td>10.6</td>
<td>5190</td>
<td>583</td>
<td>0.112</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>2. Q-Sepharose Anion-Exchange</td>
<td>2.13</td>
<td>28.2</td>
<td>60.0</td>
<td>224</td>
<td>3.64</td>
<td>38.5</td>
<td>32.4</td>
</tr>
<tr>
<td>3. Sephacyl S-200</td>
<td>18.2</td>
<td>0.30</td>
<td>5.46</td>
<td>98.4</td>
<td>18.0</td>
<td>16.9</td>
<td>161</td>
</tr>
<tr>
<td>4. Anion-Exchange FPLC</td>
<td>1.50</td>
<td>0.12</td>
<td>0.18</td>
<td>35.0</td>
<td>194</td>
<td>6.01</td>
<td>1730</td>
</tr>
</tbody>
</table>

**Note:**
Initial concentrations in the reactions mixture were 0.22 mM PABA and 1.00 mM AcCoA.
Fig. 19. Chromatographic Purification of PAT from Homozygous Rapid Acetylator Hamster Liver. Polymorphic PABA NAT activity in nmoles/min/ml is plotted on the left ordinate versus fraction number on the abscissa. The top panel represents Q-Sepharose anion-exchange chromatography, the center panel represents Sephacryl S-200 gel filtration chromatography, and the bottom panel represents anion-exchange FPLC chromatography. The right ordinate indicates % KCl of the elutant. For further details see Materials and Methods.
Table 7. Purification of Liver AcCoA-Dependent MAT from Homozygous Rapid Acetylator Hamsters.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Total Activity (moles/min)</th>
<th>Specific Activity (moles/min/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
<th>INH NAT</th>
<th>PABA NAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 105,000 x g</td>
<td>235</td>
<td>9.46</td>
<td>26,200</td>
<td>11.8</td>
<td>100</td>
<td>0.60</td>
<td>90.5</td>
<td>67.8</td>
</tr>
<tr>
<td>2. Q-Sepharose Anion-Exchange</td>
<td>2.50</td>
<td>83.4</td>
<td>1,480</td>
<td>7.09</td>
<td>5.63</td>
<td>0.60</td>
<td>300</td>
<td>380</td>
</tr>
<tr>
<td>3. Sephacryl S-200 Gel filtration</td>
<td>30.0</td>
<td>0.36</td>
<td>1,350</td>
<td>125</td>
<td>5.13</td>
<td>10.6</td>
<td>300</td>
<td>380</td>
</tr>
<tr>
<td>4. Anion-Exchange FPLC</td>
<td>2.0</td>
<td>0.75</td>
<td>282</td>
<td>1760</td>
<td>1.08</td>
<td>150</td>
<td>7,800</td>
<td>7,800</td>
</tr>
</tbody>
</table>
Fig. 20. Chromatographic Purification of MAT from Homozygous Rapid Acetylator Hamster Liver. Monomorphic INH NAT activity in nmoles/min/ml is plotted on the ordinate versus fraction number on the abscissa. The top panel represents Q-Sepharose anion-exchange chromatography, the center panel represents Sephacryl S-200 gel filtration chromatography, and the bottom panel represents anion-exchange FPLC chromatography. The right ordinate indicates % KCl of the elutant. For further details see Materials and Methods.
N-ACETYLMYLTRANSFERASE ACTIVITY

(nmoles/min/ml)

FRACTION NUMBER

IKC%
Fig. 21. Native Gel Analysis of Purified PAT from Homozygous Rapid Acetylator Hamster Liver. The PAT isozyme was applied to native PAGE as described in Materials and Methods. Following electrophoresis, gels were sliced and the protein band was extracted and assayed for PABA NAT activity. The NAT activity in nmoles/min/ml is plotted on the ordinate versus gel slice number on the abscissa. The specific activity of gel slice number 9 was 1190 nmoles/min/mg.
Following SDS-PAGE, a single protein band was detected after silver staining (Fig. 22). In contrast, the MAT isozyme was not purified to homogeneity using the procedure described in this study (data not shown).

The molecular weight of the reduced PAT isozyme was estimated from SDS-PAGE using standard proteins. An apparent molecular weight of 37–38 KD was obtained for the PAT isozyme (Fig. 22).

Comparison of PAT and MAT Acetyl Transfer Reactions

Partially purified Sephacryl S-200 PAT and MAT isozymes of homozygous rapid acetylator hamster liver cytosol were analyzed for a diverse set of acetyl transfer reactions as shown in Table 8. The relative specificity of the two isozymes to catalyze the various acetyl transfer reactions appears to be substrate and activity-dependent (Table 8). Most of the AcCoA-dependent N-acetylation of PABA and AF, and the AcCoA-dependent O-acetylation of N-OH-AF are catalyzed by the PAT isozyme. In striking contrast, essentially all of the AcCoA-independent arylhydroxamic acid N,O-AT activity for either N-OH-AAF or N-OH-AABP is catalyzed by the MAT isozyme. These results substantiate studies done with more crude preparations.

Heat Inactivation of NAT and OAT Activities

The heat inactivation rates of NAT and OAT activities in the PAT and MAT isozymes of homozygous rapid acetylator hamsters were determined to assess whether the PAT and MAT isozymes are single
Fig. 22. SDS-PAGE of Purified PAT from Homozygous Rapid Acetylator Hamster Liver. SDS-polyacrylamide slab gels (12.5%) were prepared and run as described under Materials and Methods. Protein bands were detected by silver stain. Lane 1 and 4 contain molecular weight markers as described under Materials and Methods. Lane 2 contains 16 ug of liver cytosol. Lane 3 contains 1.4 ug of the PAT isozyme.
Table 8. Relative Capacity of Homozygous Rapid Acetylator Hamster Liver Cytosol PAT and MAT Acetyltransferase Isozymes to Catalyze Various Acetyl Transfers.

<table>
<thead>
<tr>
<th>Acetyl Transfer Reaction/Substrate</th>
<th>PAT</th>
<th>MAT</th>
<th>PAT/MAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcCoA-dependent NAT&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PABA</td>
<td>50,500 ± 828</td>
<td>383 ± 35</td>
<td>132</td>
</tr>
<tr>
<td>AF</td>
<td>21,100 ± 1230</td>
<td>550 ± 24</td>
<td>38</td>
</tr>
<tr>
<td>AcCoA-dependent OAT&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-OH-AF</td>
<td>32,200 ± 710</td>
<td>550 ± 24</td>
<td>59</td>
</tr>
<tr>
<td>AcCoA-independent N,O-AT&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-OH-AAF</td>
<td>15.7</td>
<td>3380</td>
<td>0.005</td>
</tr>
<tr>
<td>N-OH-AABP</td>
<td>18.4</td>
<td>3670</td>
<td>0.005</td>
</tr>
</tbody>
</table>

<sup>a</sup>PAT and MAT isozyme partially purified through Sephacryl S-200 chromatography. Table values represent Mean ± S.D. n = 3.

<sup>b</sup>Enzyme activity expressed as nmoles/min/mg protein.

<sup>c</sup>Enzyme activity expressed as pmoles bound/min/mg DNA/mg protein.

<sup>d</sup>An average of duplicate determinations from a single enzymatic preparation.
proteins capable of catalyzing both the NAT and OAT activities. Partially purified Sephacryl S-200 PAT and MAT liver preparations were used, as well as the purified PAT liver preparation obtained by anion-exchange FPLC chromatography (Mono Q). PABA (1 mM), AF (2.5 mM), and N-OH-AF (5.0 mM) served as substrates. Thermal stability was determined after preincubation at 55°C for various time points (Figs. 23-24). The PAT and MAT heat inactivation rates followed first order kinetics. The MAT heat inactivation rate constants of PABA and AF NAT and N-OH-AF OAT activities did not differ significantly (P > 0.05) (Table 9). The heat inactivation half-lives were 4.46 min for PABA NAT activity, 4.38 min for AF NAT activity, and 4.97 min for N-OH-AF OAT activity.

The partially purified Sephacryl S-200 PAT isozyme exhibited similar heat inactivation rates for AF NAT and N-OH-AF OAT activities; however, these rates differed significantly (P < 0.05) from PABA NAT activity. Similar results were obtained upon further purification of this isozyme on anion-exchange chromatography FPLC (Mono Q). The heat inactivation half-lives were 3.76 min for PABA NAT activity, 1.99 min for AF NAT activity, and 2.25 min for N-OH-AF OAT activity.

After preincubation at 55°C for 5 min, a heated/control (H/C) ratio was used to determine the remaining proportion of enzyme activity. The H/C ratios for the NAT and OAT activities catalyzed via the PAT and MAT isozymes are shown in Table 10. The results suggested that PAT-catalyzed AF NAT and N-OH-AF OAT activities are
Fig. 23. Thermal inactivation of the MAT isozyme from Homozygous Rapid Acetylator Hamster Liver. Partially purified Sephacryl S-200 MAT isozyme was analyzed for PABA NAT, AF NAT, and N-OH-AF OAT activities following incubations at 55°C for the times indicated. The percent residual activity is plotted on the ordinate versus time of incubation at 55°C on the abscissa. The top panel (A) represents PABA NAT activity, the center panel (B) represents AF NAT activity, and the lower panel (C) represents N-OH-AF OAT activity. The inset figures represent linear single exponential transformations of the data.
Fig. 24. Thermal Inactivation of the PAT Isozyme from Homozygous Rapid Acetylator Hamster. Partially purified Sephacryl S-200 and the homogenous polymorphic acetyltransferase isozyme were analyzed for PABA NAT, AF NAT, and N-OH-AF OAT activities following incubations at 55°C for the times indicated. The percent residual activity is plotted on the ordinate versus time of incubation at 55°C on the abscissa. The left panel represents Sephacryl S-200 partially purified PAT isozyme and the right panel represents highly purified PAT isozyme. The top panels (A and D) represent PABA NAT activity, the center panels (B and E) represent AF NAT activity), and the lower panels (C and F) represent N-OH-AF OAT activity. The inset figures represent linear single exponential transformations of the data.
PERCENT RESIDUAL ACTIVITY

INCUBATION TIME AT 55°C (MIN)
Table 9. Thermal Stability of NAT and OAT Activities Catalyzed by Rapid Acetylator Hamster Liver Isozymes.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Preparation</th>
<th>Thermal Inactivation Rate Constant (Min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PABA NAT</td>
</tr>
<tr>
<td>MAT</td>
<td>Sephacryl S-200ᵃ</td>
<td>0.155 ± 0.020ᶜ</td>
</tr>
<tr>
<td>PAT</td>
<td>Sephacryl S-200ᵃ</td>
<td>0.185 ± 0.012ᵈ</td>
</tr>
<tr>
<td>PATᵇ</td>
<td>Mono Qᵇ</td>
<td>0.152 ± 0.028ᵈ</td>
</tr>
</tbody>
</table>

Table values represent Mean ± S.D. n = 3.

ᵃIsozyme partially purified through Sephacryl S-200 chromatography.
ᵇIsozyme highly purified through anion-exchange FPLC chromatography.
ᶜRate constants not significantly different (P > 0.05) for MAT isozyme.
ᵈRate constants of PABA NAT significantly different from AF NAT and N-OH-AF OAT (P < 0.05) for PAT isozyme.
ᵉRate constants of AF NAT and N-OH-AF OAT not significantly different (P > 0.05).
ᶠTable values obtained from a single assay.
Table 10. Thermal Inactivation Heated/Control Ratio of NAT and OAT Activities Catalyzed by Rapid Acetylator Hamster Liver Isozymes.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>PABA NAT</th>
<th>AF NAT</th>
<th>N-OH-AF-OAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATa</td>
<td>0.463 ± 0.88</td>
<td>0.343 ± 0.051</td>
<td>0.320 ± 0.078</td>
</tr>
<tr>
<td>PAT</td>
<td>0.430 ± 0.044b</td>
<td>0.257 ± 0.021c</td>
<td>0.217 ± 0.025c</td>
</tr>
</tbody>
</table>

The effects of preincubation at 55°C for 5 min on Sephacryl S-200 PAT and MAT isozymes. Table values represent Mean ± S.D. n = 3.

aH/C ratios not significantly different (P > 0.05) for MAT isozyme.
bH/C ratio of PABA NAT significantly different (P < 0.05) from AF NAT and N-OH-AF OAT for PAT isozyme.
cH/C ratio of AF NAT and N-OH-AF OAT not significantly different (P > 0.05).
more thermolabile than PABA NAT activity. The H/C ratios for the various MAT acetyl transfer reactions, however, were not significantly different (P > 0.05) (Table 10).

**Determination of PAT Amino Acid Sequence**

Homogenous PAT samples were taken to the Microchemical Facilities at Emory University (Atlanta, GA) for sequencing analysis. Determination of protein sequence from their amino terminal by automatic Edman degradation was unsuccessful because the N-terminal was blocked. Subsequent PAT samples have been subjected to trypsin digestion. The tryptic peptides are presently being isolated, and purified by HPLC for sequence analysis.
CHAPTER V

DISCUSSION

The acetylation polymorphism is a heritable trait of two alleles at a single gene locus (Weber and Hein, 1985). Genetic variability in acetylation capacity has been associated with differences in susceptibility to arylamine-induced carcinogenesis (Weber 1987; Hein 1988a). Several studies have demonstrated that metabolic activation is a required factor for the initiation of arylamine-induced carcinogenesis. Acetylation reactions have been identified as key metabolic steps in the activation/deactivation of arylamine carcinogens and their metabolites (Miller and Miller, 1981b). Arylamine-induced bladder and colorectal carcinogenic models have suggested that variability in the levels of NAT and OAT activities are important factors in the genetic predisposition of individuals to these cancers (Hein, 1988a). Consequently, animals which exhibit the acetylation polymorphism are useful models for elucidating the mechanism(s) involved in arylamine-induced carcinogenesis.

The objective of this project was to investigate the genetic and biochemical characteristics of AcCoA-dependent NAT and OAT cytosolic enzymes with respect to genotype-dependent activity, kinetic constants, and chromatographic properties utilizing inbred Syrian hamsters of defined acetylator genotype.
Arylamine NAT and N-hydroxyarylamine OAT activities exhibited a similar pattern of acetylation polymorphism for the substrates tested. An acetylator gene-dose relationship was observed for PABA, AF, and ABP in hamster liver cytosol (Table 1). The highest levels of NAT activity were expressed in homozygous rapid acetylator hamsters and the lowest levels in homozygous slow acetylator hamsters. These results indicate the existence of an acetylator genotype-dependent expression of NAT activity in hamster liver. Similar results have also been obtained with cytosol derived from hamster bladder (Hein et al., 1987c), peripheral blood (Hein et al., 1986b), intestine, kidney, and lung (Hein et al., 1987b), and colon (Ogolla et al., 1988).

The magnitude of the difference in NAT activity between rapid and slow acetylator genotypes was substrate-dependent with the polymorphic PABA NAT activity in rapid acetylators about 82-fold greater than in the slow acetylators, while AF and ABP were 7- and 5-fold higher, respectively (Table 1). In contrast, INH exhibited similar levels of NAT activity across acetylator genotypes.

AcCoA-dependent metabolic activation of N-OH-AF in hamster liver cytosol also showed an acetylation polymorphism (Table 2). Higher activity was exhibited in homozygous rapid acetylators than in the homozygous slow acetylators. An acetylator genotype-independent expression was observed for the AcCoA-dependent metabolic activation of N-OH-ABP and N-OH-DMABP in crude liver cytosol (Table 2). The polymorphic expression of N-OH-AF and the monomorphic expression of
N-OH-ABP and N-OH-DMABP illustrate the substrate-dependent expression of the OAT polymorphism. The O-acetylation of N-hydroxyarylamine substrates has also been reported in other mammalian species including human liver, colon (Flammang and Kadiiubar, 1986; Flammang et al., 1987), and bladder (Kirlin et al., 1989) cytosols. Human OAT-mediated metabolic activation of N-OH-AF and N-OH-DMABP expressed in these tissues was acetylator genotype-dependent. Unlike in humans, previous studies with liver cytosol derived from rapid and slow acetylator inbred mice (Hein et al., 1988) and the present hamster study demonstrated that the metabolic activation of N-OH-DMABP exhibited acetylator genotype-independent expression.

The expression of multiple forms of acetyltransferase activity in hamster liver has been reported previously (Hein et al., 1985b; Smith and Hanna, 1986; Kato and Yamazoe, 1988). Partial purification of hamster liver cytosol, by anion-exchange chromatography indicated the presence of two distinct NAT isozymes in each acetylator genotype. These isozymes differed in chromatographic properties, substrate specificity, and genetic control. The PAT isozyme(s) was acetylator genotype-dependent and exhibited higher catalytic activity in rapid acetylators than in slow acetylators for all substrates tested except INH (Figs. 8-10). The MAT isozyme(s) was acetylator genotype-independent and exhibited similar catalytic activity in rapid and slow acetylators (Figs. 8-10). Recently, Grant et al. (1989) also demonstrated the presence
of two arylamine NATs in human liver cytosol. These NATs exhibited indistinguishable molecular masses (31 KD); however, they could be separated by anion-exchange chromatography. In addition, they were functionally distinguished by their difference in apparent affinities for the acceptor amine sulfamethazine.

The present data indicates that both the PAT and MAT isozymes in hamster liver cytosol catalyze the AcCoA-dependent N-acetylation of arylamines and the O-acetylation of N-hydroxyarylamines (Figs. 8-13, Table 3 and 8). Saito et al. (1986) reported the purification of a single enzyme from the liver cytosol of outbred Syrian golden hamsters. The molecular mass of the purified acetyltransferase was estimated to be 33 KD by gel filtration and SDS-PAGE. The acetyltransferase purified by Saito et al. (1986) appears to be the same or similar to the MAT isozyme characterized in the present study, since both enzymes exhibit high levels of N,O-AT activity. In contrast, that purified enzyme appears to differ from the PAT isozyme, since very low N,O-AT activity is exhibited by the latter enzyme.

In comparison to crude cytosol, the PAT isozyme(s) exhibited levels of AcCoA-dependent OAT and NAT activities that were consistent with the acetylation polymorphism for all substrates tested except INH (Table 3, Figs. 8-13). However, the MAT isozyme(s) showed levels of OAT and NAT activities that did not vary with acetylator genotype (Table 3, Figs. 8-13). These results demonstrate that the levels of expression of both NAT and OAT
activities are consistent with acetylator genotype as reflected by the PAT isozyme.

To further characterize the NAT activities expressed in liver cytosol and partially purified PAT and MAT isozymes, Michaelis-Menten kinetic constants were determined using homozygous rapid and slow acetylator hamster liver preparations.

In crude cytosol, the apparent Vmax for PABA, AF, and ABP NAT activities were significantly higher in the rapid acetylator genotype than the slow acetylator genotype. In contrast, the apparent Vmax for INH NAT activity was similar across acetylator genotypes (Table 4). These findings are consistent with the polymorphic expression of PABA (Andres and Weber, 1986) and AF (Hein et al., 1982b) NAT activity reported in inbred rabbit liver cytosol, but dissimilar to the polymorphic expression of INH NAT activity observed in rabbit liver cytosol (Hein et al., 1982c).

Determinations of the apparent Vmax catalytic activity of the partially purified PAT and MAT isozymes of rapid and slow acetylator genotypes clearly show that the PAT isozyme reflects the polymorphic expression of the NAT activity. This suggests that the PAT isozyme plays a significant role in the metabolism of arylamine carcinogens. The cytosolic differences in NAT activity observed between rapid and slow acetylators for the N-acetylation of PABA, AF, and ABP are primarily due to the PAT isozyme(s). By comparison, the lack of catalytic difference in cytosolic INH NAT activity between rapid and slow acetylators is due to the MAT isozyme(s)
Pat has been designated as the gene symbol for the PAT locus in the hamster, with Pat<sup>r</sup> representing the rapid acetylator allele and Pat<sup>s</sup> representing the slow acetylator allele (Hein, 1988b). The results of this investigation support the existence of a single gene locus in hamster liver which regulates the PAT isozyme(s). This isozyme(s) exhibits genetic variability in the N-acetylation of arylamine and in the O-acetylation of N-hydroxyarylamine carcinogens (Tables 3-5). However, the MAT isozyme(s) shows levels of NAT and OAT activities that do not vary with acetylator genotype.

The NAT activity of crude cytosol and of the partially purified PAT and MAT isozymes was further characterized by determination of the apparent Km for various substrates. The relationship between acetylator genotype and apparent Km values was substrate dependent. The apparent Km of cytosolic NAT activity was higher in homozygous rapid than in homozygous slow acetylators for all substrates tested, except PABA (Table 5). This result is consistent with similar reports in inbred rabbit liver cytosol (Andres and Weber, 1986), inbred hamster bladder cytosol (Yerokun et al., 1989) and human liver cytosol (Kilbane et al., 1988). The apparent Km for AcCoA in rapid and slow acetylator hamster liver cytosol (Table 5) is consistent with observations in rabbits (Reeves et al., 1988). The physiological basis for the relatively low affinity of NAT for its endogenous co-factor AcCoA is not known. However, recent studies by Kato and Yamazoe (1988) and the present study suggest that
relatively high levels of AcCoA concentrations are required for the N-acetylation reaction in vitro. In addition, in vivo studies in humans and isolated rat liver cells have demonstrated that an increase in the cellular concentration of AcCoA, via ethanol induction, increases the acetylation of drugs (Olsen and Morland, 1978; 1982). Therefore, AcCoA concentration appears to be a rate-limiting factor in the acetylation reaction in vivo. The present findings are also consistent with recent studies by Grant et al. (1989). They isolated and purified two NATs from human liver cytosol which differed in their affinity for AcCoA. It is interesting that the apparent Kms observed for the PAT and MAT isozymes of homozygous rapid acetylator are higher than those in the slow acetylator hamster. At present, this can not be accounted for; however, the data from the crude cytosol repeatedly indicated that the rapid acetylator exhibited a lower affinity for the co-factor than the slow acetylator (Table 5).

The apparent Km of the PAT isozyme differed significantly between homozygous rapid and slow acetylators for each substrate tested (Table 5). In contrast, the apparent Km for the MAT isozyme for AF and ABP did not differ, suggesting that the MAT isozyme(s) catalyzing the N-acetylation of these carcinogens is the same or very similar in homozygous rapid and slow acetylators.

The apparent Km values for PABA were lower in homozygous rapid than slow acetylators in crude cytosol and in both the PAT and MAT isozymes. The apparent Km for PABA was particularly high in slow
acetylator cytosol and in the slow acetylator MAT isozyme. This observation suggests that the N-acetylation of PABA occurs almost exclusively via the PAT isozyme.

Kinetic characterization of the partially purified NAT isozymes provide evidence for an intrinsic structural difference between the PAT and MAT isozymes in both rapid and slow acetylators. The kinetic data also suggests that rapid and slow acetylators express structural variants of the PAT isozyme (Pat and PatS allozymes) rather than quantitative differences in a single PAT isozyme. However, the purification of the PatS allozyme needs to be accomplished to strengthen this conclusion. Furthermore, the data indicate that the MAT isozymes are not expressed as structural variants in rapid and slow acetylators. Thus, the results support the hypothesis that there exists at least two isozymes of NAT in hamster liver cytosol, the PAT isozyme which is polymorphically regulated by the acetylator gene locus Pat and the MAT isozyme which may be monomorphically regulated, yielding catalytically similar NAT isozymes in rapid and slow acetylators.

To fully elucidate the biochemical mechanism(s) of the PAT and MAT isozymes and determine whether the NAT, OAT, and N,O-AT activities reside on the same protein, the purification of these isozymes is required. It is of special interest to determine the following: the definitive number of NAT isozymes; the precise differences in amino acid sequence between the PAT allozymes; and the nature of the gene mutation responsible for the existence of
structurally variant Pat$^r$ and Pat$^s$ allozymes. It is expected that the cloning and analysis of cDNA and genomic DNA encoding the PAT allozymes will provide this information. Consequently, it is of the utmost importance to purify the NAT isozymes. A combination of gel filtration and anion-exchange chromatographic procedures were employed to purify the PAT and MAT isozymes from the 105,000 x g supernatant of homozygous rapid acetylator hamster liver homogenate. The PAT isozyme was purified to apparent homogeneity as demonstrated by SDS-PAGE after silver staining. However, the MAT isozyme was not purified to apparent homogeneity under the same conditions. It is expected that slight modifications of the present procedure, coupled with a 4-aminoazobenzene Sepharose 6-B affinity chromatography step, will achieve the purification of the MAT isozyme. The 4-aminoazobenzene ligand exhibits a strong binding to the MAT isozyme (Smith and Hanna, 1986). The MAT isozyme from hamster liver has been purified to electrophoretic homogeneity by Saito et al. (1986). The highly purified MAT isozyme catalyzes the AcCoA-dependent arylamine N-acetylation and N-hydroxyarylamine O-acetylation, as well as the AcCoA-independent arylhydroxamic acid N,O-acyltransfer.

The PAT and MAT isozymes are capable of catalyzing several acetyl transfer reactions (Table 8). The relative specificity of the two isozymes appear to be reaction- and substrate-dependent. Most of the AcCoA-dependent NAT and OAT activities are eluted with the PAT isozyme. These activities could not be separated by protein
purification techniques which detect differences in ionic charge and molecular size. As shown in Table 8, the PAT/MAT ratios clearly indicate that the PAT isozyme plays an important role in the acetylator genotype-dependent expression of both NAT and OAT activities. In contrast, the PAT isozyme exhibited little or no AcCoA-independent N,O-AT activity. The similarities between NAT and OAT activities do not necessarily mean that both activities are catalyzed by the same protein. However, the results of this investigation strongly support the existence of a PAT isozyme which is under the genetic control of the acetylator gene and catalyzes both the NAT and OAT activities. The MAT isozyme catalyzes the AcCoA-dependent NAT and OAT activities, as well as the AcCoA-independent N,O-AT activity. MAT plays the predominant role in the expression of the N,O-AT activity and appears to be independent of the acetylator gene locus.

Studies in outbred hamster (Saito et al., 1986) and inbred mouse (Mattano et al., 1989) liver cytosols have indicated the purification of a single enzyme capable of catalyzing the N-acetylation of arylamines, the O-acetylation of N-hydroxyarylamines, and the N,O-acyltransfer of arylhydroxamic acids. It is possible that the enzyme purified by Saito et al., (1986) and Mattano et al. (1989) is analogous to the MAT isozyme isolated in this study.

One of the most commonly measured properties of enzymes in biochemical genetics is thermal stability (Weinshilboum, 1981).
Differences in thermal stability is a sensitive indicator of variation in enzyme structure (Paigen, 1971; Campbell et al., 1986). The physical basis of variation in protein thermal stability is a subject of controversy (Stellwagen and Wilgus, 1978). The sensitivity of thermal inactivation, as a test for structural changes, is illustrated by the findings on carboxypeptidase A. Two forms of this enzyme differ only in the antepenultimate C-terminal amino acid residue. One form with leucine in this position is twice as labile as the other with valine (Walsh et al., 1966). Therefore, variation in thermal stability is a potentially useful method for the detection of protein structural differences. In addition, the enzyme activity remaining after the heat inactivation treatment divided by the basal enzyme activity is used to calculate a so-called "heated/control" or "H/C" ratio. This ratio serves as a measure of the thermal stability for an enzyme (Weinshilboum, 1981; Campbell et al., 1986).

The heat inactivation rate constants of the partially purified MAT isozyme towards each of the substrates tested (Table 9, Fig. 23) exhibited no significant differences (P > 0.05). Furthermore, the H/C ratios for the MAT isozyme towards the various substrates tested exhibited similar degrees of thermostability. The data suggest that the NAT, OAT, and N,O-AT activities are carried out by the same MAT isozyme in hamster liver cytosol.

The heat inactivation rate constants of AF NAT and N-OH-AF OAT activities catalyzed by PAT were very similar, suggesting that the
same or a very similar enzyme catalyzes the N-acetylation of AF and the O-acetylation of N-OH-AF. However, these rates differed from that obtained with PABA NAT. This observation suggests that the active site of the PAT isozyme distinguishes between the structural configuration of PABA and that of AF and N-OH-AF carcinogens. The PAT isozyme has a higher affinity for PABA than AF. Consequently, the PABA molecule may force the PAT isozyme into its maximal active form at a faster kinetic rate and with less steric hindrance than AF and N-OH-AF. It may be possible that the interaction between PABA and the PAT isozyme is different from that of AF and N-OH-AF. This may account for the resulting difference in the heat inactivation rate constants between PABA and the carcinogens (AF, N-OH-AF). Alternatively, PABA NAT is catalyzed by a distinct enzyme that is also regulated by acetylator genotype. Further research is required to fully understand the heat inactivation rate differences between the arylamine drug PABA, and the arylamine and N-hydroxyarylamine carcinogens AF and N-OH-AF.

Although the regulation of the N-acetylation polymorphism at the enzymatic level has been well studied, the molecular mechanism(s) that control the acetylation polymorphism have not been elucidated. It is not certain whether the different PAT allozymes originate from different alleles or from the post-transcriptional or post-translational processing of a single gene. Several questions regarding the expression of genetic information can only be answered after the detection and isolation of a gene-specific messenger RNA.
In order to elucidate the molecular basis that controls the acetylation polymorphism, it is essential to understand the structure, function, and regulation of the acetylator gene(s) and its product(s).

This project has provided some direction towards the elucidation of the molecular mechanism(s) that regulate the acetylation polymorphism. The purified Pat$^R$ allozyme could be used to develop monoclonal antibodies. These monoclonal antibodies could be used as probes for the isolation of a cDNA clone encoding the Pat$^R$ allozyme. If monoclonal antibodies are not available, the Pat$^R$ allozyme amino acid sequence could be used to synthesize $^{32}$P-labeled oligonucleotides. These oligonucleotides could serve as a probe to screen a cDNA library to identify a cDNA clone encoding the Pat$^R$ allozyme and a genomic library to isolate the acetylator gene(s).

Susceptibility to arylamine-induced carcinogenesis has been associated with the enzyme system acetyltransferase(s) which metabolizes arylamine carcinogens into ultimate carcinogenic species that bind to DNA. The PAT isozyme, isolated and purified in this project, plays an important role in the biotransformation of arylamines and their N-hydroxyarylamine metabolites. The results of this investigation strongly support the existence of structural variant Pat$^R$ and Pat$^S$ allozymes. The precise differences in amino acid sequence between the Pat allozymes and the nature of the gene mutation are currently under investigation in our laboratory.
CHAPTER VI

SUMMARY AND CONCLUSIONS

The relationship between acetylator genotype and the occurrence of bladder and colorectal cancer in humans has generated increased interest in the genetic and biochemical basis responsible for this relationship. The present investigation revealed that AcCoA-dependent arylamine NAT and N-hydroxyarylamine OAT activities are genetically co-regulated by an acetylator gene. Two distinct forms of NAT were identified in hamster liver and designated as PAT and MAT. Biochemical and kinetic characterization studies revealed functional and structural differences between the PAT and MAT isozymes in both rapid and slow acetylators. Furthermore, the kinetic data suggested that the PAT isozyme is expressed as structural variant allozymes regulated by the acetylator gene locus (Pat). The PAT isozyme of rapid acetylators was purified to apparent homogeneity. The purified protein catalyzes both the N-acetylation of arylamine and the O-acetylation of N-hydroxyarylamine carcinogens but little or no N,O-acyltransfer of arylhydroxamic acids. The conclusions from this investigation follow:

1. The expression of arylamine NAT and N-hydroxyarylamine OAT activities are regulated by acetylator genotype. However, the expression of arylhydroxamic acid N,O-AT activity is not regulated by acetylator genotype.
2. The acetylator genotype-dependent expression of NAT and OAT activities in hamster liver cytosol is reaction- and substrate-dependent.

3. Partial purification of inbred hamster liver cytosol by anion-exchange chromatography reveals the presence of two distinct forms of NAT activity. Both forms of the enzyme are expressed in homozygous rapid and slow acetylators. One form of the enzyme is termed PAT because its expression is acetylator genotype-dependent whereas, the other form is termed MAT because its expression is acetylator genotype-independent.

4. Michaelis-Menten kinetic determinations reveal that the PAT isozyme exhibits variations in apparent Km and Vmax towards the arylamine carcinogens whereas, the MAT isozyme does not show genotype-related variations in apparent Km and Vmax. This suggests that the PAT allozymes are structural variants.

5. The PAT isozyme catalyzes the N-acetylation of arylamines and the O-acetylation of N-hydroxyarylamines at catalytic levels which are consistent with the acetylation polymorphism.

6. The MAT isozyme catalyzes the NAT, OAT, and N,O-AT activities at catalytic levels which are acetylator genotype-independent.

7. The acetylator genotype-dependent expression of NAT activity in hamster liver cytosol towards arylamine carcinogens is attributable to structural variants (allozymes e.g., enzymes encoded via the Pat^r and Pat^s alleles) of the PAT isozyme.
8. The acetylation of arylamine and N-hydroxyarylamine carcinogens is catalyzed by a common acetyltransferase isozyme in hamster liver regulated by the acetylator gene (Pat).
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