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THE BIOCHEMICAL BASIS OF THE N-ACETYLATION POLYMORPHISM AND ITS TOXICOLOGICAL CONSEQUENCES

by
David William Hein

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in The University of Michigan
1982

Doctoral Committee:

Professor Wendell W. Weber, Chairman
Professor George J. Brewer
Professor Herbert H. Cornish
Professor Bert N. La Du

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DEDICATION

This dissertation is dedicated to the five individuals who were most critical to my scientific development and future career.

William and Ruth Hein, my parents, for understanding the importance of an education and providing me with the encouragement and finances to pursue it.

Karla Hein, my wife, for the patience needed for the spouse of a doctoral student.

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PREFACE

This dissertation is a collection of closely related manuscripts separated by chapter or subchapter. Each chapter is circumscribed, and enough details are provided within the introduction, methods, discussion, and references of each individual chapter to make the results meaningful and to provide a complete conclusion. Unnecessary repetition of methods from one chapter to another has been eliminated by cross-reference between chapters, and a single bibliography is provided at the end of the dissertation. Since this is a collection of individual, but closely related studies, there is some repetition within the introduction and discussion sections from one chapter to another. Thus, this dissertation is perhaps somewhat shorter than it appears. Every effort was made to write each subsection of a chapter as succinctly as possible, but written with sufficient detail and completeness to be reproducible and suitable for publication. Many of the chapters have already met this criterion, as they were published during the course of the dissertation research. CHAPTERS III B, IV, VIII, and IX have been published essentially as they appear in this dissertation, and portions of CHAPTERS II, III A, and V have also been published. The remaining portions of the dissertation will be submitted for publication in the near future.

The dissertation is divided into two major divisions, as befits its title. The first portion involves development of animal models and their use in elucidating the biochemical basis of the N-acetylation polymorphism (CHAPTERS II - V). The second portion of the dissertation uses both humans and animal models to investigate its toxicological consequences (CHAPTERS VI - VIII). Finally, CHAPTER IX

contains experiments of importance to both of these major purposes of the study. The overall organization of the dissertation is constructed for the majority of individuals who will examine one or two chapters at a time as a reference source to acquire information about a particular topic. Hopefully, the organization of the data in this manner will improve the readability and usefulness of the text to future researchers.

As requested by the committee at my final defense, the list of publications derived from this thesis are as follows:

WEBER. W.W., HEIN, D.W., HIRATA, M., PATTERSON, E.: Genetics of drug acetylation: Molecular nature of the INH acetylation polymorphism. In: Conjugation Reactions in Drug Biotransformation, A. Aitio, Editor, Elsevier/North Holland Biomedical Press, New York, pp. 145-153, 1978.

HEIN, D.W., HIRATA, M., WEBER, W.W.: An enzyme marker to ensure reliable determinations of isoniazid acetylator phenotype in vitro. Pharmacology 23: 203-210, 1981.

HEIN, D.W., HIRATA, M., GLOWINSKI, I.B., WEBER, W.W.: Biochemical evidence for the coexistence of monomorphic and polymorphic N-acetyltransferase activities on a common protein in rabbit liver. J. Pharmacol. Exp. Ther. 220: 1-7, 1982.

HEIN, D.W., OMICHINSKI, J.G., BREWER, J.A., WEBER, W.W.: A unique pharmacogenetic expression of the N-acetylation polymorphism in the inbred hamster. J. Pharmacol. Exp. Ther. 220: 8-15, 1982.

WEBER, W.W., HEIN, D.W., GLOWINSKI, I.B., KING, C.M., FOX, R.R.: Association of arylhydroxamic N,O-acyltransferase and genetically polymorphic N-acetyltransferase in established inbred rabbit strains, In: Host Factors in Human Carcinogenesis, H. Bartsch and W. Davis, Editors, International Agency for Research on Cancer (IARC Scientific Publications), Lyon, (in press).

HEIN, D.W., WEBER, W.W.: Polymorphic N-acetylation of phenelzine and monoacetylhydrazine by highly purified rabbit liver isoniazid N-acetyltransferase. Drug Metab. Disp. 10: 1982 (in press).

HEIN, D.W., SMOLEN, T.N., FOX, R.R., WEBER, W.W.: Identification of homozygous rapid and slow acetylators of drugs and environmental carcinogens among established inbred rabbit strains. J. Pharmacol. Exp. Ther. (submitted).

In addition, manuscripts are in preparation for portions of CHAPTER IV (Biochem. Genetics), CHAPTER V (J. Pharmacol. Exp. Ther.), CHAPTER IIIC (Drug Metab. Disp.), and CHAPTER VII (J. Clin. Invest.).

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LIST OF ABBREVIATIONS

AcCoA	acetyl coenzyme A
2AF	2-aminofluorene
AHAT	arylhydroxamic acid N,O-acyltransferase
BNA	beta-napthylamine
CNS	central nervous system
DEAE	diethylaminoethyl
DMAB	p-dimethylaminobenzaldehyde
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GABA	gamma-aminobutyric acid
GC/MS	gas chromatography / mass spectometry
INH	isoniazid
K	first order rate constant
m	milli-
M	molar
MAH	monoacetylhydrazine
min	minutes
n	nano-
NAT	N-acetyltransferase
nm	nanometer
O.D.	optical density
PA	procainamide
PABA	p-aminobenzoic acid
PAS	p-aminosalicylic acid
PHZ	phenelzine

S.D.	standard deviation
SDZ	sulfadiazine
S.E.	standard error
SGPT	serum glutamic-pyruvic transaminase
SLE	systemic lupus erythematosus
SMZ	sulfamethazine
sod	small optical density unit (O.D. / 1000)
t 1/2	half-life
TNBS	trinitrobenzene sulfonic acid (picrylsulfonic acid)
u	micro-
w/v	weight / volume
X^2	chi square value

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CHAPTER I

GENERAL INTRODUCTION AND PURPOSE

Pharmacogenetics is a subdiscipline of pharmacology which involves investigation of genetic factors that influence the therapeutic and toxicological actions of chemicals. Differences in drug response or toxicity between individuals are related to either environmental or hereditary factors. The importance of hereditary factors has only recently become appreciated as the first reviews of pharmacogenetic studies were reported only 20 years ago (Kalow, 1962; Evans, 1963). In the past two decades, however, the field has rapidly expanded and the importance of genetic factors in drug response and toxicity has recently been the subject of numerous reviews (e.g., Cohen and Weber, 1972; La Du, 1972a,b; Weber, 1972; Vesell, 1977; Evans, 1980; Harris et al., 1980; Kouri, 1980; Nebert, 1980a,b, 1981; and Ritchie et al., 1980).

It is somewhat surprising that the importance of genetic factors in drug response was not recognized earlier. If one assumes that all proteins and other macromolecules within an individual are either direct or indirect products of a gene, it would follow that interactions of chemicals with these constituents of the individual would be under genetic control. For example, gene products could affect absorption, distribution, metabolism, or excretion of the chemical (pharmacokinetics), or its interaction at a receptor

(pharmacodynamics). Genetically determined differences between individuals in any one of these parameters might substantially alter the therapeutic or toxicological action of the chemical.

One well known example of an instance in which genetically determined differences in metabolism of a drug have been reported is the N-acetylation polymorphism. In this instance, the term polymorphism simply means that variation between individuals in N-acetylation capacity is due to the frequent occurrence of two alleles in humans which results in a bimodal (FIGURE 1) or trimodal (Sunhara, 1962) distribution rather than a normal or unimodal one. The initial observations were made by Hughes et al. (1955) who showed that certain individuals developed drug toxicity when receiving ordinary doses of INH. Furthermore, these same individuals were found to excrete lower amounts of acetyl-INH, the major metabolite, in the urine. Subsequently, other investigators confirmed these findings (Biehl, 1957; Knight et al., 1959; Evans et al., 1960, Peters et al., 1965). The study of Evans et al. (1960) measured serum levels of INH in individuals of 57 complete families. As shown in FIGURE 1, six hours after drug administration, the amount of unmetabolized INH in serum showed a bimodal distribution. Approximately half of the individuals were classified as rapid acetylators, and the other half as slow acetylators. As also shown in FIGURE 1, and confirmed by a host of investigators, the acetylator phenotype of an individual is a simple autosomal Mendelian trait determined by two major alleles (R,r) at a single locus. Rapid acetylators are either homozygous (RR) or heterozygous (Rr) for the rapid acetylator allele (R), while slow acetylators are homozygous (rr) for the slow acetylator allele.

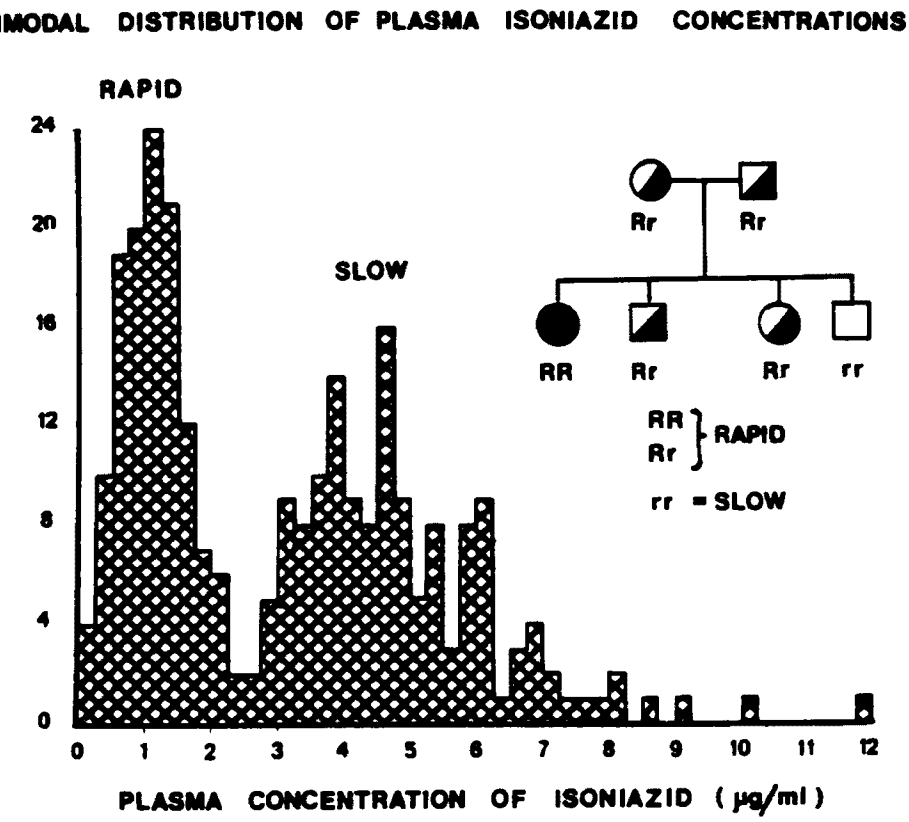


FIGURE 1. Distribution and genetic inheritance pattern of INH excretion in humans.

(from Evans et al., 1960).

The data of FIGURE 1 were obtained primarily in American individuals. In further racio-geographic studies of this polymorphism, allele frequencies and thus the proportions of rapid and slow acetylator phenotypes were shown to be quite variable. For example, the proportion of slow acetylators in a population ranges from about 5% in Canadian Eskimos to about 83% in Egyptians (TABLE 1). In general, most populations of European and African origin (including Caucasians and Blacks of the United States) contain approximately equal proportions of rapid and slow acetylators, which indicates a frequency of 0.72 for the r allele and 0.28 for the R allele. In contrast, populations of Asiatic origin are primarily rapid acetylators (TABLE 1). Thus, in circumstances where acetylator phenotype is important in drug response or toxicity, various populations of the world will be affected differently.

The major metabolic step in the biotransformation of INH is N-acetylation via the enzyme NAT (E.C. 2.3.1.5) located primarily in liver and small intestine (e.g., Weber, 1973). As recently reviewed by Weber and Hein (1979), the polymorphism in INH plasma levels is due to differences in activity levels of NAT between rapid and slow acetylators. The first order rate constants for the individual reaction steps in INH metabolism are illustrated in FIGURE 2 for one rapid and one slow acetylator (Ellard and Gammon, 1976). A 3-4-fold difference is found in the metabolic conversion rate of INH to acetyl-INH via polymorphic NAT, while subsequent hydrolysis, conjugation, and excretion rates are approximately equal in the two phenotypes. It has been proposed that MAH, a metabolite of INH (FIGURE 2), is also metabolized by N-acetylation, and this subject is

TABLE 1. Distribution of acetylator phenotype in the world[a].

Population	Number studied	Proportion of slow acetylators (q^2)	Slow gene frequency (q)	Reference
Asiatic origin				
Japanese	1806	0.12	0.34	Sunhara et al. (1961)
USA-Japanese	209	0.10	0.32	Dufour et al. (1964)
Chinese	85	0.15	0.39	Evans (1962)
Chinese (Taiwan)	121	0.22	0.47	Sunhara et al. (1963)
Chinese (Singapore)	375	0.22	0.47	Ellard (1976a)
Chinese (Hong Kong)	186	0.22	0.47	Ellard (1976a)
Burmese	121	0.38	0.62	Smith and Kyi (1968)
Thai	108	0.28	0.53	Sunhara et al. (1961)
Thai	100	0.58	0.76	Schloot et al. (1967)
Korean	65	0.11	0.33	Sunhara et al. (1961)
Ryukuyan	124	0.15	0.38	Sunhara et al. (1961)
Ainu	86	0.13	0.36	Sunhara et al. (1961)
American Indian	15	0.21	0.46	Mitchell et al. (1960)
Athebaskan Indian	47	0.38	0.62	Scott et al. (1969)
Hindu Indian	143	0.58	0.76	Devadatta et al. (1960)
Hindu Indian	299	0.60	0.77	Gangadharam and Selkon (1962)
South Indian (Madras)	1497	0.59	0.77	Tuberculosis Centre (1973)
Canadian Indian	46	0.37	0.61	Jeanes et al. (1972)
Canadian Eskimo	216	0.05	0.22	Armstrong and Pert (1960)
Northern Alaskan Eskimo	45	0.27	0.52	Scott et al. (1969)
Southern Alaskan Eskimo	112	0.18	0.43	Scott et al. (1969)
Polynesian	111	0.07	0.26	Hayward (1975)
Filipino	50	0.28	0.53	Peters et al. (1972)
European origin				
USA Caucasian	200	0.52	0.72	Evans et al. (1960)
USA Caucasian	106	0.58	0.76	Dufour et al. (1964)
USA Caucasian	39	0.59	0.77	Eidus et al. (1979)
USA Scandinavian	70	0.67	0.82	Harris (1962)
USA Italian	14	0.64	0.80	Harris (1962)
USA Greek	10	0.60	0.77	Harris (1962)
USA Spanish (Salt Lake City)	23	0.60	0.77	Harris (1962)
USA Spanish (Denver)	131	0.30	0.55	Harris (1962)
Canadian Caucasian	102	0.59	0.77	Eidus et al. (1974b)
Canadian Caucasian	105	0.58	0.76	Eidus et al. (1979)
German	524	0.44	0.66	Schmeidel (1962)
Finn	91	0.64	0.80	Mattila and Tiitinen (1967)
Finn	341	0.58	0.76	Tiitinen (1969)
Briton	135	0.62	0.79	Evans (1969)
Swede	130	0.68	0.82	Hanngren et al. (1970)
Costa Rican	60	0.38	0.62	Peters et al. (1976)
Latin American	131	0.30	0.55	Mitchell et al. (1960)
Norwegian	100	0.56	0.75	Walstad et al. (1975)
Skolt Lapp	26	0.50	0.71	Tiitinen et al. (1968)
Czech	410	0.60	0.77	Vuzherova et al. (1973)
African origin				
American Black	81	0.48	0.69	Evans et al. (1960)
American Black	31	0.48	0.69	Mitchell et al. (1960)
American Black	116	0.51	0.71	Dufour et al. (1964)
American Black	63	0.40	0.63	Eidus et al. (1979)
Sudanese Black	102	0.65	0.80	Evans (1962)
South African Black	108	0.41	0.64	Bach et al. (1976)
South African Black	100	0.27	0.52	Buchanan et al. (1976)
South African Black	337	0.41	0.64	Eidus et al. (1979)
West African Black	120	0.60	0.77	Seleiko and Aderounmo (1977)
East African	204	0.55	0.74	Ellard et al. (1975)
Nigerian	109	0.49	0.70	Fawcett and Gammon (1975)
Egyptian	50	0.83	0.91	Hashem et al. (1969)
Jewish origin				
USA Ashenazi	11	0.55	0.75	Harris (1962)
Israeli Ashenazi	100	0.67	0.82	Szenberg et al. (1963)
Israeli non-Ashenazi	179	0.69	0.83	Szenberg et al. (1963)
Israeli Baghdad	60	0.75	0.87	Szenberg et al. (1963)

a Up-dated from Weber (1973).

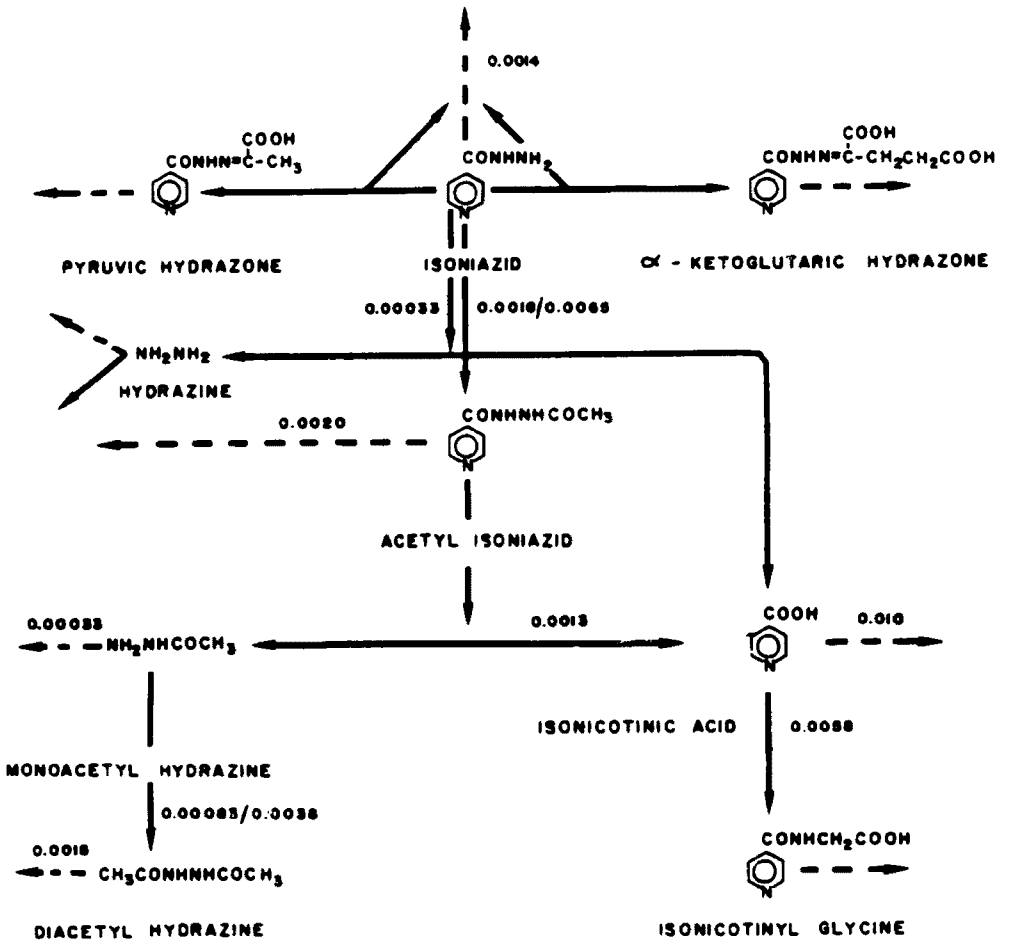


FIGURE 2. Metabolism of INH.

First order rate constants $(min)^{-1}$ for the polymorphic acetylation steps were determined for one slow and one rapid acetylator, respectively (solid lines). Renal elimination is represented by dotted lines. (modified from Ellard and Gammon, 1976). In instances in which two rate constants are listed, the first is for a rapid acetylator human and the second is for a slow acetylator human.

examined in CHAPTER VIII.

In addition to INH, many other arylamine and hydrazine drugs and environmental carcinogens are also known to be acetylated by the polymorphic enzyme NAT. Some typical examples are illustrated in FIGURE 3. Although N-acetylation is a major step in the metabolism of each of these compounds, some of them do not show differences in N-acetylation rate between rapid and slow acetylator individuals. For example, variation in the N-acetylation rate between humans for PABA and PAS is monomorphic (unimodal) rather than polymorphic (Evans, 1963; Jenne, 1965; Glowinski et al., 1978; see CHAPTER IX). Hence, substrates which show a polymodal distribution, such as INH and SMZ, are termed polymorphic while substrates which show a unimodal distribution, such as PABA and PAS, are termed monomorphic. The biochemical basis of these findings have heretofore remained unknown.

The N-acetylation polymorphism has acquired greater clinical significance over the past two decades because of the variety of reports which implicate increased susceptibility to disease, drug toxicity, or chemical carcinogenesis with an acetylator phenotype. As summarized in FIGURE 4, slow acetylators have been shown to have increased susceptibility to peripheral neuritis induced by INH, hydralazine, or phenelzine; these findings are discussed in CHAPTERS VII and VIII. Slow acetylators have also been shown to be more susceptible to a drug-induced SLE syndrome produced by PA, hydralazine, and INH; these findings are discussed in CHAPTER VI. In addition, unconfirmed findings by Lower et al. (1979) suggest that slow acetylators are also more susceptible to the induction of bladder

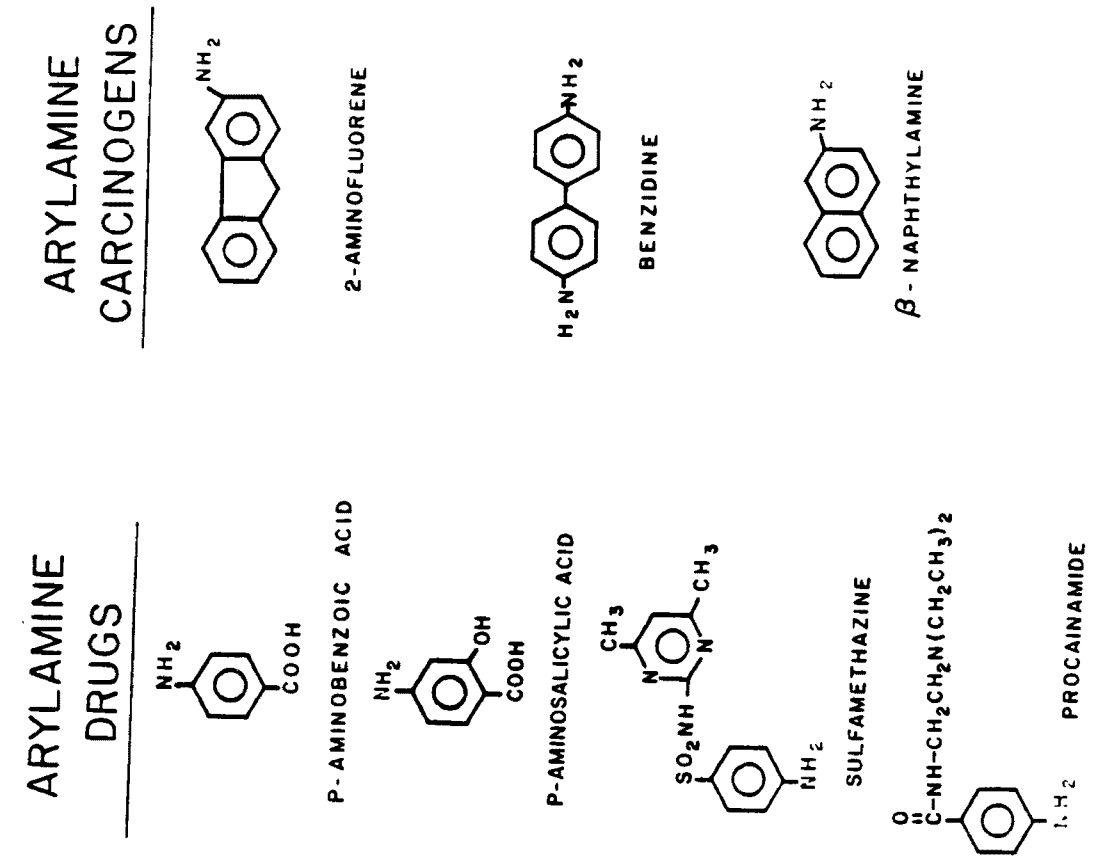


FIGURE 3. Representative structures of NAT substrates.

DRUG-INDUCED TOXICITY POSSIBLY RELATED TO ACETYLATOR PHENOTYPE

TOXICITY	TOXIC AGENT
I. PERIPHERAL NEURITIS	ISONIAZID
	HYDRALAZINE
	PHENELZINE
II. HEPATOTOXICITY	ISONIAZID
	METHYLENEDIANILINE
III. LUPUS ERYTHEMATOSIS	PROCAINAMIDE
	HYDRALAZINE
	ISONIAZID
IV. BLADDER CANCER	2-AMINOFLUORENE
	BENZIDINE
	2-NAPTHYLAMINE

FIGURE 4 Drug-induced toxicity possibly related to acetylator phenotype.

cancer by environmental carcinogenic arylamines such as 2AF, BNA, and benzidine. In contrast, some investigators have shown that INH-induced hepatotoxicity is more frequent in rapid acetylators, and these findings are discussed in CHAPTER VIII. Finally, the significance of acetylator phenotype with respect to hepatotoxicity produced by methylenedianiline is currently being investigated in animal models in our laboratory.

The N-acetylation polymorphism was discovered in humans and its toxicological consequences have also been described in human studies. The major purposes of this dissertation were to elucidate the biochemical basis of the N-acetylation polymorphism, and to investigate its toxicological consequences, many of which have been studied to some extent in man but remain controversial. In order to best accomplish both of these goals and to advance beyond information already obtained in humans, the development of animal models is critical. For example, investigation of the biochemical basis of the N-acetylation polymorphism requires isolation of NAT enzyme from the liver. NAT is very unstable after death (see CHAPTER IX), however, and thus, human liver autopsy specimens are usually unsuitable. Also, human liver biopsy samples are quite difficult to obtain. Thus, an animal model of the N-acetylation polymorphism would be advantageous for several reasons: 1) it provides a readily available source of NAT, 2) it allows the use of inbred subjects in which the genetic composition of the animal is fixed at nearly every gene locus and genetic noise between subjects is minimized, and 3) it provides the opportunity to design and carry out genetic crosses important to a genetic analysis. Development of animal models is also important to

investigate the second purpose of this dissertation, namely to investigate some of the toxicological consequences of the N-acetylation polymorphism. Human toxicological studies often cannot be conducted for ethical reasons, and thus they often require compilation of epidemiological data from different patient or population groups. These studies, though valuable, have often led to unresolved controversies regarding the significance of acetylator phenotype with respect to INH-induced hepatotoxicity, PHZ-induced toxicity, and susceptibility to spontaneous SLE and arylamine-induced bladder cancer. To our knowledge, only one study has examined the toxicological consequences of the N-acetylation polymorphism in an animal model (Tannen and Weber, 1980b). This dissertation represents the second.

The first documentation of the N-acetylation polymorphism in another species was reported by Frymoyer and Jacox (1963a). They examined the N-acetylation rate of SDZ in vivo in 162 rabbits and found a bimodal frequency distribution (FIGURE 5) much like that observed in man with INH (FIGURE 1). Approximately half of the rabbits could be classified as rapid acetylators and half as slow acetylators. In addition, the acetylator phenotype was genetically determined by the same mechanism as in humans. Subsequent investigations by Weber et al. (1976) further characterized the rabbit as an animal model of the N-acetylation polymorphism and showed that its expression was quite similar to that in man.

Thus, the rabbit was chosen as the animal model for experiments to elucidate the biochemical basis of the N-acetylation polymorphism (CHAPTERS II and III) and its toxicological consequences (CHAPTERS VII

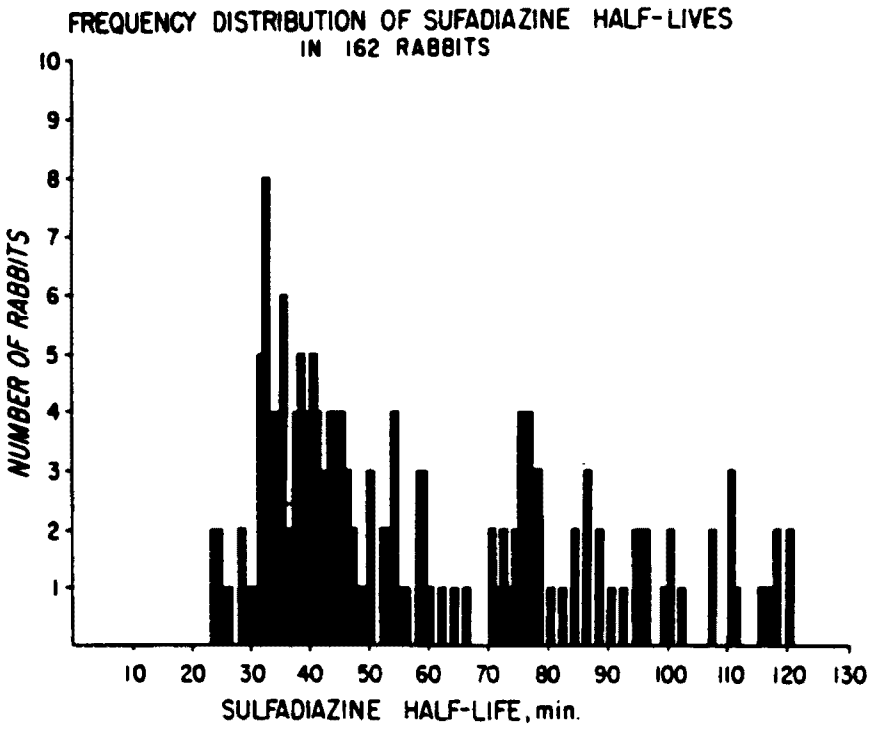


FIGURE 5. Distribution of SDZ N-acetylation rates in rabbits. (From Frymoyer and Jacox, 1963a).

and VIII). A new animal model, the inbred hamster, was also developed and partially characterized (CHAPTER IV). This model, unique in some respects, served to elucidate further the biochemical basis of the N-acetylation polymorphism (CHAPTER V). Finally, human liver samples (CHAPTER IX) and human subjects (CHAPTER VI) were also utilized in those investigations where ethical considerations did not preclude their use.

CHAPTER II

DEVELOPMENT OF AN INBRED RABBIT MODEL OF THE N-ACETYLATION POLYMORPHISM

Introduction

An important metabolic step in the biotransformation of arylamine and hydrazine drugs and carcinogens is N-acetylation via the cytosolic enzyme NAT (E.C. 2.3.1.5) located predominantly in liver and small intestine (Weber, 1973). Initial observations in man by Hughes et al. (1955) and later confirmed by a host of investigators have shown that differences in human N-acetylation capacity are under genetic control. Individuals can be classified as rapid or slow acetylators of arylamines and hydrazines; rapid acetylators are either homozygous (RR) or heterozygous (Rr) for a codominant rapid acetylator allele, whereas slow acetylators are homozygous (rr) for a recessive slow acetylator allele (Evans et al., 1960). The N-acetylation rate of each individual is dependent primarily upon the activity level of the liver and gut mucosal NAT (Weber, 1973).

In the metabolism of many carcinogenic arylamines such as 2AF, there are actually two steps that involve N-acetyl transfer. The first N-acetyl transfer is AcCoA-dependent, inter-molecular, and catalyzed by the NAT described above. Following a microsomal enzyme catalyzed hydroxylation step, another N-acetyl transfer occurs which

is AcCoA-independent and intra-molecular, catalyzed by the cytosolic enzyme AHAT (Bartsch et al., 1972; King, 1974). A previous comparison of the properties of NAT and AHAT activities suggested that the enzymes were remarkably similar in many respects. For example, both were present in tissues of rabbit, monkey, rat, and mouse, and notably absent in dog. Distribution in tissues did not appear to differ as both occurred in liver and gut mucosa, but were negligible (polymorphic activity) in blood, brain, and skeletal muscle. Both NAT and AHAT activities were exquisitely sensitive to sulfhydryl inhibitors, insensitive to organophosphates, and uninducible by barbiturates. Both could be partially purified by similar procedures involving ultracentrifugation (105,000 g), ammonium sulfate precipitation (45-65%), gel filtration (G-100), and ion exchange chromatography (DEAE-cellulose).

In view of the potential participation of AHAT activity as a host factor capable of affecting arylamine carcinogenesis (Bartsch et al., 1972; King, 1974), a detailed comparative study of the biochemical properties and genetic control of NAT and AHAT was undertaken by Glowinski et al. (1980) to discern whether a relationship between them could be demonstrated. The occurrence of large genetic variation in rabbit liver NAT activity (Frymoyer and Jacox, 1963a,b; Weber et al., 1976) offered a distinct advantage which led them to choose this animal model over other species for that study. They found that both activities were probably properties of the same enzyme because 1) both activities were high in rapid acetylator rabbit liver cytosol and low in slow acetylator rabbit liver cytosol, and 2) rapid acetylator rabbit liver NAT and AHAT activities were inseparable by techniques

which exploit differences in solubility, charge, and size, as the two activities copurified to electrophoretic homogeneity (Glowinski et al., 1980).

Animal models of the N-acetylation polymorphism have also been demonstrated in the inbred mouse (Tannen and Weber, 1980a; Glowinski and Weber, 1982a), and inbred hamster (see CHAPTER IV). Yet, the rabbit has most often been used as an animal model of the acetylation polymorphism for several reasons: 1) there are large differences in NAT activity levels in liver and small intestine between rapid and slow acetylators (Hearse and Weber, 1973), 2) high levels of NAT activity are readily detectable in liver and small intestine (Hearse and Weber, 1973), 3) liver NAT and AHAT activity have recently been purified to electrophoretic homogeneity in the rabbit Glowinski et al., 1980), and 4) the qualitative expression of the substrate N-acetylation polymorphism in rabbit is similar to that in man. (Weber, 1973). For example, in man and rabbit large differences exist between rapid and slow acetylators for INH, SMZ, and 2AF NAT activities (Glowinski et al., 1978; see CHAPTER III) while much smaller differences in NAT activity exist for PAS (Jenne, 1965; see CHAPTER III and TABLE 8) and PABA (Glowinski et al., 1978; see CHAPTERS III and IX and TABLE 8). In contrast, in the inbred hamster, PABA and PAS show large differences in NAT activity between rapid and slow acetylators, while SMZ, INH, and 2AF show little difference (see CHAPTER IV and FIGURES 14-19). Similarly, the inbred mouse also differs from man since rapid and slow acetylator mice do not differ with respect to liver PA, 1-napthylamine (Tannen, 1978) and INH (D. W. Hein and W. W. Weber, unpublished observations) NAT

activity levels.

Thus, the primary limitation of the currently available rapid and slow acetylator rabbit model is the genetic variability inherent in non-isogenic animals. The mouse and hamster models avoid this limitation because they are inbred. Indeed, inbred animals have been likened to biological "analytical reagents" because they have achieved homozygosity at nearly every gene locus (Festing, 1979). Consequently, with the establishment of 17 inbred and partially inbred rabbit strains at The Jackson Laboratory, Bar Harbor, Maine, we decided to investigate many of these lines for possible identification of homozygous rapid and slow acetylators of a variety of arylamine drugs and carcinogens. In addition, since these 17 inbred lines are derived from at least four independent origins (Altman and Katz, 1979), they are a potentially useful tool for assessing further the hypothesis that liver NAT and AHAT activities are properties associated with a single liver enzyme protein.

Methods

Chemicals and reagents:

(see TABLE 33)

Source of inbred and partially inbred rabbits:

Male inbred and partially inbred rabbits were provided by The Jackson Laboratory, Bar Harbor, ME. A description of the genetic origins and characteristics of each of these lines has been published (Altman and Katz, 1979). The appropriate coefficient of inbreeding for each of these rabbits used in the study are given in TABLES 2-4.

Source of selectively bred rapid and slow acetylator rabbits:

Selected lines of mature New Zealand White rabbits (identified by number in this chapter and all chapters of the dissertation) of known acetylator phenotype (males and females) were bred and housed in our animal care facilities. Each rabbit was caged individually at room temperature on a light-dark schedule with lights on from 8:00 AM to 8:00 PM and fed sulfonamide-free rabbit chow with water available ad libitum. Acetylator phenotype was confirmed by in vitro measurement of liver SMZ NAT activity, and by pedigree information.

NAT purification:

Rabbits were killed by carbon dioxide intoxication or a sharp blow to the head. Immediately after death, the liver was rapidly removed, washed, minced, and homogenized (Waring blender; 25% w/v) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. The duodenum was also rapidly removed, washed, and incised longitudinally. The mucosal tissue was scaped away with a scalpel from the first twelve inches of the duodenum and hand homogenized in

7 ml of 0.05 M sodium pyrophosphate buffer, pH 7.0, containing 1 mM DTT. This resulted in a protein concentration of approximately 32 mg/ml. The muscular gut-wall tissue was discarded. Liver and gut mucosal preparations were spun at 10,000 g for 20 min followed by 105,000 g for 60 min. The cytosolic supernatant fraction was assayed for NAT activity as described below.

PABA and SMZ NAT activity determinations:

PABA and SMZ NAT activities were determined at 37°C by a micromodification of the Bratton-Marshall (1939) procedure as previously published (Weber and King, 1981). The colorimetric procedure measures the AcCoA-dependent disappearance of the arylamine substrate. Final concentrations in the reaction mixture were 44.4 uM for PABA or SMZ, and 2.22 mM for AcCoA. Absorbance was measured at 540 nm against a water blank in a Beckman Model 35 spectrophotometer. The amount of acetylated product formed was determined by subtracting the experimental from the control absorbance reading. Controls received no AcCoA. Under the conditions of the assay, a decrease of one sod absorbance unit corresponds to the acetylation 6.08 pmoles of PABA or 5.75 pmoles of SMZ.

INH NAT activity determinations:

INH NAT activity determinations were carried out by a modification of the procedure previously described by Weber (1971). The assay measures the AcCoA-dependent formation of acetyl-INH. Assays were carried out in 1.5 ml capped polypropylene microsample tubes. To 50 ul of suitably diluted enzyme preincubated at 37°C was added 20 ul of 50 mM INH. The reaction was started with the addition of 20 ul of 10 mM AcCoA. Final concentrations in the

FIGURE 6. Standard curve for assay of INH NAT activity.

In panel A, wavelength on the abscissa is plotted versus sod on the ordinate for equal concentrations of INH (circles) and acetyl-INH (squares) under the conditions described in the INH NAT assay (Methods). In panel B, nmoles of acetyl-INH added to the assay as described in Methods is plotted on the abscissa versus absorbance (sod) at 303 nm on the ordinate. This standard curve yields a constant of 0.222 nmoles acetyl-INH / sod.

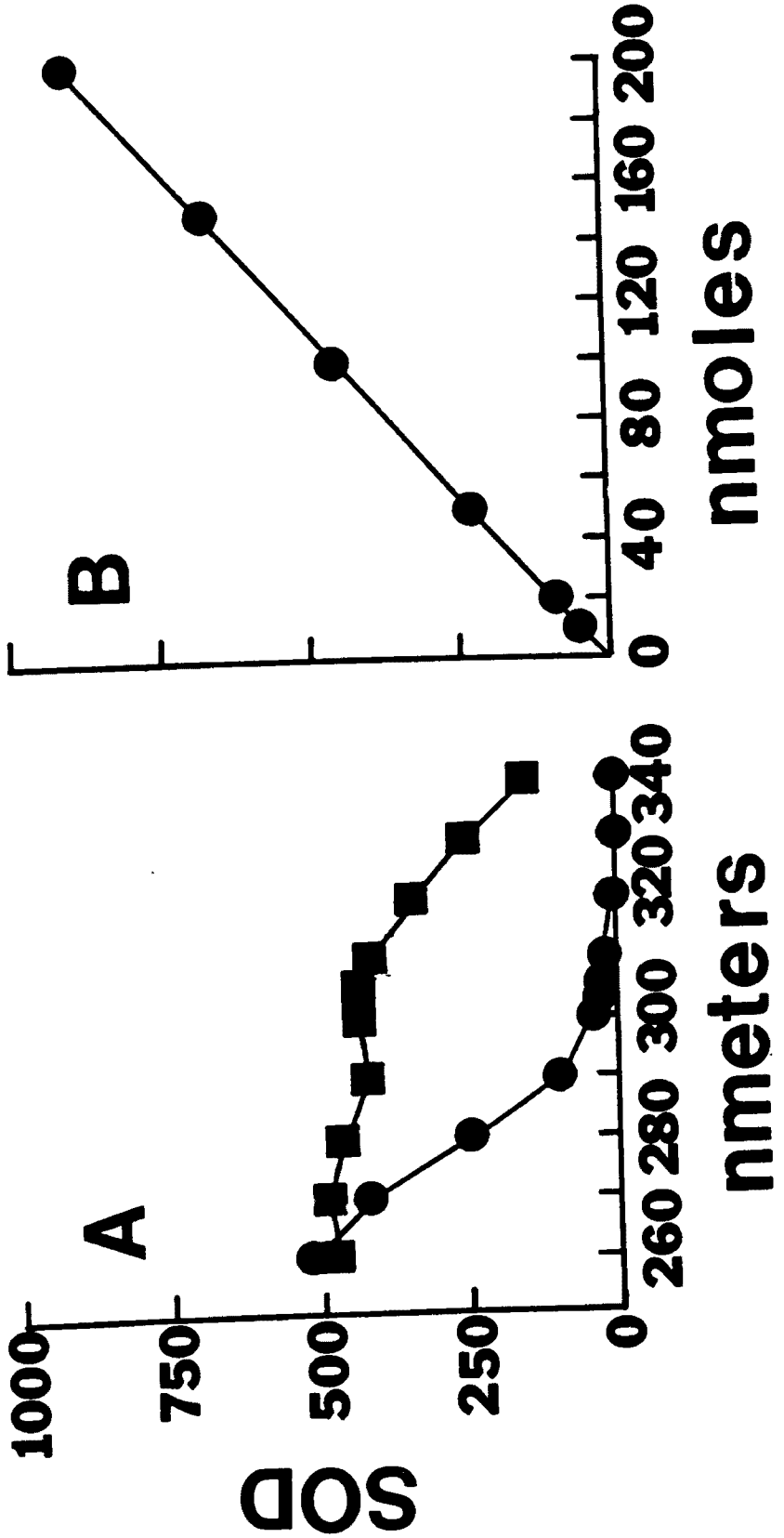

A
SOD
1000
750
500
250
0
260 280 300 320 340
nmeters
B
0 40 80 120 160 200
nmoles

FIGURE 6

reaction mixture were 11.1 mM for INH, and 2.22 mM for AcCoA. The reaction was stopped at specified time points by the addition of 50 ul of trichloroacetic acid (10% w/v) to give linear time-activity plots. After centrifugation in a Brinkmann Eppendorf centrifuge 3200, 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 (UV) against a water blank in a Beckman Model 35 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 of AcCoA. All assays were carried out in duplicate or triplicate at 37°C. Under the conditions of this assay, an increase of one sod absorbance unit corresponds to the acetylation of 0.222 nmoles of INH.

DMAB assay of PA, PAS, and 2AF NAT activity:

Assays were carried out in 1.5 ml capped polypropylene microsample tubes. To 50 ul of suitably diluted enzyme preincubated at 37°C was added 20 ul of 1 mM arylamine substrate (PA and PAS were dissolved in water; 2AF in dimethylsulfoxide). The reaction was started with the addition of 20 ul of 10 mM AcCoA. Final concentrations in the reaction mixture were 0.22 mM for arylamine substrate, and 2.22 mM for AcCoA. 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 3200, 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

FIGURE 7. Standard curves for DMAB assay of NAT activity.

The number of nmoles of 2AF or PA added under conditions described for DMAB assay of NAT activity (Methods) is plotted on the abscissa versus absorbance (sod) at 460 nm on the ordinate. These standard curves yield constants of 0.027 nmoles 2AF/sod and 0.024 nmoles PA/sod.

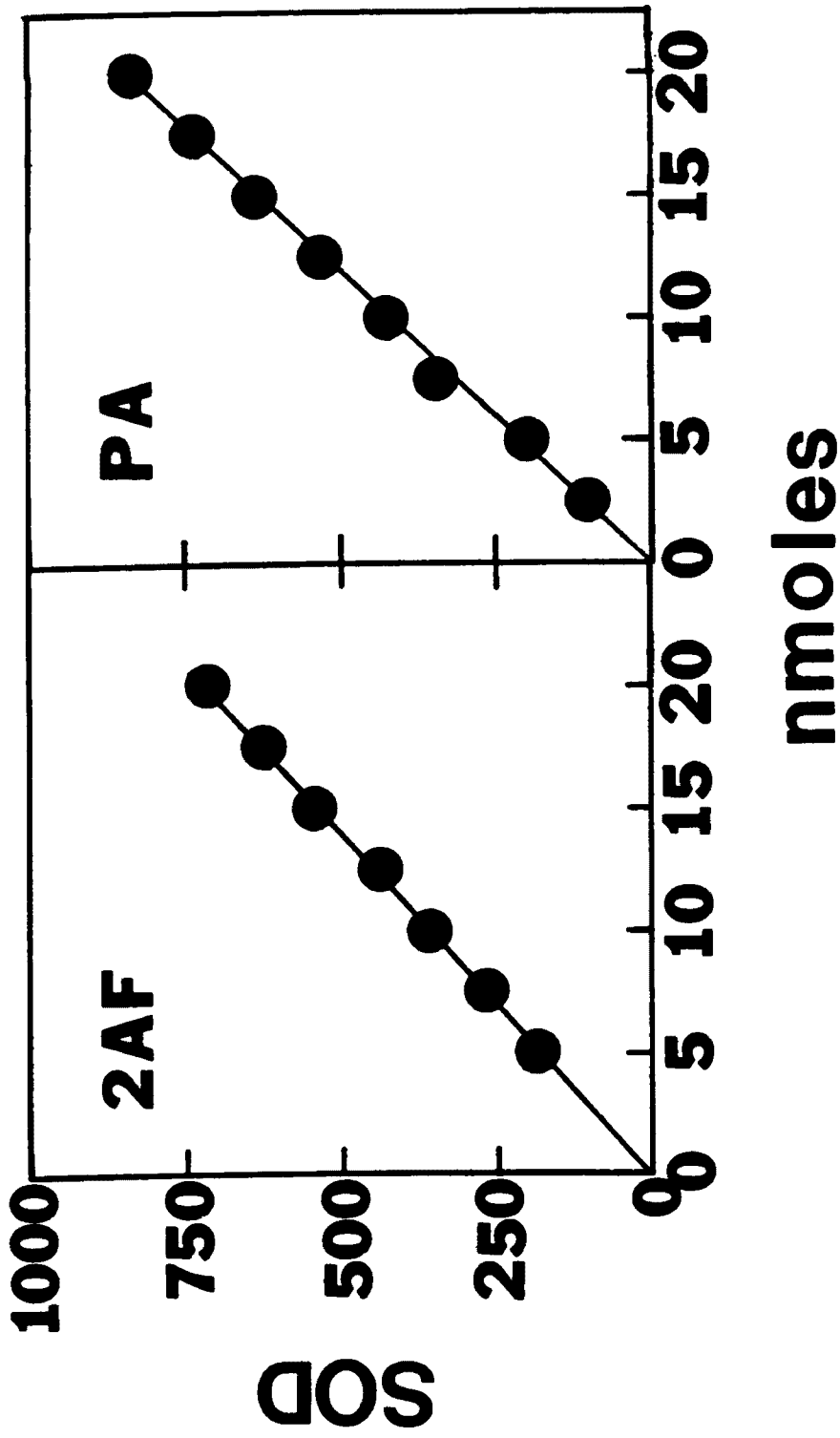


FIGURE 7

final mixing and recentrifugation, the tubes were allowed to stand for at least 30 min. Absorbance was measured at 460 nm against an ethanol blank in a Beckman Model 35 spectrophotometer. The amount of acetylated product formed was determined by subtracting the experimental from the control absorbance reading. Controls received 20 ul of water instead of 20 ul of AcCoA. All assays were carried out in duplicate at 37°C. Under the conditions of this assay, a decrease of one sod absorbance unit corresponds to the acetylation of 27.0 pmoles of 2AF or PAS, and 24.0 pmoles of PA.

Liver AHAT activity determinations:

N-hydroxy-2-acetylaminofluorene AHAT activity was determined at 37°C by a a radiolabel assay procedure previously published (Weber and King, 1981).

Protein determinations:

Total protein was determined by a microbiuret method (Gornall et al., 1949).

Data analysis:

NAT activity determinations were conducted at protein concentrations and over time periods that yielded linear plots of activity with time. Initial velocities were calculated by extrapolation of time activity curves to the addition of AcCoA (time zero) and measuring the slope to the tangent of the curve.

Results

Liver NAT and AHAT activity survey:

A survey of NAT and AHAT activities in 17 inbred and partially inbred rabbit cytosols (105,000 g) shows that 11 strains are identified as rapid acetylators while 6 strains are slow acetylators for both NAT and AHAT activities (TABLE 2). In the rapid acetylator strains, liver INH NAT activity ranged from 2.1 to 4.5 units (average of 3.1) and the liver AHAT activity ranged from 3.1 to 24.5 units (average of 11.7). Among the slow acetylator strains, INH NAT activity was undetectable while AHAT activity ranged from 0.5 to 2.0 units (average of 1.5). Although the INH NAT and AHAT activities in TABLE 2 are lower than we have usually observed (e.g. see footnote to TABLE 2) encountered in the shipment of samples from Bar Harbor to Ann Arbor, they nevertheless provide unequivocal phenotype classification since there is no overlap of NAT, or AHAT, activity between the rapid and slow acetylator groups.

Gene dose-response of liver NAT activity:

PABA, PAS, PA, SMZ, INH, and 2AF NAT activities were determined in liver NAT preparations obtained from rabbits of each acetylator genotype. A gene dose-response was demonstrated for each of the six substrates (TABLES 3-4). Highest NAT activity levels were always observed in homozygous rapid acetylator inbred strains (B/J, III/J, IIIC/J, III/DwJ, IIIEP/J, and IIIVO/J), lower levels in obligate heterozygous rapid acetylator rabbits (287, 289, 291, and 292), and lowest in homozygous slow acetylator (ACEP/J, III/cdJ, IIIVO/ahJ, and IIIVO/vptJ) inbred strains. The differences in magnitude of NAT activity level between acetylator genotypes was dependent upon the

TABLE 2. NAT and AHAT activities in rapid and slow acetylator inbred rabbits.

Rabbit Strain	Coefficient of Inbreeding	NAT Activity[a] (Units acetylated/min/mg)	AHAT Activity[a,b] (Units acetylated/min/mg)
AC/J	0.85	4.0	24.5
AX/J	0.88	2.7	5.0
B/J	INBRED	2.6	3.1
WH/J	0.85	4.2	21.3
X/J	0.91	2.1	3.8
Y-1/J	0.86	3.2	4.5
III/J	INBRED	2.6	8.0
IIIC/J	0.96	3.5	22.3
III/DwJ	0.95	4.5	20.9
IIIEP/J	0.98	2.3	5.9
IIIVO/J	0.98	2.6	9.7
ACEP/J	0.96	N.D.[c]	1.8
AXBU/J	0.83	N.D.	0.5
OS/J	0.90	N.D.	1.1
III/cdJ	0.97	N.D.	2.0
IIIVO/ahJ	0.97	N.D.	0.8
IIIVO/vptJ	0.97	N.D.	0.8

[a] Liver INH NAT and N-hydroxy-2-acetylaminofluorene AHAT activities in the 105,000 g cytosol were lower than usually obtained. The animals were sacrificed in Bar Harbor, ME. The livers were isolated and homogenized there, and subsequently mailed in dry ice to Ann Arbor, where centrifugation was completed.

[b] Data supplied by Dr. Irene Glowinski in collaborative study.

[c] Non-detectable.

substrate employed, progressively increasing in the following order: PABA, PAS, PA (TABLE 3), SMZ, INH, 2AF (TABLE 4).

Gene dose-response of liver AHAT activity:

A gene dose-response was demonstrated for gut mucosa 2AF NAT activity derived from duodenum (TABLE 5). Highest 2AF NAT activity levels were found in homozygous rapid acetylators (average of 27.7 $\pm$ 2.0 nmoles/min/mg), intermediate 2AF NAT activity level were found in heterozygous rapid acetylators (average of 12.0 $\pm$ .5 nmoles/min/mg), and lowest activity levels were found in homozygous slow acetylators, both inbred and non-inbred (average of 0.232 $\pm$ .033 nmoles/min/mg).

TABLE 3. Gene dose-response of liver PABA, PAS, and PA NAT activity in rapid and slow acetylator inbred rabbits.

RABBIT	COEFFICIENT OF INBREEDING	ACETYLATOR GENOTYPE	NAT Activity (nMoles/min/mg) PABA	PAS	PA
B/J	Inbred	RR	4.23	10.9	2.08
III/J	Inbred	RR	7.23	14.9	1.95
IIIC/J	0.96	RR	4.28	14.9	2.38
IIIDw/J	0.96	RR	4.08	10.0	2.19
IIIEP/J	Inbred	RR	3.43	11.3	3.11
IIIVO/J	0.97	RR	3.85	13.5	2.38
Mean ± S.E.		RR	4 52 ± .56	12.6 ± .87	2.35 ± .17
287	------	Rr	4.97	8.62	0.95
289	------	Rr	2.24	8.41	1.45
291	------	Rr	1.60	6.70	0.92
292	------	Rr	2.55	7.75	0.64
Mean ± S.E.		Rr	2.84 ± .74	7.87 ± .43	0.99 ± .17
ACEP/J	0.94	rr	1.26	1.22	0.016
III/cdJ	0.95	rr	1.86	2.04	0.062
IIIVO/ahJ	0.97	rr	2.98	2.17	0.038
IIIVO/vptJ	0.97	rr	1.97	1.99	0.064
Mean ± S.E.		rr	2.02 ± .36	1.86 ± .22	0.045 ± .011

TABLE 4. Gene dose-response of liver SMZ, INH and 2AF NAT activity in rapid and slow acetylator inbred rabbits.

RABBIT	COEFFICIENT OF INBREEDING	ACETYLATOR GENOTYPE	NAT Activity (nMoles/min/mg) SMZ	INH	2AF
B/J	Inbred	RR	4.26	17.6	52.1
III/J	Inbred	RR	4.64	19.1	34.9
IIIC/J	0.96	RR	3.04	19.5	48.6
III/DwJ	0.96	RR	3.75	22.2	36.2
IIIEP/J	Inbred	RR	2.55	18.1	52.2
IIIVO/J	0.97	RR	3.14	18.1	52.1
Mean ± S.E.		RR	3.56 ± .32	19.1 ± .68	46.0 ± 3.4
287	------	Rr	1.84	8.75	15.2
289	------	Rr	1.36	9.08	18.3
291	------	Rr	0.94	8.55	13.6
292	------	Rr	1.32	6.78	7.67
Mean ± S.E.		Rr	1.37 ± .18	8.29 ± .52	13.7 ± 2.2
ACEP/J	0.94	rr	0.009	N.D.*	0.094
III/cdJ	0.95	rr	0.044	N.D.	0.109
IIIVO/ahJ	0.97	rr	0.053	N.D.	0.189
IIIVO/vptJ	0.97	rr	0.014	N.D.	0.112
Mean ± S.E.		rr	0.030 ± .011	N.D.	0.126 ± .021

* Non-detectable with assay employed (less than 0.1 nMoles/min/mg)

TABLE 5. Gut mucosa 2AF NAT activity in rapid and slow acetylator rabbits*.

Rabbit[a]	Acetylator Genotype	2AF NAT Activity (nMoles/min/mg)
B/J	RR	25.8
IIIC/J	RR	23.7
IIIEP/J	RR	32.9
IIIVO/J	RR	28.2
Mean ± S.E.	RR	27.7 ± 2.0
152	Rr	13.0
289	Rr	11.6
291	Rr	11.3
Mean ± S.E.	Rr	12.0 ± .5
ACEP/J	rr	0.180
III/cdJ	rr	0.183
IIIVO/vptJ	rr	0.196
139	rr	0.245
140	rr	0.356
Mean ± S.E.	rr	0.232 ± .033

[a]Coefficients of inbreeding for inbred rabbits same as in TABLE 3.

*Data supplied by Dr. Toni Smolen in a collaborative study.

Discussion

In our survey of liver NAT and AHAT activities, we have identified eleven homozygous rapid acetylator and six homozygous slow acetylator inbred rabbit strains (TABLE 2). Five rapid acetylator strains (AC/J, AX/J, WH/J, X/J, and Y-1/J) and two slow acetylator strains (AXBU/J and OS/J) were not selected for more extensive studies. Instead, we chose to look in more detail at the six rapid and four slow acetylator strains that are the most highly inbred at this time. Liver NAT activity for each of these ten strains was determined for a number of arylamine and hydrazine substrates. Each of these substrate NAT activities exhibited a gene dose-response, with highest NAT activity levels in homozygous rapid acetylators, intermediate levels in heterozygous rapid acetylators, and lowest levels in homozygous slow acetylators. It should be pointed out that although the heterozygous rapid acetylator animals are not derived from inbred animals, the results obtained are, nevertheless, obtained from obligate (by pedigree information) heterozygotes. These findings are consistent with the trimodal distribution in SDZ N-acetylation capacity in vitro in rabbits originally reported by Frymoyer and Jacox (1963b). Although a similar gene dose-response of liver NAT activity levels in vitro has yet to be demonstrated in humans, Chapron et al. (1980) have been able to show a trimodal distribution in acetylator capacity, presumably corresponding to the three acetylator genotypes, based on pharmacokinetic parameters of SMZ obtained in vivo. This confirms the trimodal distributions of acetylation capacity found in earlier epidemiological studies (Sunhara et al., 1961; Dufour et al., 1964; Scott et al., 1969).

In addition to the liver NAT activity described above, we have also shown the gene dose-response relationship for 2AF NAT activity in the gut mucosa of the duodenum. The high levels of NAT activity in the duodenum have been previously reported by Hearse and Weber (1973) who carried out an extensive tissue distribution study in rabbits. The genotype-dependent level of NAT activity in gut mucosa may have pharmacokinetic significance due to the well-known first pass effect in INH metabolism (e.g., Weber and Hein, 1979). Similar gene dose-response relationships for NAT activity in liver and gut mucosa are consistent with the hypothesis that the two enzymes are highly similar or identical. It is of interest that Patterson et al. (1980) have subjected rabbit liver and gut mucosa NAT to Ouchterlony double diffusion immunological studies and found that the NATs from the two tissues yielded reactions of complete identity, while both were immunologically distinct from rabbit erythrocyte and lymphocyte NATs.

Each of the six arylamine and hydrazine substrates (PABA, PAS, PA, SMZ, INH, and 2AF) exhibited the gene dose-response relationship for liver NAT activity. However, the magnitude of the genetically determined differences between acetylator phenotypes, as well as between acetylator genotypes , was dependent upon the substrate employed. The smallest differences were observed with PABA and PAS, two substrates that are often termed monomorphic substrates in man and rabbit. Progressively larger differences in liver NAT activity were observed for PA, SMZ, INH, and 2AF, four substrates usually identified as polymorphic substrates in man and rabbit. These results suggest that there is a continuum in expression of the magnitude of the

acetylator difference between acetylator phenotypes and genotypes and this subject is examined extensively in CHAPTER III C. Previous studies have shown that liver PABA (monomorphic) and SMZ (polymorphic) NAT activities coexist on a common liver protein in rabbit liver (see CHAPTER III B). Our present findings support this conclusion, suggesting that a single liver NAT within each phenotype catalyzes the acetylation of a number of substrates (both monomorphic and polymorphic), and that the magnitude of the NAT activity level difference between genotypes is dependent upon the substrate structure (see CHAPTER III C). Comparisons of liver NAT between acetylator phenotypes, however, show qualitative, structural differences (see CHAPTER III A).

The hypothesis that liver NAT and AHAT activities are under common genetic control and are properties of a common protein in rabbit liver (Glowinski et al., 1980) is well supported by our inbred rabbit survey of NAT and AHAT activities since no discordance was noted in NAT and AHAT activity across the 17 inbred rabbit strains.

Experimental uses for homozygus rapid and slow acetylator rabbits are numerous. For example, the significance of the N-acetylation polymorphism in INH- and MAH-induced toxicity has previously been shown in a rabbit model (see CHAPTER VII), and the rabbit has been used in studies to investigate the role of acetylator status in INH-induced hepatotoxicity (see CHAPTER VIII). In addition, recent in vitro studies with hepatocytes in primary culture isolated from rapid and slow acetylator rabbits have shown that rates of unscheduled DNA repair produced by hydrazines capable of causing a drug-induced SLE syndrome and by carcinogenic arylamines are dependent upon acetylator

phenotype. (McQueen et al., 1982). Our study now identifies and characterizes isogenic rabbits of known acetylator genotype that show large differences in N-acetylating capacity in both liver and gut mucosa for carcinogenic and therapeutic arylamines and hydrazines. Our initial characterization of these inbred rabbits may substantially enhance the opportunities for using this animal species in future biochemical and toxicological investigations of the N-acetylation polymorphism.

CHAPTER III

BIOCHEMICAL BASIS OF THE N-ACETYLATION POLYMORPHISM IN THE RABBIT

A.

Biochemical Basis of Rapid and Slow Acetylator N-Acetyltransferase

Introduction

Individual differences in the N-acetylation rate of arlyamines and hydrazines are genetically determined in several mammalian species including man and rabbit (Weber, 1973), hamster (see CHAPTER IV), and mouse (Tannen and Weber, 1980a; Glowinski and Weber, 1982a). These differences, reflected both in vivo and in vitro, are primarily determined by the liver activity level of the cytosolic enzyme NAT. In man and rabbit, differences in NAT activity levels form the basis of the INH acetylation polymorphism which has important clinical significance in man (Weber and Hein, 1979). Rapid acetylators are either homozygous (RR) or heterozygous (Rr) for a codominant rapid acetylator gene (R), while slow acetylators are homozygous (rr) for a slow acetylator gene (Weber, 1973).

The rabbit has been used as an animal model for studying the INH acetylation polymorphism, and a review of the biochemical properties of rabbit liver NAT has recently been reported (Weber and Glowinski, 1980). Although the original discoveries of the individual differences in N-acetylator capacity in man (Hughes et al., 1955) and rabbit (Frymoyer and Jacox, 1963a,b) were made many years ago, its

molecular basis has not been clearly elucidated. One basic question remaining to be answered is whether these genetically determined N-acetylation differences are due to quantitative differences in the amount of NAT enzyme between rapid and slow acetylators, or, alternatively, due to qualitative structural differences in liver NAT between the two phenotypes. The purpose of this study together with a companion study recently reported (Patterson et al., 1980) was to discern which of these two possibilities is correct.

Methods

Chemicals and reagents:

(see TABLE 33)

Source of liver NAT:

Mature New Zealand White rabbits of known acetylator phenotype maintained in our animal care facilities at the University of Michigan and inbred rabbits of known acetylator phenotype obtained from The Jackson Laboratory, Bar Harbor, Maine were used as source of liver NAT. (For full description, see CHAPTER II).

Liver NAT purification:

Rabbits were killed by injection of air into a peripheral ear vein or by carbon dioxide intoxication. The liver was rapidly removed, minced and homogenized (25% w/v) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT and EDTA. The homogenate was centrifuged at 10,000 g for 20 min followed by 105,000 g for 60 min. In the heat inactivation experiments, the liver NAT was further purified by ammonium sulfate fractionation (45-60%) and column chromatography on Sephadex G-100 as previously described (Weber, 1971). All purification steps were conducted at 4°C.

PABA NAT activity determinations:

PABA NAT activity was determined at room temperature (22-23°C) by a micromodification of the Bratton-Marshall procedure as described in CHAPTER II.

PAS NAT activity determinations:

PAS NAT activity was determined at 37°C using the DMAB assay as described in CHAPTER II when carrying out Lineweaver-Burk analyses requiring PAS concentrations of 0.4 to 0.1 mM. A modified DMAB assay

in which the final addition of DMAB was 300 ul instead of 1 ml was used when carrying out Lineweaver-Burk analyses requiring PAS concentrations of 0.02 to 0.4 mM. Under the conditions of the modified DMAB assay, a decrease of one sod absorbance unit corresponded to the acetylation of 8.60 pmoles of PAS.

Protein determinations:

Protein was determined by a microbiuret method (Gornall et al., 1949).

Liver NAT heat inactivation procedure:

Liver NAT from rapid and slow acetylator rabbits was purified through Sephadex G-100 as previously described (Weber, 1971) and subjected to heat inactivation at 50°C. At predetermined time points, PABA NAT activity was measured as described above.

Data analysis:

PABA and PAS NAT activity determinations were conducted and analyzed as described in CHAPTER II. Kinetic constants for PAS NAT activity were calculated with a modified Hyper-program by Cleland (1963) which uses a least squares linear regression method.

Results

Rapid and slow acetylator NAT heat inactivation:

Sephadex G-100 purified liver NAT preparations from two rapid and two slow acetylator rabbits were subjected to heat inactivation at 50°C. As shown in TABLE 6, the heat inactivation rates for PABA NAT activity from the slow acetylator preparations (half-lives of 10 and 11 min) were significantly ($p < .001$) faster than the heat inactivation rates from the rapid acetylator preparations (half-lives of 20 and 23 min).

Kinetic constants of liver PAS NAT activity:

Lineweaver-Burk analyses were carried out on 105,000 g liver NAT preparations from rabbits of all three acetylator genotypes. As shown in TABLE 7, the apparent Km for PAS ranged from 0.42 to 1.30 mM in the homozygous rapid (RR) acetylator genotype for an average of 0.75 mM. This was higher, but not significantly higher ($p > .05$) than the apparent Km in the heterozygous rapid acetylator (Rr) genotype, which ranged from 0.22 to 1.01 mM for an average of 0.63 mM. However, the apparent Km in the combined rapid acetylator phenotype (0.69 mM) was significantly ($p < .001$) higher (23-fold) than the apparent Km in the slow acetylator phenotype, which ranged from 0.019 to 0.053 mM for an average of 0.030 mM.

The apparent Vmax for PAS NAT activity differed significantly between all three acetylator genotypes. It ranged from 45 to 102 (average of 74) nmoles/min/mg in the homozygous rapid acetylator genotype, a value significantly higher ($p < .001$) than that of the heterozygous rapid acetylator genotype, which ranged from 16 to 44 (average of 32) nmoles/min/mg. The apparent Vmax in the slow

TABLE 6. Heat inactivation of liver PABA NAT activity in rapid and slow acetylator rabbits.*

RABBIT NUMBER	ACETYLATOR PHENOTYPE	PABA NAT INACTIVATION HALF-LIFE AT 50° (min)
74	Rapid	20
111	Rapid	23
89	Slow	10
94	Slow	11

* Heat inactivation experiments were conducted with Sephadex G-100 purified liver NAT. PABA NAT heat inactivation rates were calculated using semi-log linear least squares regression analysis.

acetylator genotype ranged from 1.4 to 2.0 (average of 1.8) nmoles/min/mg, which was significantly lower ($p < .001$) than either of the two rapid acetylator genotypes.

TABLE 7. Kinetic constants of rabbit liver PAS NAT activity in the three acetylator genotypes.[a]

RABBIT	GENOTYPE	Km(mM)	Vmax (nmoles/min/mg)
III/J	RR	1.30 ± .27	102 ± 17
III/DwJ	RR	0.42 ± .16	45 ± 10
IIIC/J	RR	0.54 ± .17	81 ± 17
IIIEP/J	RR	0.73 ± .21	69 ± 14
Mean	RR	0.75 ± .19[b]	74 ± 12[e]
287	Rr	0.71 ± .34	31 ± 11
289	Rr	0.56 ± .13	44 ± 7
291	Rr	0.22 ± .09	16 ± 3
292	Rr	1.01 ± .51	36 ± 14
Mean	Rr	0.63 ± .16[c]	32 ± 6[f]
ACEP/J	rr	0.019 ± .0001	1.4 ± .03
IIIcd/J	rr	0.036 ± .009	2.0 ± .2
IIIVO/ahJ	rr	0.018 ± .003	2.0 ± .1
IIIVO/vptJ	rr	0.053 ± .038	1.8 ± .6
139	rr	0.023 ± .006	1.7 ± .1
140	rr	0.030 ± .004	1.6 ± .1
Mean	rr	0.030 ± .005[d]	1.8 ± .1[g]

[a]Table values were calculated by means of a modified Hyper-program by Cleland (1963) as described in Methods. The coefficient of inbreeding for these animals is shown in TABLES 3-4.

b not significantly higher than c ($p > .05$)
b and c significantly higher than d ($p < .001$),
e significantly higher than f ($p < .001$)
g significantly lower than both e and f ($p < .001$).

Discussion

The differences in heat inactivation and kinetic constants reported in this chapter between rapid and slow acetylator rabbit liver NAT support the hypothesis of a qualitative, structural difference between NAT of the two acetylator phenotypes. These findings are consistent with others in hamster (see CHAPTER V) and mouse liver (Glowinski and Weber, 1982b) and human lymphocyte (McQueen and Weber, 1980) that also demonstrated differences in biochemical characteristics indicative of qualitative differences between rapid and slow acetylator NAT.

The results of this study are also consistent with our companion investigation that argued against a quantitative difference in liver NAT protein between the two phenotypes (Patterson et al., 1980). In that study, immunotitration and immunodiffusion experiments indicated that the quantity of NAT molecules in liver cytosols of rapid and slow acetylator rabbits was essentially equal despite the large difference (up to 400-fold) in SMZ NAT activity levels.

Finally, recent findings have suggested that rabbit liver NAT activity covaries and coresides on a common enzyme protein with AcCoA-independent AHAT activity (Glowinski et al., 1980; see CHAPTER II). In addition, Glowinski et al. (1980) also showed that this same liver protein has the capacity to polymorphically catalyze AcCoA-independent transacetylase reactions, such as acetyl transfer from N-hydroxy-2-acetylaminofluorene to 4-aminoazobenzene. Thus the findings of our current experiments also suggest a qualitative difference in these two AcCoA-independent activities between rapid and slow acetylators.

B.

Biochemical Basis of Monomorphic and Polymorphic Expression of N-Acetyltransferase Activity

Introduction

N-acetylation is an important metabolic step in the biotransformation of a number of arylamine and hydrazine drugs (Weber, 1973). Examples of drugs which undergo N-acetylation include INH, SMZ, PABA, PAS, and PA. In addition, several aromatic amine carcinogens such as 2AF, benzidine, and BNA (Glowinski et al., 1978) and some of the endogenous biogenic amines such as serotonin (Weisbach et al., 1960; Schloot et al., 1969; Steinberg et al., 1969; White et al., 1969) are also N-acetylated. The cytosolic liver enzyme which catalyzes the N-acetylation of this diverse group of arylamines and hydrazines has been identified as liver AcCoA-dependent NAT (E.C.2.3.1.5) (Weber, 1973).

Differences in the N-acetylation rate of these arylamines and hydrazines are under genetic control in several animal species, including man (Evans et al., 1960), rabbit (Frymoyer and Jacox, 1963a,b), mouse (Tannen and Weber, 1980a; Glowinski and Weber, 1982a) and hamster (see CHAPTER IV). In man and rabbit, this difference in N-acetylation rate forms the basis of the well-known INH acetylation polymorphism, which has important clinical significance in man (Weber

and Hein, 1979). Individuals can be classified as either rapid or slow acetylators, due to differences in NAT activity levels in liver. In rabbit, these differences are quite large, reaching several hundred-fold (Glowinski et al., 1978; see CHAPTER II), making the rabbit a frequently used animal model for studying this genetic trait. Substrates which show this polymorphism in acetylation rate between rapid and slow acetylator phenotypes are classified as polymorphic substrates, and include INH, SMZ, PA, and various carcinogenic arylamines (Weber and Glowinski, 1980). In man (Drayer and Reidenberg, 1977; Lunde et al., 1977) and rabbit (see CHAPTER VII) these genetically determined differences in acetylation rate are clearly associated with differences in drug toxicity. The importance of these differences in the relative susceptibility to arylamine-induced carcinogenesis, however, remains unknown.

In contrast to polymorphic substrates, other substrates for NAT (e.g., PABA and PAS) do not show large differences in acetylation rate between rapid and slow acetylators in both humans and rabbits (Jenne, 1965; Hearse and Weber, 1973; Glowinski et al., 1978; see CHAPTERS II and IX) and are thus classified as monomorphic substrates. This finding has suggested that more than one form of NAT may be present in the liver of each phenotype (Jenne, 1965; Hearse and Weber, 1973). Alternatively, a single NAT enzyme with diverse actions may catalyze the acetylation of both monomorphic and polymorphic substrates. This study was designed to utilize genetic and biochemical methods to investigate the two possibilities.

Methods

Chemicals and reagents:

(see TABLE 33)

Source of N-acetyltransferase:

The source of selected lines of mature New Zealand White rabbits of known acetylator phenotype is described in CHAPTER II.

Liver N-acetyltransferase purification:

Rabbits were killed by injection of air into a peripheral ear vein, cardiac puncture, or carbon dioxide intoxication. Following sacrifice, NAT was purified by one of two different procedures as described below.

Procedure I:

Immediately after death, the liver was removed, minced, and homogenized in 0.1 M potassium phosphate buffer (25% w/v), pH 7.4, followed by centrifugation at 10,000 g for 20 minutes and 105,000 g for 60 minutes. The supernatant fraction was partially purified by ammonium sulfate fractionation (45-60%) and column chromatography on Sephadex G-100 as previously described (Weber, 1971). All purification steps were conducted at 4°C.

Procedure II:

Immediately after death, the liver was removed, minced, and homogenized (20% w/v) in 50 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, followed by centrifugation at 10,000 g for 20 minutes and 105,000 g for 60 minutes. The supernatant fraction was partially purified by ammonium sulfate fractionation (45-60%), then resuspended in 2 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0 and dialyzed overnight under argon. The dialyzed fractions were

applied to a DEAE-cellulose (medium mesh) column (2.5 x 10 cm) and eluted with a linear 16 hr gradient of 2 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, to 2 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, 0.5 M sodium chloride at a flow rate of 64 ml/hr. Peak enzyme fractions were concentrated in a Millipore ultrafiltration apparatus. The concentrate was applied to a Sephacryl S-200 column (2.5 x 60 cm) and eluted with 20 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, at a flow rate of 64 ml/hr, and peak activity fractions were combined and concentrated in the Millipore ultrafiltration apparatus. All purification steps were conducted at 4°C, and a complete description of this purification procedure has been previously published (Glowinski et al., 1980).

N-acetyltransferase flatbed isoelectric focussing procedure:

Flatbed isoelectric focussing of partially purified (Sephadex G-100) rabbit liver preparations of NAT was conducted with an LKB 2117 Multiphor apparatus (Winter et al., 1975) in an attempt to separate PABA and SMZ NAT activity. Preliminary experiments with ampholines of pH range 3.5 to 10 indicated that narrow range ampholines in the range of 5.0 to 7.0 were best suited for our purpose. Due to instability of NAT in acidic conditions (below pH 6.5) several steps were taken to minimize enzyme inactivation during the isoelectric focussing procedure. The surface of the gel bed was covered by an atmosphere of cold nitrogen gas to prevent oxidation, and a methanol-water mixture refrigerated at -5°C was circulated through the cooling unit to minimize heat inactivation. DTT (0.5 mM) and AcCoA (0.1 mM) were also added to the gel matrix to stabilize the enzyme at pH's in the neutral

range. After 4.5 hours of electrophoresis, the gel was divided into 30 fractions and eluted with 1.5 ml of water. The pH of the solution was measured and then neutralized with 0.1 M phosphate buffer (pH 7.4) for subsequent assay of PABA and SMZ NAT activities. Protein was determined by absorbance at 280 nm.

N-acetyltransferase polyacrylamide gel electrophoresis procedure:

The procedure has been previously published in detail (Glowinski et al., 1980). Briefly, polyacrylamide gels were prepared to a final concentration of 7% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.14% (w/v) ammonium persulfate, 0.083% (w/v) N,N,N',N'-tetramethylethylenediamine, and 0.089 M Tris/borate buffer, pH 8.3, and pre-electrophoresed for two hours at a current of 2mA/gel. Aliquots of Sephacryl S-200 concentrate containing 20-50 ug of protein were applied to the gels, electrophoresed for 90 minutes at a current of 2mA/tube, and scanned for protein at 280 nm. The gels were sliced into 1 mm sections, one-half of each gel slice was combined with one-half of the adjacent gel slice, and homogenized in 50 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, and assayed for PABA and SMZ NAT activities.

PABA and SMZ NAT activity determinations:

PABA and SMZ NAT activities were determined at 37°C by a micromodification of the Bratton-Marshall procedure as described in CHAPTER II).

PAS and PA NAT activity determinations:

PAS and PA NAT activities were determined at 37°C by the DMAB assay of NAT activity as described in CHAPTER II.

INH NAT activity determinations:

INH NAT activity determinations were carried out at 37°C as described in CHAPTER II.

Protein determinations:

Total protein was determined in 105,000 g cytosol by a microbiuret method (Gornall et al., 1949). Protein determinations during the isoelectric focussing and polyacrylamide disc gel electrophoresis procedures were done by absorbance at 280 nm.

Data analysis:

NAT activity determinations were conducted and analyzed as described in CHAPTER II. PABA and SMZ NAT activity inactivation half-lives were calculated using semi-log linear least squares regression analysis.

Results

Genetic covariation of monomorphic and polymorphic NAT activities:

NAT activities of (105,000 g) rabbit liver preparations were determined for PAS and for the prototypic monomorphic substrate, PABA, and the prototypic polymorphic substrate, INH. As shown in FIGURE 8 and TABLE 8 , INH NAT activities ranged from 0.017 to 0.257 nmoles/min/mg in eight slow acetylator rabbits (average of 0.12 nmoles/min/mg). In ten rapid acetylator rabbits, INH NAT activities ranged from 14.3 to 23.0 nmoles/min/mg (average of 17.5 nmoles/min/mg). Thus, the average difference in INH NAT activity between the two phenotypes was approximately 150-fold. PABA NAT activities showed considerably less variation between the two phenotypes, ranging from 1.37 to 3.68 nmoles/min/mg (average of 2.66 nmoles/min/mg) in the slow acetylator rabbits. In the rapid acetylator rabbits, PABA NAT activities ranged from 3.81 to 5.61 nmoles/min/mg (average of 4.86 nmoles/min/mg) (FIGURE 8 and TABLE 8). Thus, the average PABA NAT activity difference between the two phenotypes was 1.8-fold. PAS NAT activities in six slow acetylator rabbits ranged from 0.53 to 1.93 nmoles/min/mg (average of 1.09 nmoles/min/mg), whereas they ranged from 12.4 to 18.3 nmoles/min/mg (average of 15.8 nmoles/min/mg) in six rapid acetylator rabbits (TABLE 8). Thus, the average difference in PAS NAT activity between the two phenotypes was 14-fold.

Copurification of monomorphic and polymorphic NAT activities:

PABA and SMZ NAT activities copurified through purification procedure I. The final step involved isoelectric focussing as shown in FIGURE 9. After 4.5 hours the peak for PABA and SMZ NAT activity was

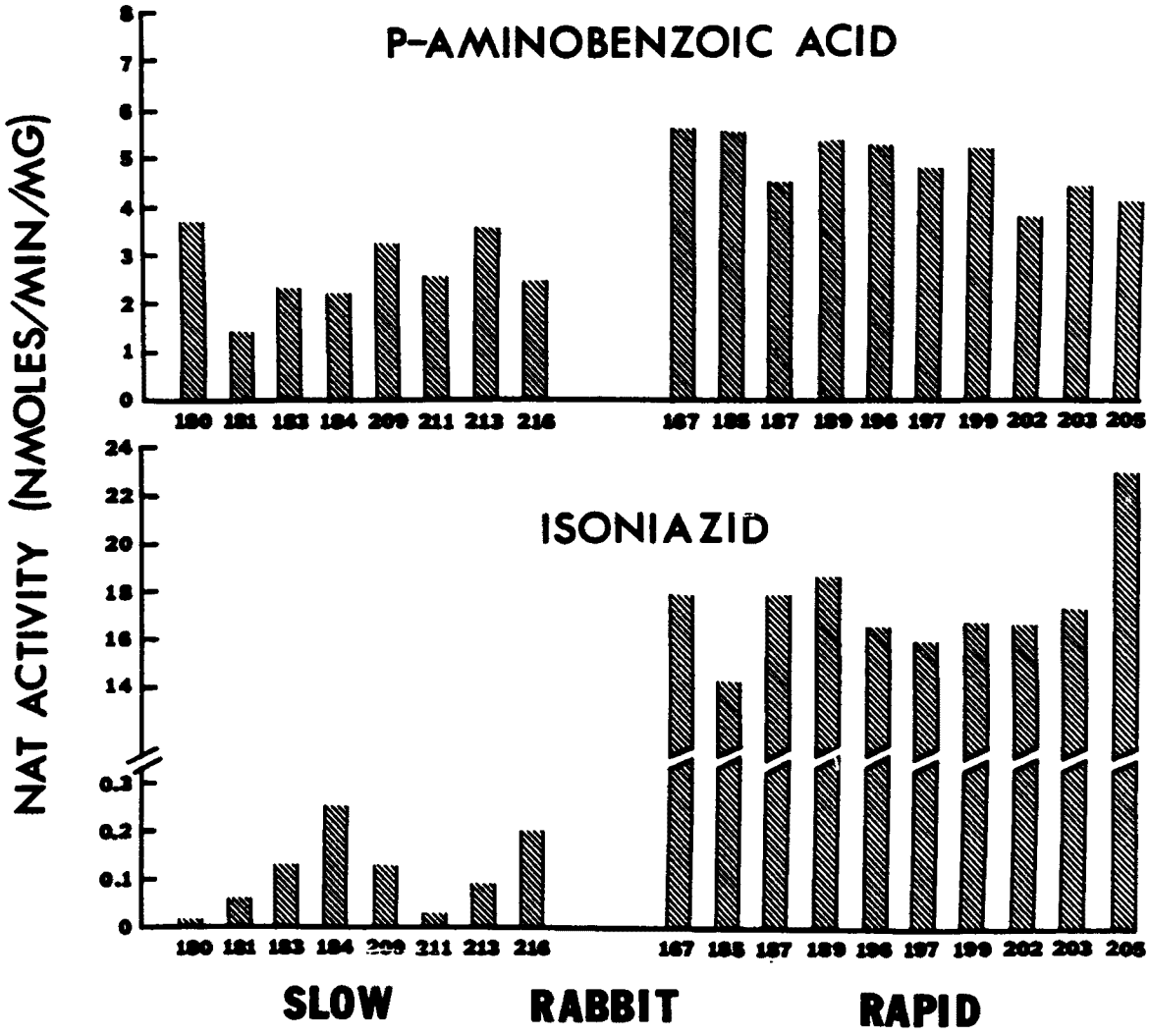


FIGURE 8. Liver PABA and INH NAT activities in rapid and slow acetylator rabbits.

TABLE 8. Liver NAT activities in rapid and slow acetylator rabbits.[a]

Slow Acetylators				
Rabbit No.	SDZ Half-Life[b]	PABA	PAS	INH
	min		*nmol/min/mg*	
180	93	3.68	1.01	0.017
181	87	1.37	0.54	0.062
183	71	2.30	1.93	0.135
184	73	2.19		0.257
209	122	3.19	0.53	0.128
211	99	2.56	0.83	0.031
213	109	3.56		0.088
216	115	2.46	1.72	0.203
Average ± S.E.		2.66 ± 0.27	1.09 ± 0.24	0.12 ± 0.03
Rapid Acetylators				
Rabbit No.	SDZ Half-Life	PABA	PAS	INH
	min		*nmol/min/mg*	
167	26	5.61	12.4	17.9
185	37	5.55	16.6	14.3
187	34	4.51		17.9
189	33	5.34	15.2	18.7
196	43	5.26	15.9	16.6
197	43	4.81		16.0
199	43	5.22		16.8
202	38	3.81	16.3	16.8
203	38	4.42	18.3	17.4
205	38	4.09		23.0
Average ± S.E.		4.86 ± 0.20	15.8 ± 0.80	17.5 ± 0.72

[a] PABA, PAS, and INH NAT activities are each significantly higher in rapid *vs.* slow acetylators ($P < .001$).
[b] Sulfadiazine half-life determined *in vivo*.

FIGURE 9. Flatbed isoelectric focusing patterns of rabbit liver NAT.

Flatbed isoelectric focusing patterns of PABA (●) and SMZ (*) NAT activities. Sephadex G-100 purified fractions were applied to the gel, and isoelectric focusing was conducted for 4.5 hours (A) and 1.5 hours (B) at 18 watts (1300 volts). The ordinates from left to right indicate NAT activity in units acetylated per minute per milliliter, optical density at 280 nm (○), and pH (□). The abscissa indicates the gel fraction number. The experiment was carried out in collaboration with Dr. Masaharu Hirata.

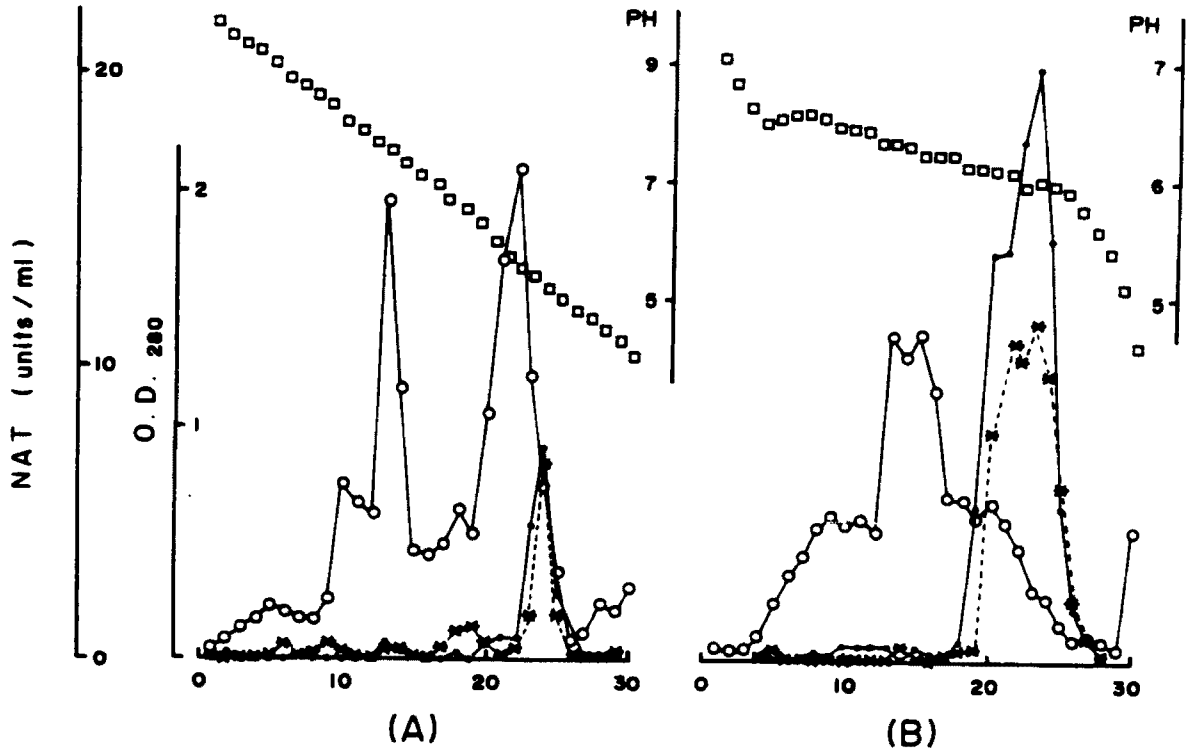


FIGURE 9

found at pH 5.3 (FIGURE 9A). The recovery of protein, as judged by absorbance at 280 nm, was quantitative, although only a low percentage (10%) of NAT activity was recovered. Similar isoelectric focussing results were obtained with both 105,000 g supernatant and Sephadex G-100 enzyme preparations. Although comigration of PABA and SMZ NAT activity was always observed, low recoveries made interpretation of results difficult. Consequently, shorter periods of isoelectric focussing were carried out to increase recovery of NAT activity. Although this produced an incomplete pH gradient, recovery of PABA and SMZ NAT enzyme activities was greater than 99% (FIGURE 9B). It is evident that PABA and SMZ NAT activities comigrate to the anode in a single symmetrical protein peak.

PABA and SMZ NAT activities were also not resolved through purification procedure II. Copurification of PABA and SMZ NAT activity, together with PA and PAS NAT activities through the Sephacryl S-200 chromatography step is shown in FIGURE 10. Peak activity was found in Sephacryl fraction No. 40 for each of the four substrates. A complete summary of the copurification of PABA and SMZ NAT activities through purification procedure II shows that percent recovery and fold purification for PABA and SMZ NAT activities across each purification step were highly similar (TABLE 9). Total fold purification of PABA NAT activity was 73.9, whereas it was 73.5 for SMZ NAT. Total percent recovery of enzyme activity was 7.24 for PABA NAT and 7.19 for SMZ NAT activity (TABLE 9). The copurification of PABA and SMZ NAT activities in the subsequent analytical polyacrylamide gel electrophoresis step is shown in FIGURE 11. After Sephacryl S-200 chromatography, electrophoresis of the combined,

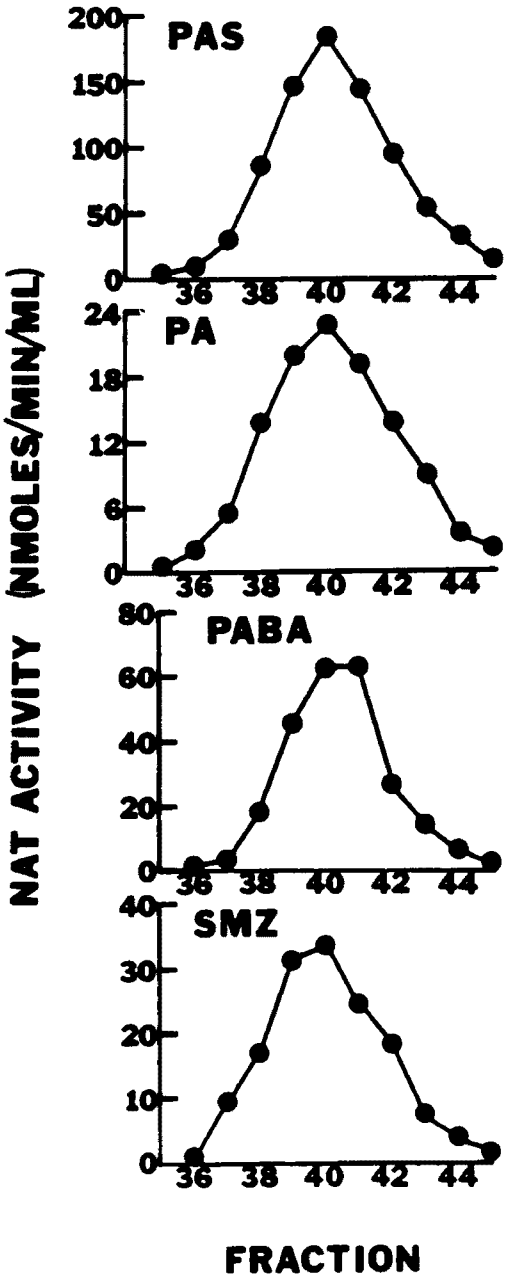


FIGURE 10. Co-purification of PAS, PA, PABA, and SMZ NAT activity of rabbit liver through Sephacryl S-200 chromatography.

The liver NAT activity in nmoles/min/ml is listed on the ordinate Sephacryl fraction numbers 35 to 45 listed on the abscissa, for four substrates; PABA, SMZ, PAS, and PA. Peak NAT activity levels were found in fraction 40 for each substrate. No activity was found in fractions below 35 or above 45.

TABLE 9. Copurification summary for PABA and SMZ NAT activities in rapid acetylator rabbit liver.[a]

Purification Step	Protein Concen.	Volume	PABA NAT			SMZ NAT		
			Specific activity	% Recovery	Fold purification	Specific activity	% Recovery	Fold purification
	mg/ml	*ml*	*nmol/min/mg*			*nmol/min/mg*		
105,000 × *g* supernatant	24.6	170	2.82	100		3.93	100	
					2.3			2.3
Ammonium sulfate precipitation (45-60%)	43.0	26	6.60	62.6		9.03	61.4	
					20.7			19.4
DEAE-cellulose chromatography	1.49	4.2	136.9	30.2		176.4	28.3	
					1.52			1.64
Sephacryl S-200 chromatography	0.48	2.5	208.6	38.3		289.1	41.4	
Total				7.24	73.9		7.19	73.5

[a]Raw data furnished by Dr. Irene Glowinski in a collaborative study.

FIGURE 11. Polyacrylamide disc gel electrophoresis of rabbit liver NAT.

Concentrated Sephacryl S-200 purified liver NAT was applied to polyacrylamide disc gels as described in Methods, and electrophoresed at 2 mA/tube for 90 min. After scanning at 280 nm, gels were sliced and assayed for PABA and SMZ NAT activity. The NAT activity in pmoles per min is plotted on the ordinate versus the gel slice number on the abscissa. Peak PABA and SMZ NAT activity levels were found in gel slices 39-40, associated entirely with the single homogeneous protein peak B. No NAT activity was found in slices corresponding to protein peak A. Raw data was supplied by Dr. Irene Glowinski and Dr. Jim Vaught in a collaborative study.

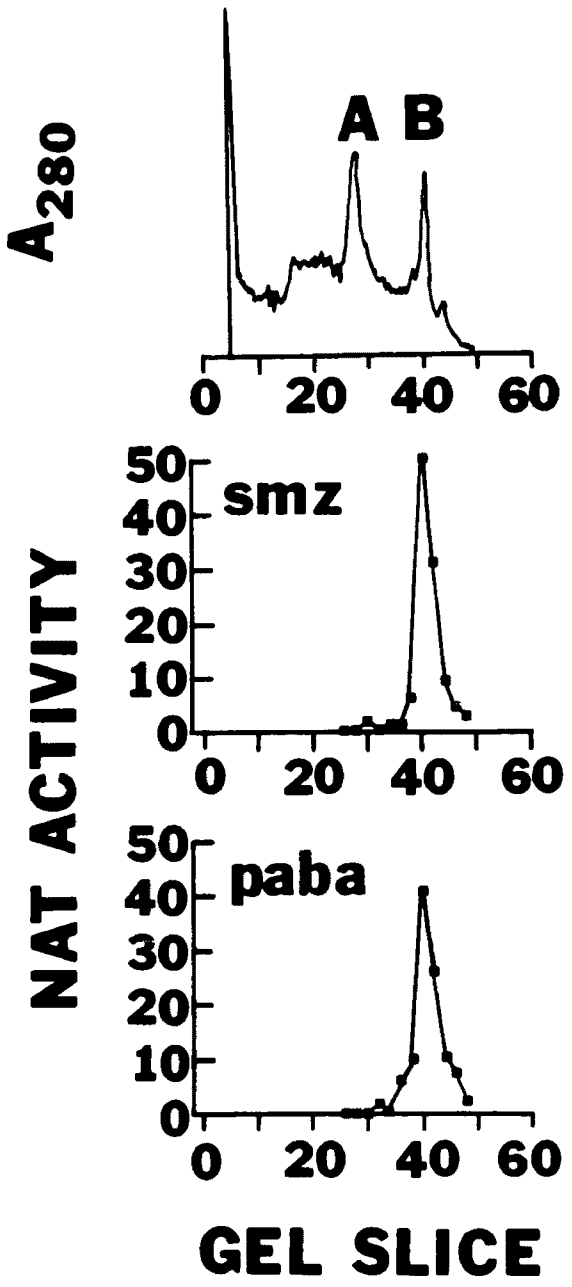

A B
A_{280}
0 20 40 60
smz
paba
50
40
30
20
10
0
NAT ACTIVITY
GEL SLICE

FIGURE 11

concentrated, peak activity fractions revealed two major, symmetrical, protein peaks (A and B). PABA and SMZ NAT activities were both associated with protein B and not with protein A.

Heat inactivation studies of NAT:

Heat inactivation rates of PABA and SMZ NAT activities in three rapid acetylator liver preparations (Sephadex G-100) were determined at 50°C (FIGURE 12). Initial PABA NAT activities in the preparations were 11.9, 8.0, and 16.8 nmoles/min/mg protein, while initial SMZ NAT activities were 12.9, 9.6, and 11.9 nmoles/min/mg, respectively. PABA and SMZ NAT activity heat inactivation rates were first order and indistinguishable within the rapid acetylator preparations.

Heat inactivation rates of PABA and SMZ NAT activities were also conducted in a highly purified (Sephacryl S-200) preparation from a rapid acetylator rabbit at 55°C (FIGURE 13). The heat inactivation rates were first order for both activities. The heat inactivation half-life was 3.77 min for PABA NAT activity and 3.63 min for SMZ NAT activity.

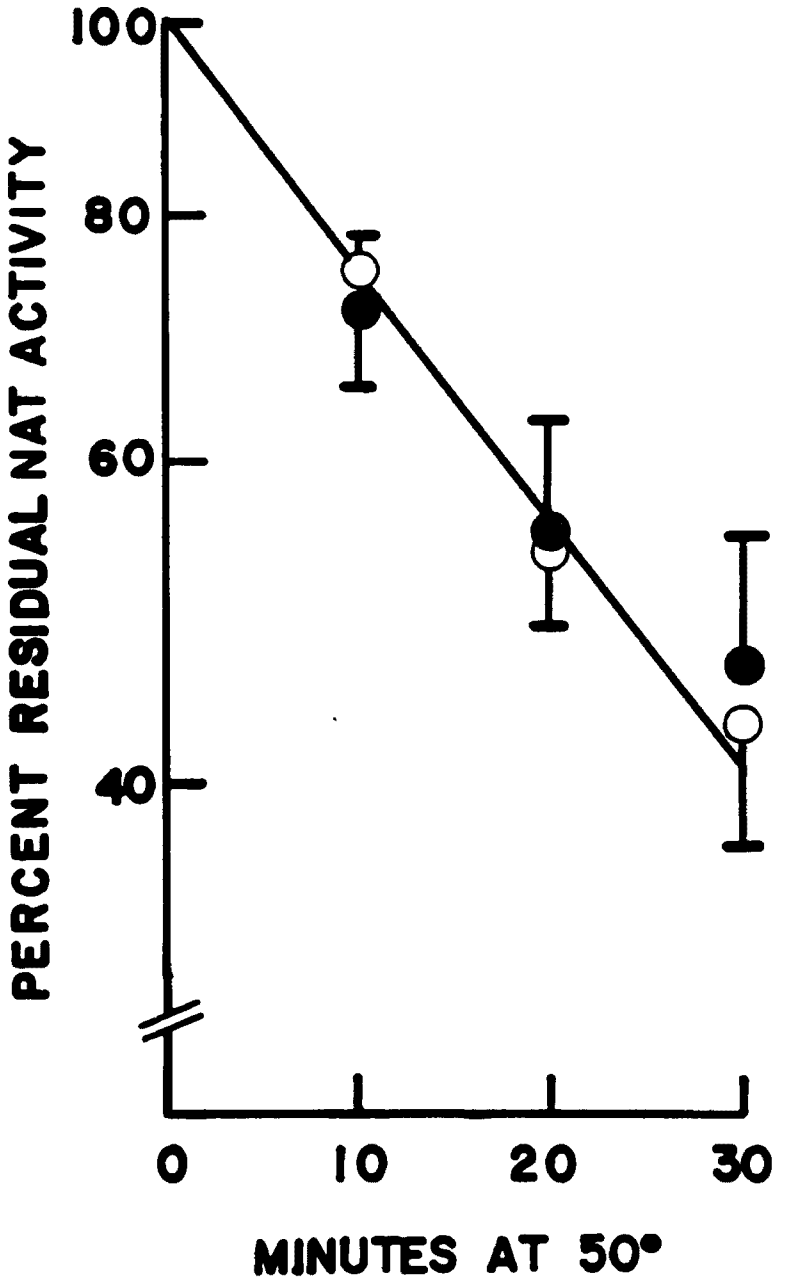


FIGURE 12. Heat inactivation of Sephadex G-100 purified rabbit liver PABA and SMZ NAT activities at 50°C.

The percent residual PABA (○) and SMZ (●) NAT activity is plotted on the ordinate versus time of incubation at 50°C on the abscissa. Each data point represents the mean of three different rapid acetylator rabbits; the lines above and below each point represent the standard error.

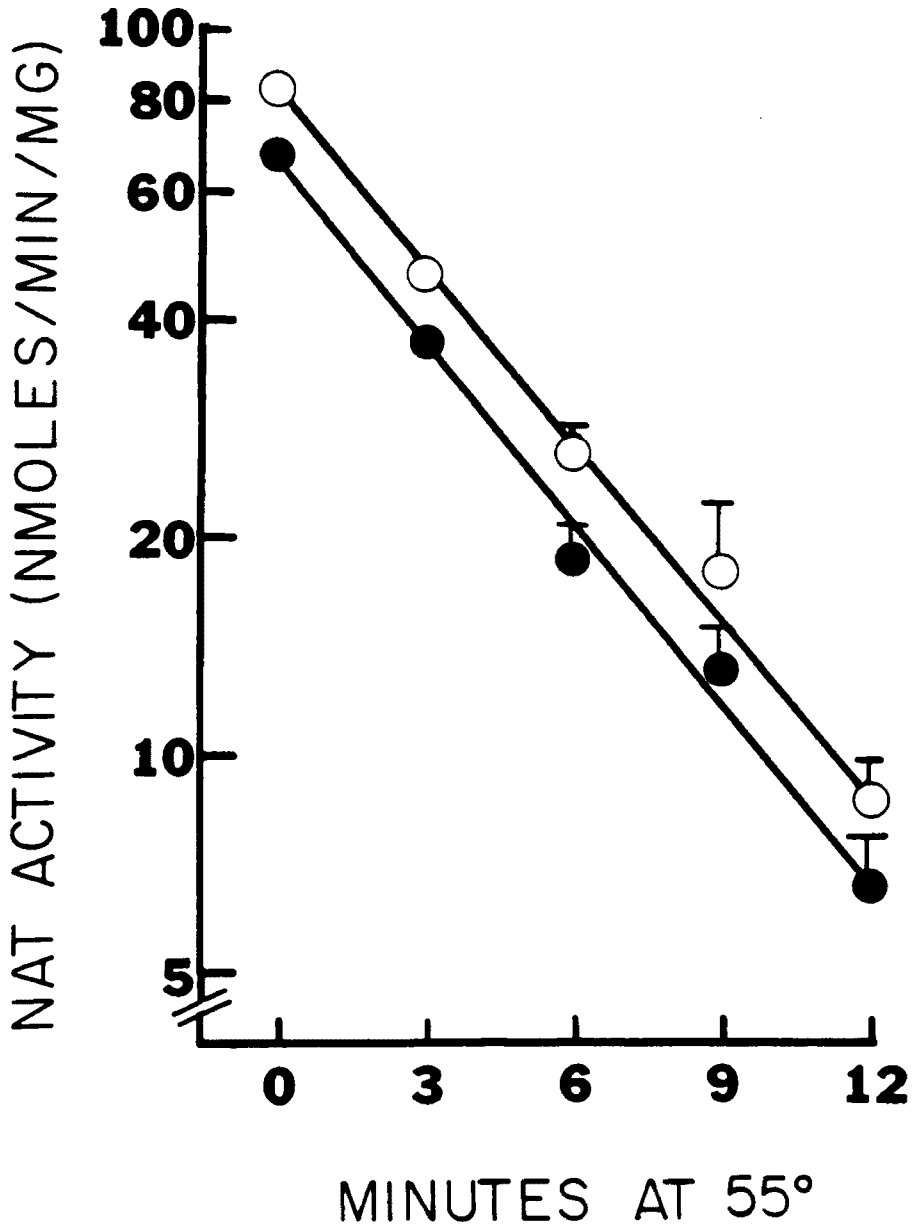


FIGURE 13. Heat inactivation of Sephacryl S-200 purified rabbit liver PABA and SMZ NAT activities at 55°C.

The specific PABA (open circles) and SMZ (closed circles) NAT activity is plotted on the ordinate versus time of incubation on the abscissa. Each data point is the mean of three determinations; lines above the point indicate S.E. In instances where no S.E. is shown, the S.E. lies within the dot. The half-life for PABA NAT inactivation was 3.77 min (k = .184) r = -.9969. The half-life for SMZ NAT activity was 3.63 min (k = .191) r = -.9962.

Discussion

A wide variety of structurally diverse arylamine and hydrazine drugs and carcinogens undergo N-acetylation via the liver enzyme NAT. Of special interest is the fact that individual differences in N-acetylation of these compounds is under genetic control, with polymorphic substrates such as INH and SMZ showing large differences in rates of N-acetylation between rapid and slow acetylators. In contrast, other substrates such as PABA show approximately equal activity in the two phenotypes and are considered monomorphic substrates. A major objective of this dissertation is to understand the molecular nature of NAT enabling us to predict a priori whether a novel arylamine drug or carcinogen will show a monomorphic or polymorphic N-acetylation expression. The immediate question to be addressed is whether different NAT enzymes mediate monomorphic and polymorphic expression, or, alternatively, whether a single enzyme protein with multiple catalytic capacities is responsible. Taken in total, the biochemical and genetic results of these experiments support the latter hypothesis.

Large in vitro differences in liver NAT activity have been found between rapid and slow acetylator rabbits for other polymorphic substrates such as SMZ, and several carcinogenic arylamines (Glowinski et al., 1978 ; see CHAPTER II). We now show that PABA and PAS NAT activity also showed genetically-determined differences between the phenotypes (1.8-fold for PABA and 14-fold for PAS). These results demonstrate common genetic control of PABA, PAS, and other polymorphic (e.g., SMZ, INH) NAT activities, and suggest a progression from polymorphic to monomorphic expression in the rabbit.

Each of these NAT activities, with decreasing degrees of difference between the activities in rapid versus slow acetylators (i.e., INH > PA > PAS > PABA) were found to copurify through two different purification procedures which exploit differences in solubility, size and charge. Purification procedure I involved sequential centrifugation, fractional precipitation with ammonium sulfate, and gel filtration on Sephadex G-100. Flatbed gel isoelectric focussing was the final step in this sequence to attempt a separation of PABA and SMZ NAT activities. Although both activities comigrated to pH 5.3, the acidic condition inactivated a large fraction of the enzyme and made a firm conclusion regarding their separation difficult (FIGURE 9A). Consequently, isofocussing was carried out for a shorter period of time (1.5 hrs) to minimize losses of activity. This resulted in incomplete focussing to a pH of approximately 5.9 (FIGURE 9B) , but demonstrated that the two activities comigrated as both activities were recovered quantitatively.

Polymorphic and monomorphic NAT activities also copurified through purification procedure II, involving sequential centrifugation, fractional precipitation with ammonium sulfate, ion exchange chromatography on DEAE-cellulose, gel filtration on Sephacryl S-200, and polyacrylamide disc gel electrophoresis. The PABA and SMZ NAT activities copurified to electrophoretic homogeneity. After Sephacryl S-200 chromatography, analytical polyacrylamide disc gel electrophoresis of the combined, concentrated, peak activity fractions revealed two major protein bands (A and B; FIGURE 11). PABA and SMZ NAT activities were both associated with the single, symmetrical protein peak B; no NAT activity was associated with

protein peak A. The molecular weight of protein peak B has been documented as 33,000 daltons by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis in a previous study (Glowinski et al., 1980).

Indistinguishable heat inactivation patterns of SMZ and PABA NAT activity also support the coexistence of these two activities on a common protein. Heat inactivation differences have frequently been used as a sensitive test for demonstrating small qualitative differences in protein structure, when other methods, such as extensive separation techniques have failed (Paigen, 1971). PABA and SMZ NAT activity heat inactivation patterns were indistinguishable in both Sephadex G-100 purified and the highly purified Sephacryl S-200 preparations. To obtain further evidence for the hypothesis that the two activities coexist on a common enzyme protein, the heat inactivation studies were conducted at two different temperatures. It is pertinent to note that heat inactivation studies of this same type have shown qualitative structural differences between rapid and slow acetylator NAT (see CHAPTER III A).

Recently, it has been shown that the NAT enzyme has an AcCoA-independent catalytic action in addition to this widely diverse set of AcCoA-dependent catalytic actions. Glowinski et al. (1980) have shown that the electrophoretically homogenous protein peak B has the capacity for intramolecular N-O transfer of acetyl groups, an enzyme activity identified as AHAT. Furthermore, that study and those of CHAPTER II have shown that differences in NAT and AHAT activity levels were under common genetic control in the rabbit. This is of particular interest as both NAT and AHAT enzymes are important metabolic steps in the bioactivation of carcinogenic arylamines to

reactive derivatives and both of these enzymatic activities show genetically determined differences based on acetylator phenotype. It has been suggested that the acetylator phenotype of an individual is important in susceptibility to arylamine-induced carcinogenesis (Lower et al., 1979). Currently, however, the precise toxicological implications of acetylator phenotype with respect to relative susceptibility of arylamine-induced carcinogenesis remains unknown.

Finally, our finding that PABA and SMZ NAT activities coexist on a common enzyme protein in rabbit liver may be of clinical importance. In human liver, PABA NAT activity is high in all individuals regardless of acetylator phenotype, and is less stable than liver polymorphic (SMZ) NAT activity after death (see CHAPTER IX). Consequently, we have recently reported that PABA NAT activity could serve as an enzyme marker to ensure reliable determinations of liver SMZ NAT activity and acetylator phenotype post-mortem (see CHAPTER IX). Although the coexistence of PABA and SMZ NAT activities on a common enzyme protein in human liver has yet to be confirmed, the findings of this investigation further suggest that PABA NAT activity may be particularly suitable as an enzyme marker in this regard.

C.

Relationship of Substrate Structure to Monomorphic and Polymorphic Expression of N-Acetyltransferase Activity

Introduction

The relationship of substrate structure to N-acetylation rate by NAT (E.C. 2.3.1.5) has been addressed by several previous studies. Lipmann (1945) and Riddle and Jencks (1971) first studied the acetylation of a limited number of substrates by pigeon liver NAT. Similarly, other investigators have studied the acetylation of different substrates by human liver NAT (Steinberg, 1970), rat liver NAT (Weber et al., 1968; Tannen and Weber, 1979; Yu and Boulton, 1979; Gollamudi et al., 1980), rat brain NAT (Yang and Neff, 1976), rat blood NAT (Tannen and Weber, 1979; Gollamudi et al., 1980), deer mouse liver NAT (Tannen and Weber, 1979), monkey liver NAT (Weber et al., 1968), and rabbit blood NAT (Drummond et al., 1980). Although a few studies have also been carried out with rabbit liver NAT, Weber et al. (1968) and Cohen and Weber (1969) examined a few substrates in rapid acetylators only, and Glowinski et al. (1978) examined only PABA, SMZ, and a few carcinogenic arylamines. Thus, a complete and systematic study of the relationship of substrate structure to rate of catalysis by rapid and slow acetylator NAT has yet to be reported.

The examination of this relationship has acquired increased

significance because of recent findings that monomorphic substrates such as PABA and polymorphic substrates such as SMZ are acetylated by the same liver NAT protein (see CHAPTER III B). These studies suggest that the structure of the substrate is critical in determining whether the NAT enzyme catalyzes it in a monomorphic or polymorphic manner. Thus, a complete and systematic examination of the relationship between substrate structure and the magnitude of polymorphic expression may result in sufficient understanding of this relationship to permit a priori predictions of this type with respect to novel arylamine and hydrazine drugs and carcinogens, based entirely on their chemical structure. This ability would be of great importance in the design of new therapeutic agents, especially those which might show increased susceptibility to drug-induced toxicity related to acetylator status.

Methods

Chemicals and reagents:

(see TABLE 33)

Animals:

Two male inbred rabbits were provided by The Jackson Laboratory, Bar Harbor, ME. The homozygous rapid acetylator (RR) was a IIIC/J (inbreeding coefficient of 0.96) and the homozygous slow acetylator (rr) was a IIIVO/vptJ (inbreeding coefficient of 0.97). A full description of the origins and characteristics of these lines has previously been published (Altman and Katz, 1979).

Liver NAT purification:

Rabbits were sacrificed by carbon dioxide intoxication. The liver was rapidly removed, minced, and homogenized (25% w/v) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT and EDTA. The homogenate was centrifuged at 10,000 g for 20 min followed by 105,000 g for 60 min. The cytosolic supernatant was stored at -20°C until the purification as described below was initiated. The unfrozen cytosol was dialyzed overnight against a 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM DTT and EDTA, and applied to a DEAE-cellulose (medium mesh) column (2.5 x 10 cm) which had been thoroughly washed with the dialysis buffer. The column was eluted with a linear 24 hour gradient of 20 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA to 20 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, 0.5 M KCl. Five ml fractions were collected and assayed for NAT activity. Peak fractions were combined and concentrated in a Millipore ultrafiltration apparatus. The concentrate (3 ml) was applied to a Sephacryl S-200 column (2.5 x 60

cm) and eluted with 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. Five ml fractions were collected and NAT activity determinations were carried out. Fractions containing highest levels of NAT activity were again combined and concentrated in a Millipore ultrafiltration apparatus. All purification steps were conducted at 4°C.

NAT activity determinations:

All arylamine NAT activity determinations were carried out with a modified DMAB assay and all hydrazine NAT activity determinations were carried out with a modified TNBS assay. Concentrations of reagents used in each of these assays were identical for all the substrates of this study. Thus, the PABA, SMZ, and INH NAT activity determinations were done at conditions different from those commonly published. The specifics of these assays are described below.

DMAB assay of NAT activity:

The modified DMAB assay was used for NAT activity determinations of 4-aminoantipyrine, and all arylamines of this study as listed in TABLE 10. In each assay, a 1 mM solution of arylamine was made up in water, or dimethylsulfoxide for 2AF, 2-aminoanthracene, and 6-aminochrysene. In all other respects, this assay is identical to the DMAB assay described in CHAPTER II, except that 0.5 ml of DMAB is added at the final step instead of 1 ml. Thus, reactions were carried out at 37°C and final concentrations in the assay mixture were always 0.22 mM for arylamine substrate and 2.22 mM for AcCoA. A standard curve was constructed for each arylamine substrate under the conditions of this assay, which generated an assay constant of amount substrate acetylated/decrease in absorbance units at 460 nm. The assay

TABLE 10. Substrate constants determined for DMAB assay of NAT activity.

SUBSTRATE	FACTOR*
aniline	.041
p-aminoacetophenone	.015
p-phenetidine	.024
p-anisidine	.023
p-isopropylaniline	.029
anthranilic acid	.038
p-aminophenylethylalcohol	.026
p-aminobenzoic acid	.015
p-aminophenol	.025
p-chloroaniline	.021
p-aminobenzoylhydrazide (amine)	.013
procainamide	.014
beta-napthylamine	.017
2-aminofluorene	.016
6-aminochrysene	.051
2-aminoanthracene	.017
sulfadiazine	.016
sulfamethazine	.015
sulfapyridine	.016
4-aminoantipyrine	.025

* nmoles substrate acetylated / decrease of one sod absorbance unit.

constants generated from these standard curves are listed in TABLE 10 for each arylamine substrate. NAT assays for each substrate were shown to be enzyme- and AcCoA-dependent, and multiple readings and time points were carried out only at protein concentrations which gave linear plots of NAT activity with time.

TNBS assay of NAT activity:

A modified TNBS assay of NAT activity was conducted for all hydrazines in this study as listed in TABLE 11. In each assay, a 1 mM solution of hydrazine substrate was made up in water. In all other respects this assay is identical to the TNBS assay of NAT activity described in CHAPTER VIII, except that color development was achieved with the additions of 300 ul of potassium phosphate dibasic solution and 50 ul of TNBS solution, rather than 1 ml and 100 ul, respectively. Thus, reactions were carried out at 37°C and final concentrations in the assay were always 0.22 mM for hydrazine substrate and 2.22 mM for AcCoA. A standard curve for each hydrazine substrate was constructed under the conditions of this assay, and assay constants as described above were generated (TABLE 11). All assays were conducted as described above for arylamines.

Protein determinations:

Protein was determined in the Sephacryl S-200 fractions by the method of Lowry et al. (1951).

TABLE 11. Substrate constants determined for TNBS assay of NAT activity.

SUBSTRATE	FACTOR*
isoniazid	.024
phenelzine	.028
monoacetylhydrazine	.035
benzoylhydrazine	.031
nicotinic acid hydrazide	.026

*nmoles substrate acetylated / decrease of one sod absorbance unit.

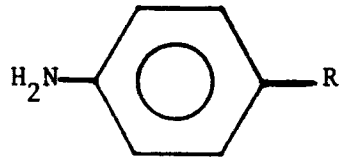
Results and Discussion

NAT activity determinations of aniline and derivatives:

NAT activity was determined for aniline and several of its derivatives in both the rapid and slow acetylator preparations. As shown in TABLE 12, rapid acetylator NAT activities ranged from 7909 nmoles/min/mg for p-isopropylaniline to 184 nmoles/min/mg for PA. Arylamines with carbonyl substituents para to the amine group (i.e., PA, p-aminobenzoyl hydrazide, p-aminoacetophenone, and PABA) produced the lowest rapid acetylator NAT activities. Substrates with relatively nonpolar aliphatic substituents para to the amine group (i.e., p-isopropylaniline, p-phenetidine, p-anisidine, and p-aminophenylethylalcohol) produced the highest rapid acetylator NAT activities, in direct concordance with delocalization of electron density of the substituent group para to the amine (i.e.,p-isopropyl-aniline > p-phenetidine > p-anisidine > p-aminophenylethyl-alcohol). As also shown in TABLE 12, slow acetylator NAT activities ranged from 300 nmoles/min/mg for PABA to 1.12 nmoles/min/mg for PA. As was the case for rapid acetylator NAT, arylamines with carbonyl substituent groups para to the amine group (PA, p-aminoacetophenone, and p-aminobenzoyl hydrazide) produced the lowest slow acetylator NAT activities, with the striking exception of PABA, which actually produced the highest. Except for PABA, the highest slow acetylator NAT activities were again produced by arylamines with relatively nonpolar substituents para to the amine in direct concordance with delocalization of electron density of the para substituent group (i.e., p-isopropylaniline > p-phenetidine > p-anisidine).

The ratio of rapid acetylator NAT activity to slow acetylator

TABLE 12. Rapid and slow acetylator inbred rabbit liver NAT activities for aniline and derivatives.



SUBSTRATE	R SUBSTITUENT	GENOTYPE RR	GENOTYPE rr	RR/rr
p-aminobenzoic acid	--COOH	1314*	300	4
p-phenetidine	$--OCH_2CH_3$	5838	90.5	65
p-anisidine	$--OCH_3$	4973	71.8	69
p-isopropyl-aniline	$--CH(CH_3)_2$	7909	104	76
p-aminophenol	--OH	3919	50.4	78
p-aminobenzoyl hydrazide	$--CONHNH_2$	604	6.28	96
aniline	--H	1465	11.6	126
procainamide	$--CONHCH_2CH_2N(C_2H_5)_2$	184	1.12	164
p-aminoaceto-phenone	$--COCH_3$	1176	4.50	261
p-chloroaniline	--Cl	3122	11.2	279
p-aminophenyl-ethylalcohol	$--CH_2CH_2OH$	3865	9.53	406

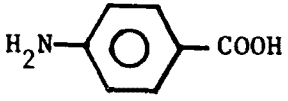
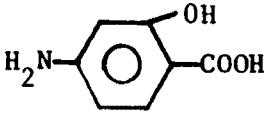
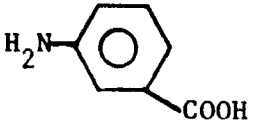
* Table values are liver NAT activities in nmoles/min/mg for Sephacryl S-200 purified enzyme in a homozygous rapid acetylator (RR) and a homozygous slow acetylator (rr) inbred rabbit.

NAT activity did not fit the above patterns, however. The highest ratio (most polymorphic) was found with p-aminophenylethylalcohol (406-fold), while the lowest ratio was found with PABA (4-fold). Alteration of the para carboxylic acid substituent of PABA to a ketone, ether, alcohol, halide, alkane, or complete removal resulted in a pronounced increase in polymorphic expression. Thus, a high electron density para to the amine group appears to be critical for monomorphic expression, and any amount of electron delocalization at this substituent appears to revert the expression to polymorphic.

NAT activities of PABA and derivatives:

As shown in TABLE 13, rapid acetylator NAT activities were 1314, 890, and 19.9 nmoles/min/mg for PABA, PAS, and anthranilic acid (m-aminobenzoic acid), respectively. The rates of slow acetylator NAT activities were in the same order; 300, 115, and 0.081 nmoles/min/mg for PABA, PAS, and anthranilic acid, respectively. In both phenotypes, NAT activity decreased in concordance with steric hindrance of the amine group. Thus, PABA has no functional group meta to the amine group, PAS has a hydroxyl substituent, and anthranilic acid has a carboxyl substituent. With respect to polymorphic expression of NAT activity, PAS showed a more polymorphic expression that PABA (8-fold), but only slightly so, whereas anthranilic acid showed a much larger difference. It is interesting to note that PABA and PAS are both relatively monomorphic both because their rates of catalysis by rapid acetylator NAT are lower than those for similar arylamines, and because their rates of catalysis by slow acetylator NAT are higher than those of similar arylamines. Thus, the substrate chemical structure of PABA and PAS produces marked, but

TABLE 13. Rapid and slow acetylator inbred rabbit liver NAT activities for PABA and derivatives.

SUBSTRATE	STRUCTURE	ACETYLATOR GENOTYPE RR	rr	RR/rr
p-Aminobenzoic acid		1314*	300	4
p-Aminosalicylic acid		890	115	8
Anthranilic acid		19.9	0.081	246

* Table values are liver NAT activities in nmoles/min/mg of Sephacryl S-200 purified enzyme in a homozygous rapid acetylator (RR) and a homozygous slow acetylator (rr) inbred rabbit.

directly opposite changes in rates of catalysis by rapid and slow acetylator NAT, resulting in a more monomorphic expression. This is further evidence for a qualitative structural difference between rapid and slow acetylator NAT as described in CHAPTER III A.

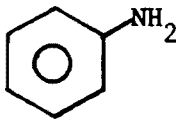
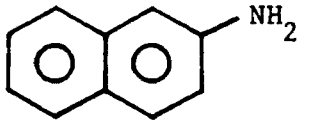
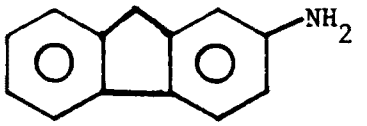
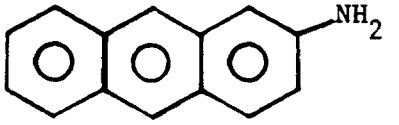
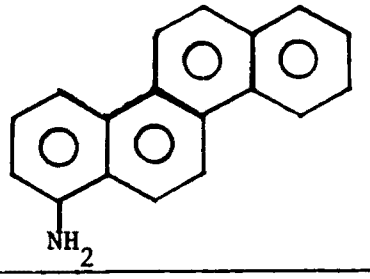
NAT activity determinations of aniline and multicyclic arylamines:

As shown in TABLE 14, rapid acetylator NAT activities ranged from 5373 nmoles/min/mg for BNA to 23.9 nmoles/min/mg for 6-amino-chrysene. Similarly, slow acetylator NAT activities followed an identical progression, ranging from 29.8 nmoles/min/mg for BNA to 0.134 nmoles/min/mg for 6-aminochrysene. Thus, both rapid and slow acetylator NAT catalyzed these compounds with respect to arylamine nucleus in the following order: (napthylamine > fluorene > anthracene> benzene >chrysene). The size of the arylamine nucleus did not have a major effect, however, on the magnitude of polymorphic expression, as the ratio of activity differences between rapid and slow acetylator NAT varied from 126-to 298-fold.

NAT activity determinations for sulfonamides:

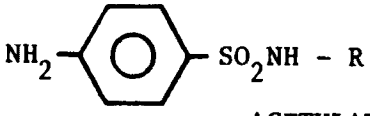
As shown in TABLE 15, rapid acetylator NAT activities were 1135, 190.3, and 132.6 nmoles/min/mg for SMZ, SDZ, and sulfapyridine, respectively. Similarly, the progression of slow acetylator NAT activities was identical; 5.50, 2,67, and 0.80 nmoles/min/mg for each of these substrates, respectively. The rate of SMZ acetylation was strikingly higher than SDZ and sulfapyridine for rapid acetylator NAT, but not much higher for slow acetylator NAT. Thus, the addition of two methyl substituents to the pyrimidine nucleus results in a pronounced difference in rapid acetylator NAT catalysis rate , but not in slow acetylator NAT catalysis rate. Consequently, the degree of

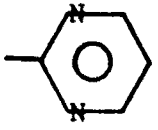
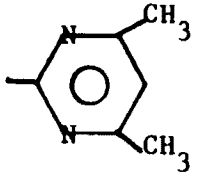
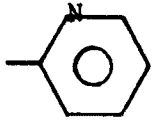
TABLE 14. Rapid and slow acetylator inbred rabbit liver NAT activities for aniline and multicyclic arylamines.

SUBSTRATE	STRUCTURE	ACETYLATOR GENOTYPE RR	rr	RR/rr
Aniline		1374*	4.60	298
BNA		5373	29.8	180
2AF		3243	16.8	193
2-aminoanthracene		1465	11.6	126
6-aminochrysene		23.9	0.134	178

* Table values are liver NAT activities in nmoles/min/mg of Sephacryl S-200 purified enzyme in a homozygous rapid acetylator (RR) and a homozygous slow acetylator (rr) inbred rabbit.

TABLE 15. Rapid and slow acetylator inbred rabbit liver NAT activities for sulfonamides.



SUBSTRATE	R SUBSTITUENT	ACETYLATOR GENOTYPE RR	rr	RR/rr
Sulfadiazine		132.6*	2.67	50
Sulfamethazine		1135	5.50	206
Sulfapyridine		190.3	0.80	238

* Table values are liver NAT activities in nmoles/min/mg of Sephacryl S-200 purified enzyme in a homozygous rapid acetylator (RR) and a homozygous slow acetylator (rr) inbred rabbit.

polymorphic expression was much higher for SMZ and sulfapyridine (206- and 238-fold) than for SDZ (50-fold).

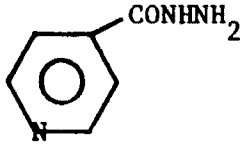
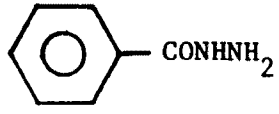
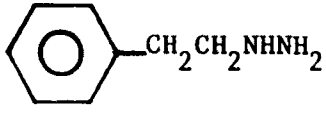
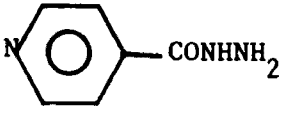
NAT activity determinations for hydrazines:

As shown in TABLE 16, rapid acetylator NAT activities for hydrazines ranged from 79.6 nmoles/min/mg for nicotinic acid hydrazide to 7.26 nmoles/min/mg for MAH. In general, the rapid acetylator NAT activities for the hydrazines were lower than found for arylamines, and the lowest NAT activity was found for the aliphatic hydrazine (MAH). It should be pointed out, however, that rabbit liver NAT has a much lower affinity for hydrazines than arylamines, and the concentrations of hydrazines used in the NAT activity determinations are below the apparent Km's reported for some hydrazines (see TABLE 31). Slow acetylator NAT activities ranged from 2.69 nmoles/min/mg for nicotinic acid hydrazine to non-detectable activity for benzoyl hydrazine. The degree of polymorphic expression for the aromatic hydrazines was approximately the same (30-54 fold), but was much higher for the aliphatic hydrazine (160-fold). We should point out, however, that when higher concentrations of INH (an aromatic hydrazine) are used in rapid and slow acetylator NAT activity determinations, the differences between the two phenotypes are commonly several hundred-fold (see TABLE 8). Thus, these results should be considered in light of the substrate concentrations used.

NAT activity determinations in non-arylamines and non-hydrazines:

NAT activity determinations were also conducted for some non-arylamine and non-hydrazine substrates. Rapid acetylator NAT activity towards 4-aminoantipyrine was 27.9 nmoles/min/mg while its activity was undetectable with slow acetylator NAT, indicating that

TABLE 16. Rapid and slow acetylator inbred rabbit liver NAT activities for hydrazines.

SUBSTRATE	STRUCTURE	ACETYLATOR GENOTYPE RR	ACETYLATOR GENOTYPE rr	RR/rr
Nicotinic acid hydrazide		79.6*	2.69	30
Benzoylhydrazine		67.0	non detectable	--
Phenelzine		27.2	0.91	30
Isoniazid		23.8	0.44	54
Monoacetylhydrazine	$CH_3CONHNH_2$	7.26	0.045	160

* Table values are liver NAT activities in nmoles/min/mg of Sephacryl S-200 purified enzyme in a homozygous rapid acetylator (RR) and a homozygous slow acetylator (rr) inbred rabbit.

it is polymorphically acetylated. No rapid or slow acetylator NAT activity was detectable for glycine. While NAT activity determinations for serotonin, histamine, and other biogenic amines were not carried out in this study, other investigators have shown that these biogenic amines are substrates for liver NAT in other species (Weissbach et al., 1960; Schloot et al., 1969; Steinberg et al., 1969; White et al., 1969; Yu and Boulton, 1979). Thus, the NAT enzyme appears to have a very broad substrate specificity, including not only arylamines and aromatic and aliphatic hydrazines, but also non-aromatic amines such as 4-aminoantipyrine and biogenic amines.

CHAPTER IV

DEVELOPMENT OF AN INBRED HAMSTER MODEL OF THE N-ACETYLATION POLYMORPHISM

Introduction

Hereditary factors are of great importance in the N-acetylation of many arylamine and hydrazine drugs and carcinogens. Original observations were made in humans by Hughes et al. (1955) who found toxicity after the administration of normal (non-toxic) doses of INH in some individuals who excreted relatively lower quantities of urinary acetyl-INH. Further studies demonstrated a polymorphic distribution of INH N-acetylation capacity within human populations (Biehl, 1957; Knight et al., 1959; Evans et al., 1960; Peters et al., 1965). In what subsequently formed the basis of the INH acetylation polymorphism, it was shown that the person-to-person differences in N-acetylation rates are genetically determined by simple autosomal Mendelian genetics. Rapid INH acetylators are either homozygous (RR) or heterozygous (Rr) for a codominant rapid acetylator gene (R), while slow acetylators are homozygous (rr) for the slow acetylator gene (Evans et al., 1960).

Differences in INH N-acetylation capacity in vivo are primarily due to in vitro differences in hepatic activity levels of the cytosolic enzyme NAT (E.C. 2.3.1.5.) (Weber, 1973). Several other arylamine substrates such as SMZ and PA also exhibit the genetically

determined polymorphism in N-acetylation rate, and are thus classified as polymorphic substrates (Weber, 1973; Drayer and Reidenberg, 1977). Other arylamine substrates, however, show a monomorphic distribution of N-acetylation activity rather than polymorphic. Examples of these substrates, which are classified as monomorphic, include PABA and PAS (Jenne, 1965; Glowinski et al., 1978; see CHAPTERS II, III, and IX).

Due, in part, to the pharmacokinetic (Weber and Hein, 1979) and toxicological (Drayer and Reidenberg, 1977; Lunde et al., 1977) significance of these genetically determined differences in N-acetylation capacity, various animal species have been investigated as possible models for studying the pharmacogenetics of arylamine N-acetylation. Early studies demonstrated that the rabbit expressed a qualitatively similar acetylation polymorphism to that in man. For example, rabbit populations are bimodally distributed as rapid and slow acetylators; the in vitro and in vivo liver N-acetylation capacity is genetically determined by simple autosomal Mendelian inheritance (Frymoyer and Jacox, 1963a,b). In addition, INH (Weber et al., 1976; see FIGURE 8) SMZ (Weber, et al., 1976; Glowinski et al., 1978; see TABLE 4) and PA (see TABLE 3) show large differences in acetylation rate between rapid and slow acetylators (polymorphic substrates). Conversely, PABA and PAS do not show these large differences in N-acetylation rate between rapid and slow acetylators, and are thus classified as monomorphic (Glowinski et al., 1978; see TABLE 3 and FIGURE 8). In further studies of rabbit liver NAT, we (see CHAPTER III B) have provided biochemical and genetic evidence for the coexistence of polymorphic (SMZ) and monomorphic (PABA) NAT activities

on a common protein.

Recently, studies involving inbred mice (Tannen and Weber, 1980a; Glowinski and Weber 1982a) and inbred rabbits (see CHAPTER II) have shown genetically determined differences between inbred strains in the N-acetylation of arylamine drugs and carcinogens in vitro. The findings of other investigations have suggested the hamster as another potentially interesting animal model for several reasons. King and Olive (1975) have previously shown that high levels of AHAT activity occur in hamster liver and small intestine, two tissues which show high levels of NAT activity in the rabbit (Hearse and Weber, 1973; see TABLES 3-5). This finding is pertinent since NAT and AHAT are under common genetic control and are properties of the same enzyme protein in rabbit liver (Glowinski et al., 1980; see CHAPTER II). In addition, hamster hepatocytes have shown a broad spectrum of carcinogen-metabolizing activity including arylamines (Poiley et al., 1979, 1980) and Nebert and Gelboin (1968) have previously shown the relative ease in success at culturing fetal hamster cells. Finally, a number of inbred hamster strains have already been established (Altman and Katz, 1979). Based on these observations, our purpose for this study was to investigate whether established Syrian inbred hamster strains also show genetically determined differences in the N-acetylation of arylamine drugs (PABA, PAS, SMZ, INH, and PA) and carcinogens (2AF). In addition, we also carried out simple genetic analyses to determine the mode of inheritance for these differences.

Methods

Chemical and reagents:

(see TABLE 33)

Animals:

Twenty-six strains of Syrian inbred hamsters (both males and females) were generously donated by the Bio Research Institute, Cambridge, MA. Each of the strains are fully inbred (greater than 20 generations) and a full description of them has previously been published (Altman and Katz, 1979). F1 and F2 animals were bred from existing stocks maintained in our animal care facilities at the University of Michigan. Hamsters of the same sex and inbred strain were caged in sets of 2 or 3 and maintained at room temperature in our animal care facilities. Purina mouse chows and water were available ad libitum. The ages of the slow acetylator hamsters at time of sacrifice for liver NAT were as follows: Bio 1.5; range 31 to 187 days, average of 162 days. Bio 82.73; range 88 to 112 days, average of 98 days. The ages of two of the rapid acetylator strains at time of sacrifice for liver NAT were as follows: Bio 4.24; range 135 to 212 days, average of 156 days. Bio 41.56; range 82 to 109 days, average of 90 days. The ages of all hamsters sacrificed for liver NAT in this study ranged from 31 to 496 days. The ages of all hamsters used for in vivo pharmacokinetic studies ranged from 81 to 407 days. The age of the animals (at least 1 month old), the time of day, and the selection sequence of animals for sacrifice were unsystematic. No age-related effects were observed.

N-acetyltransferase purification:

Hamsters were sacrificed by either decapitation or carbon dioxide

intoxification, after controls showed that the two methods had no effect on liver NAT. The livers were rapidly removed, minced, and homogenized (25% w/v) in 0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM DTT in a motor driven Kontes-Dual ground glass homogenizer. The homogenate was partially purified by centrifugation at 10,000 g for 20 minutes, followed by 105,000 g for 60 minutes. The supernatant fraction was assayed for NAT activity as described below.

PABA and SMZ NAT activity determinations:

PABA and SMZ NAT activities were determined at 37°C by a micromodification of the Bratton-Marshall procedure as previously described in CHAPTER II.

INH NAT activity determinations:

INH NAT activity was determined at 37°C as described in CHAPTER II.

2AF, PA, and PAS NAT activity determinations:

Assays were carried out at 37°C using the DMAB assay for NAT activity as described in CHAPTER II.

Protein determinations:

Total protein was determined by a microbiuret method (Gornall et al., 1949).

Urinary excretion of PABA or SMZ and N-acetyl metabolite:

Hamsters were placed in metabolic cages (Econo-Metabolism units, Model E-1100, Maryland Plastics, New York, NY) for a 12 hour period during which they were food-deprived but received water *ad libitum*. After 12 hours, the urine was collected (control urine) and the hamsters were injected i.p. with 10 mg/kg PABA in sterile water (2

ml/kg). The hamsters were immediately returned to the metabolic cage for an additional 12 hours under identical conditions, during which the post-drug urine was collected. The total amount of free drug and N-acetyl metabolite was determined by a modification of the Bratton-Marshall procedure as previously described (Evans, 1969). An identical procedure was utilized for investigations of the urinary excretion of SMZ and acetyl-SMZ.

Data analysis:

NAT activity determinations were conducted and analyzed as described in CHAPTER II. Data from male and female hamsters within the same strain were subjected to a one-way analysis of variance. Inheritance patterns of liver NAT activity were analyzed by the chi square test.

Results

Polymorphic expression of PABA NAT activity:

Determinations of liver PABA NAT specific activity among the 26 inbred strains revealed large differences in PABA NAT activity in vitro (FIGURE 14). For 24 of the inbred hamster strains, the PABA NAT activity was quite high, ranging from 55.5 nmoles/min/mg in Bio 86.93 to 36.2 nmoles/min/mg in Bio 14.6. In contrast, liver PABA NAT activity levels were over 400-fold lower in two other inbred strains (0.136 nmoles/min/mg in Bio 1.5 and 0.176 nmoles/min/mg in Bio. 82.73). Mixing experiments, in which rapid and slow acetylator hamster liver NAT were combined, yielded PABA NAT activity values precisely between those of the rapid and slow acetylator PABA NAT, indicating that no activator or inhibitor was present.

The polymorphic expression of PABA NAT activity in vivo was also demonstrated by measuring the capacity of two rapid and the two slow acetylator strains to acetylate PABA. The average percentage of the acetyl-PABA to total drug excreted in a 12-hour collection urine in the rapid acetylators was 88.0% which was significantly higher $p < .001$) than in the two slow acetylator strains (61.8%) as shown in TABLE 17.

Polymorphic expression of PAS NAT activity:

Determinations of liver PAS NAT specific activity among six inbred strains revealed large differences in PAS NAT activity in vitro (FIGURE 15). In four rapid acetylator strains, the PAS NAT activity levels ranged from 67.5 nmoles/min/mg in Bio IPI to 55.5 nmols/min/mg in Bio 4.24. The PAS NAT activity levels were about 20-fold lower in the two slow acetylator strains (3.27 nmoles/min/mg

FIGURE 14. Liver PABA NAT activities in inbred hamsters.

PABA NAT specific activity in nmoles per minute per milligram is plotted on the ordinate for each of the various 26 Bio inbred hamster strains individually identified by strain number on the abscissa. Data from an approximately equal number of males and females were pooled for each strain (indicated by the wide bar graphs) because no significant sex differences ($p > .05$) were found within any strain for this substrate activity. The number above the bar indicates the total number of animals from which the mean was determined. The line above each bar represents the standard error. The two slow acetylator strains, Bio. 1.5 and Bio. 82.73 are listed to the far left of this figure and figures 15-19.

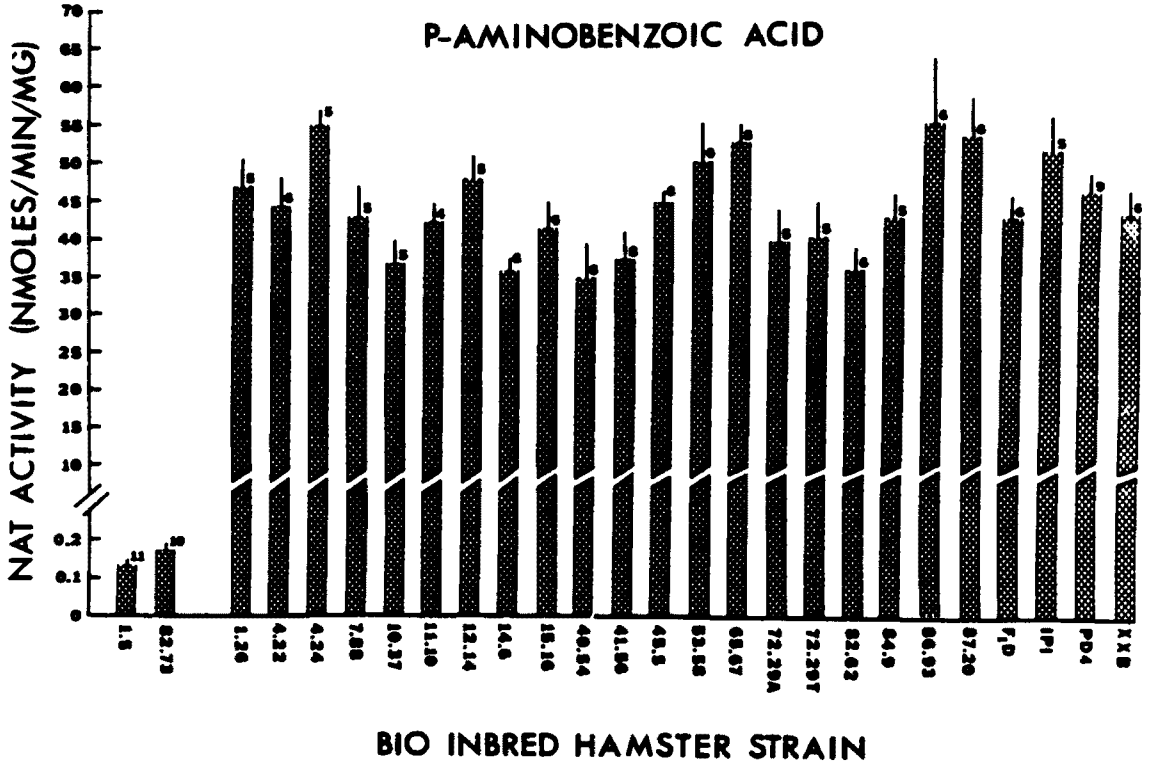

P-AMINOBENZOIC ACID
NAT ACTIVITY (NMOLES/MIN/MG)
BIO INBRED HAMSTER STRAIN

FIGURE 14

TABLE 17. Excretion of PABA or SMZ and N-acetyl metabolite in urine of rapid and slow acetylator inbred hamsters.

Bio. Inbred Strain	N	Phenotype	Percentage of acetyl-PABA to total drug in 12 hr urine	Percentage of acetyl-SMZ to total drug in 12 hr urine
1.5	5	Slow	59.9 ± 2.5	64.2 ± 2.7
82.73	5	Slow	63.6 ± 2.7	72.9 ± 4.1
4.24	5	Rapid	83.7 ± 1.1	64.2 ± 3.9
41.56	5	Rapid	92.3 ± 1.6	66.8 ± 2.6

Table values represent mean ± S.E.
Rapid acetylator hamsters excreted significantly higher percentages of acetyl-PABA ($p < .001$) than slow acetylator hamsters. The percentage of acetyl-SMZ excreted did not differ significantly ($p > .05$) between rapid and slow acetylator hamsters.

FIGURE 15. Liver PAS NAT activities in inbred hamsters.

PAS NAT specific activity on the ordinate is plotted for each of the various Bio inbred hamster strains listed on the abscissa. For further details, see the legend of FIGURE 14. *Standard errors were 0.15 for Bio 1.5 and 0.14 for Bio. 82.73.

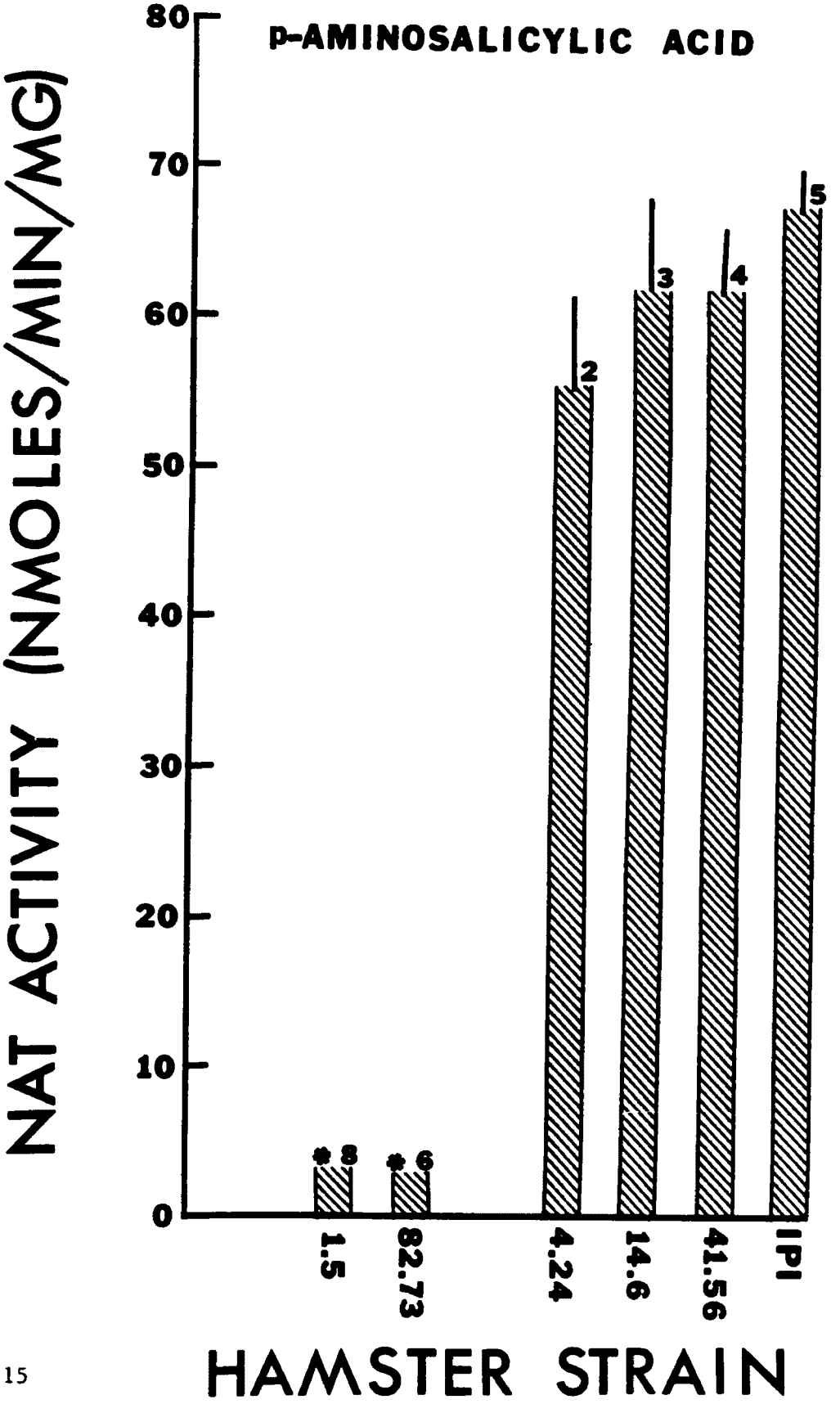

p-AMINOSALICYLIC ACID
NAT ACTIVITY (NMOLES/MIN/MG)
80
70
60
50
40
30
20
10
0
8
6
2
3
4
5
1.5
82.73
4.24
14.6
41.56
IPI
HAMSTER STRAIN

FIGURE 15

in Bio 1.5 and 3.01 nmoles/min/mg in Bio 82.73).

Monomorphic expression of INH NAT activity:

Determinations of liver INH NAT activity in vitro among the 26 inbred strains revealed a monomorphic expression of activity (FIGURE 16). Significant sex differences were observed in six of the inbred strains (Bio 82.73, 1.26, 14.6, 45.5, 53.58, and PD4). In each of these inbred strains, the males had significantly higher levels of INH NAT activity than females. Across strains, the pooled male and female data did not reveal large in vitro differences in INH NAT activity. The slow acetylator strains, Bio 1.5 and 82.73 had liver INH NAT activity levels of 34.8 and 31.9 nmoles/min/mg, respectively, values which are within the range of the rapid acetylator strains. The maximum liver INH NAT activity was 36.4 nmoles/min/mg in Bio 65.67, while the minimum was 19.7 nmoles/min/mg in Bio 14.6, yielding a maximum inter-strain difference of 1.8-fold.

Monomorphic expression of SMZ NAT activity:

Determinations of liver SMZ NAT activity among the 26 inbred strains revealed a monomorphic expression of activity in vitro (FIGURE 17). Significant sex differences were observed in 3 of the inbred strains (Bio 12.14, 65.76, and 87.20). In each of these inbred strains, the males had significantly higher levels of SMZ NAT activity than females. Across strains, the pooled male and female data did not reveal large in vitro differences in liver SMZ NAT activity. The two slow acetylator strains, Bio 1.5 and 82.73, had liver SMZ NAT activity levels of 0.076 and 0.110 nmoles/min/mg, respectively. The highest SMZ NAT activity level was found in Bio 82.73 (0.110 nmoles/min/mg) whereas the lowest was 0.049 nmoles/min/mg in Bio 14.6, yielding an

FIGURE 16. Liver INH NAT activities in inbred hamsters.

INH NAT specific activity on the ordinate is plotted for each of the various Bio inbred hamster strains listed on the abscissa. Wide bar graphs indicate data from which males and females were pooled. Significant sex differences were observed in six inbred strains. In these instances, the value for males is represented on the left by the solid black bar, and the value for females is represented on the right by the open bar. In each of the six inbred strains, males had significantly higher levels of INH NAT activity than females ($p < .05$) for Bio strains 14.6 and 45.5; ($p < .025$) for Bio strains 1.26 and 53.58, ($p < .01$) for Bio strain PD4; and ($p < .001$) for Bio strain 82.73. For further details, see the legend of FIGURE 14.

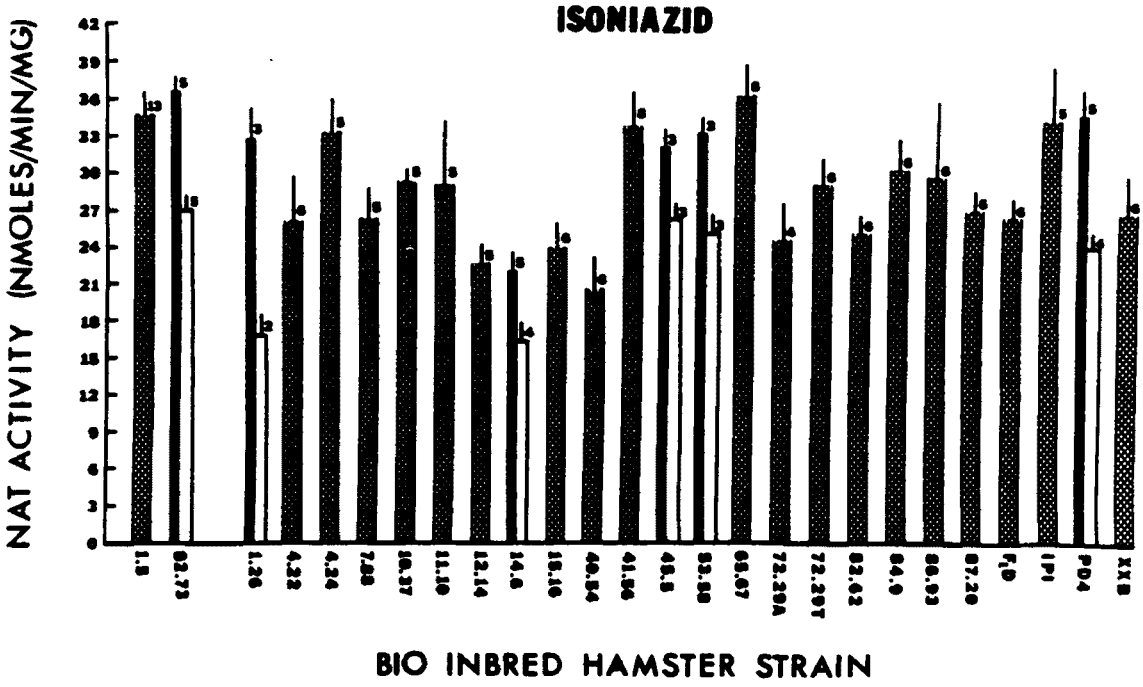

ISONIAZID
NAT ACTIVITY (NMOLES/MIN/MG)
BIO INBRED HAMSTER STRAIN

FIGURE 16

FIGURE 17. Liver SMZ NAT activities in inbred hamsters.

SMZ NAT specific activity on the ordinate is plotted for each of the various Bio inbred hamster strains listed on the abscissa. Wide bar graphs indicate data from which males and females were pooled. Significant sex differences were observed in three of the inbred strains. In these instances, the value for males is represented on the left by the solid black bar, and the value for females is represented on the right by the open bar. In each of the three inbred strains, males had significantly higher levels of SMZ NAT activity than females ($p < .01$) for Bio strain 65.67; ($p < .025$) for Bio strains 12.14 and 87.20. For further details, see the legend of FIGURE 14.

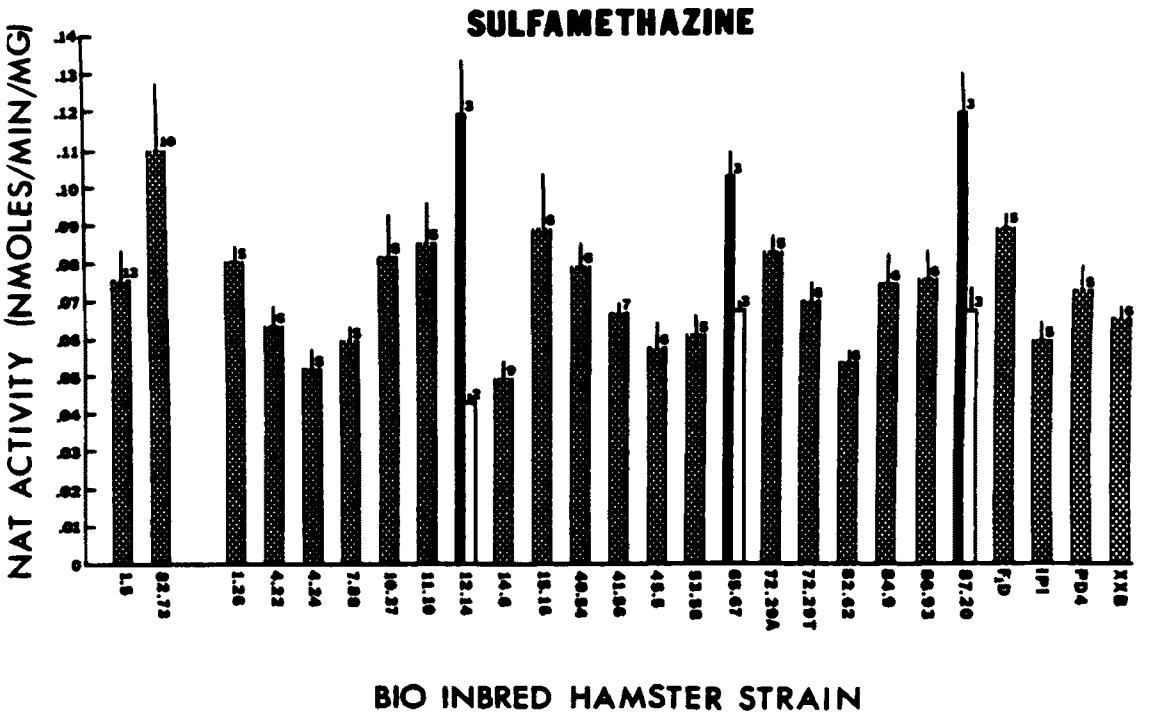

SULFAMETHAZINE
NAT ACTIVITY (NMOLES/MIN/MG)
BIO INBRED HAMSTER STRAIN

FIGURE 17

inter-strain difference of 2.2-fold.

The monomorphic expression of SMZ NAT activity was also demonstrated in vivo, by evaluating the capacity of two rapid and the two slow acetylator strains to acetylate an i.p. injection of 10 mg/kg SMZ. The percentage of acetyl-SMZ to total drug excreted in a 12 hour urine collection was 64.2 and 72.9% in the slow acetylator strains (Bio 1.5 and 82.73), whereas it was 64.2 and 66.8% in two rapid acetylator strains (Bio 4.24 and 41.56) (TABLE 17). Thus, there was no significant difference between the two phenotypes showing that SMZ behaved as a monomorphic substrate in vivo as well as in vitro.

Monomorphic expression of 2AF NAT activity:

Determinations of liver 2AF NAT specific activity among the 26 inbred strains revealed a monomorphic expression of activity in vitro (FIGURE 18). The two slow acetylator strains, Bio 1.5 and 82.73, had liver 2AF NAT activity levels of 11.9 and 10.3 nmoles/min/mg, respectively. The highest 2AF NAT activity level of all strains was 23.5 nmoles/min/mg in Bio 72.29A. The lowest 2AF NAT activity level was 10.3 nmoles/min/mg in Bio 82.73, resulting in an inter-strain difference in 2AF NAT in vitro activity of 2.3-fold.

Monomorphic expression of PA NAT activity:

Determinations of liver PA NAT specific activity among the 26 inbred hamster strains yielded a monomorphic expression of in vitro activity (FIGURE 19). The two slow acetylator strains, Bio 1.5 and 82.73, had liver PA NAT activity levels of 0.941 and 0.929 nmoles/min/mg, respectively, which were within the range of the rapid acetylator strains. The highest PA NAT activity level was 0.961 nmoles/min/mg in Bio PD4 and the lowest PA NAT activity level was

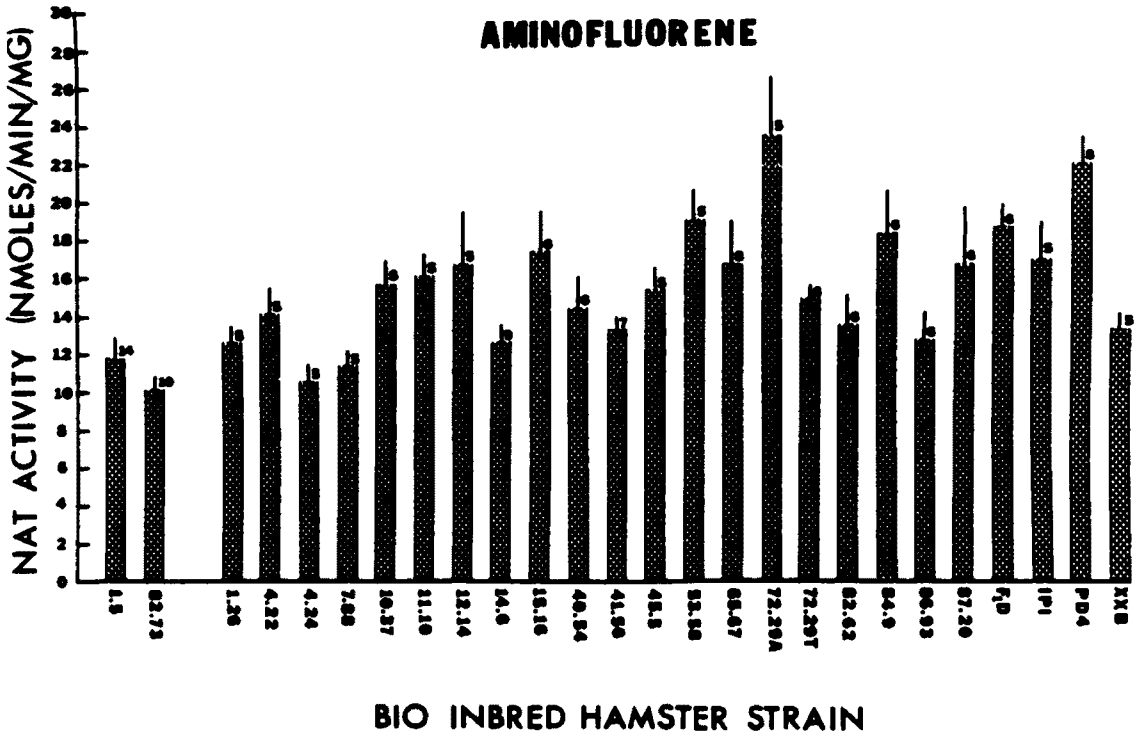


FIGURE 18. Liver 2AF NAT activities in inbred hamster.

2AF NAT activity on the ordinate is plotted for each of the various Bio inbred hamster strains listed on the abscissa. For further details, see the legend of FIGURE 14.

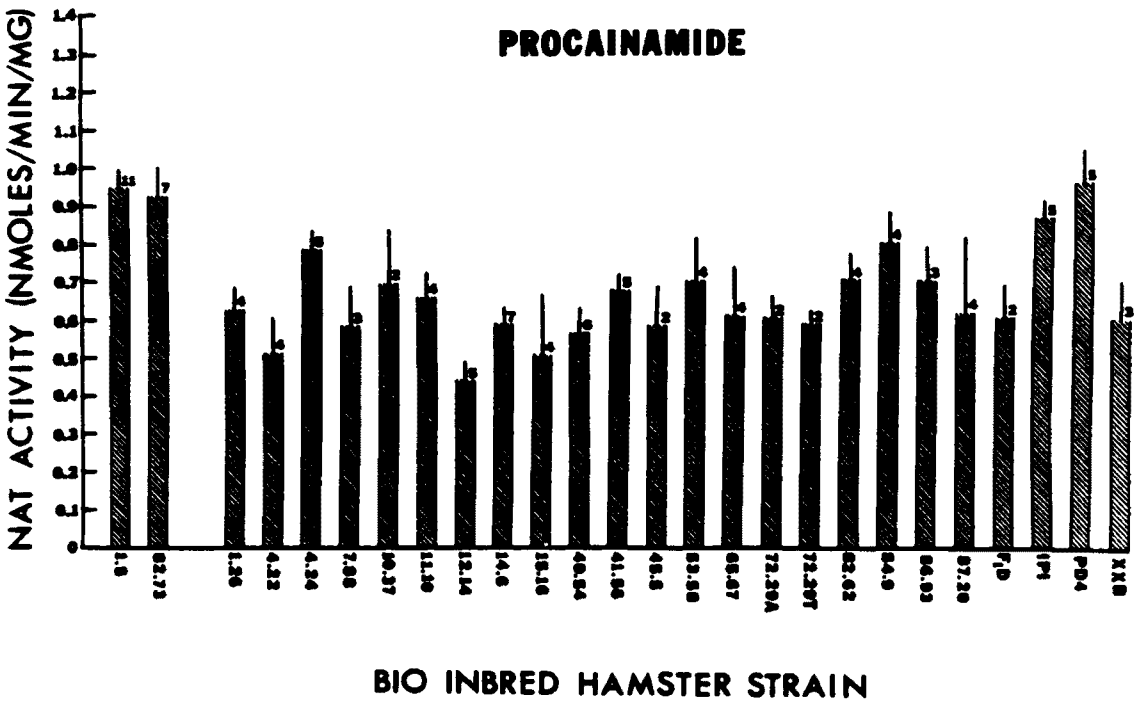


FIGURE 19. Liver PA NAT activities in inbred hamsters.

PA NAT specific activity on the ordinate is plotted for each of the various Bio inbred hamster strains listed on the abscissa. For further details, see the legend of FIGURE 14.

0.443 nmoles/min/mg in Bio 12.14, resulting in an inter-strain difference in PA NAT in vitro activity of 2.2-fold.

Inheritance patterns of liver PABA NAT activity:

Liver PABA NAT activity determinations were carried out in slow acetylator (Bio. 1.5) and rapid acetylator (Bio. 4.24, Bio. 41.56, and Bio. 65.67) parental strains and F1 and F2 progeny. F1's derived from crosses between Bio. 1.5 slow acetylators and Bio. 4.24, Bio. 41.56, and Bio. 65.67 rapid acetylators had intermediate PABA NAT activity levels averaging 26.5, 23.5, and 22.7 nmoles/min/mg, respectively (TABLE 18). F2's derived from crosses between Bio. 1.5 and Bio. 41.56 yielded a trimodal distribution of PABA NAT activity in a ratio (low/intermediate/high) of 7:22:13 (FIGURE 21) which is not significantly ($p > .05$) different from a 1:2:1 ratio ($X^2 = 1.81$). Interestingly, the first ten F2 progeny of this cross assayed yielded a ratio of 0:5:5, while the remaining 32 F2 progeny yielded a ratio of 7:17:8.

F2's derived from the cross between Bio. 1.5 and Bio. 65.67 also yielded a trimodal distribution of PABA NAT activity in the ratio of 4:9:4 (FIGURE 22), which is not significantly ($p > .05$) different from a 1:2:1 ratio ($X^2 = 0.06$).

Inheritance patterns of liver PAS NAT activity:

Liver PAS NAT activity determinations were carried out in slow acetylator (Bio 1.5) and rapid acetylator (Bio. 41.56 and Bio. 65.67) parental strains and F1 and F2 progeny. F1's derived from crosses between Bio. 1.5 and either Bio. 41.56 or Bio. 65.67 had intermediate PAS NAT activity levels averaging 38.2 and 45.5 nmoles/min/mg, respectively (TABLE 19). F2's derived from each of these crosses

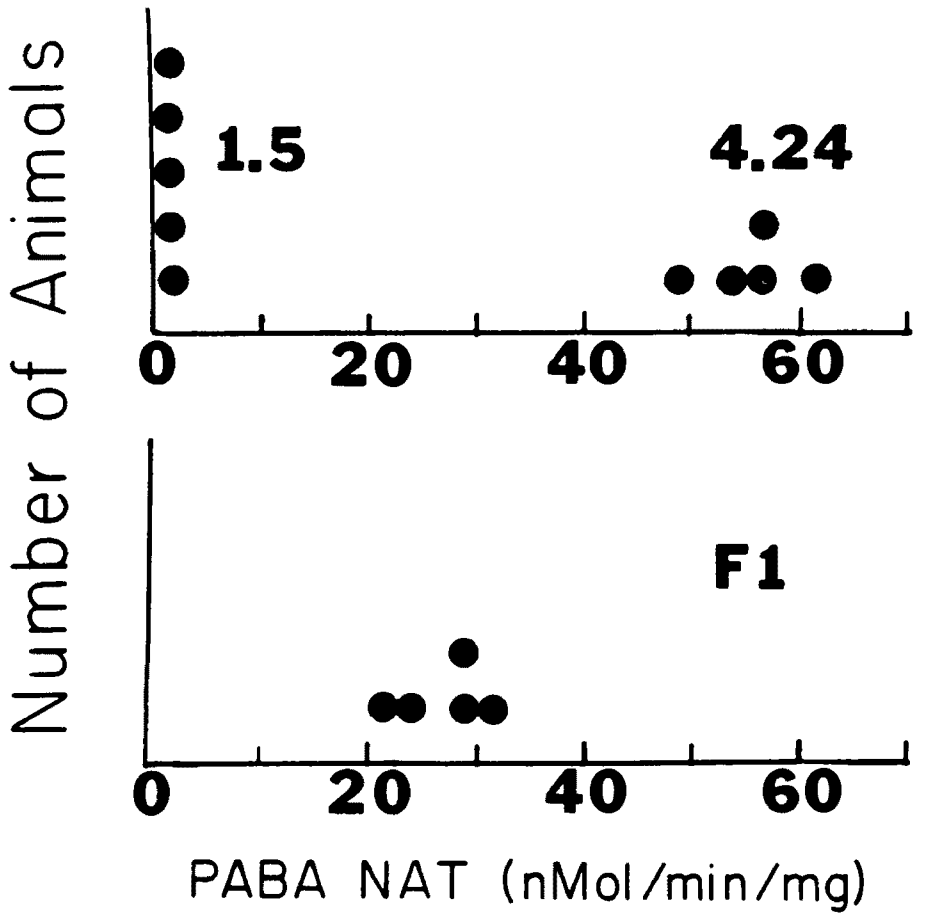


FIGURE 20. Inheritance patterns of liver PABA NAT activity in inbred hamsters Bio. 1.5 and Bio. 4.24.

Each dot represents one animal. The average PABA NAT activity and total number of animals within each mode are shown in TABLE 18.

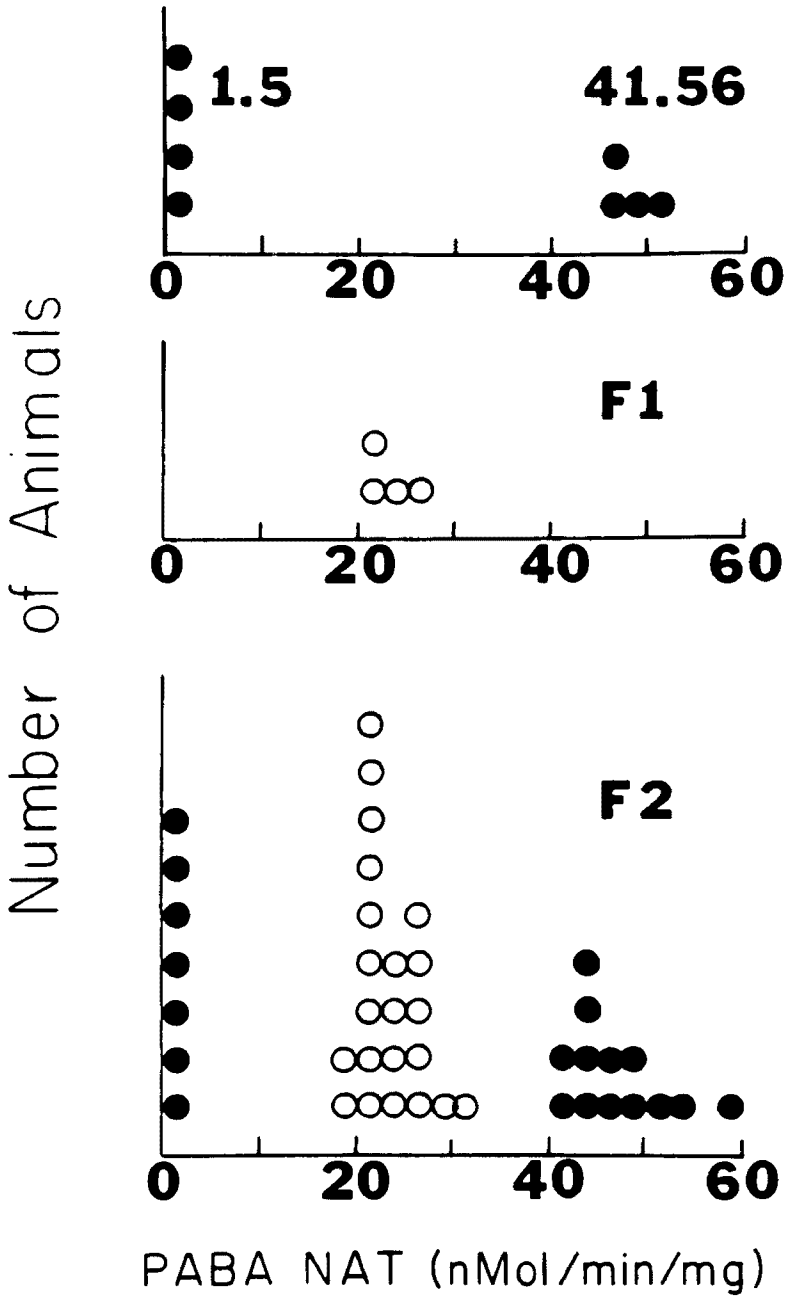


FIGURE 21. Inheritance patterns of liver PABA NAT activity in inbred hamsters Bio. 1.5 and Bio. 41.56

Each dot represents one animal. The average PABA NAT activity and total number of animals within each mode are shown in TABLE 18. Solid circles represent homozygous (RR) and (rr) acetylator genotypes. Open circles represent heterozygous (Rr) acetylator genotype.

FIGURE 22. Inheritance patterns of liver PABA NAT activity in inbred hamsters Bio. 1.5 and Bio. 65.67.

Each dot represents one animal. The average NAT activity and number of animals within each mode is shown in TABLE 18.
Solid circles represent homozygous rapid (RR) and slow (rr) acetylators. Open circles represent heterozygous rapid acetylator (Rr) animals.

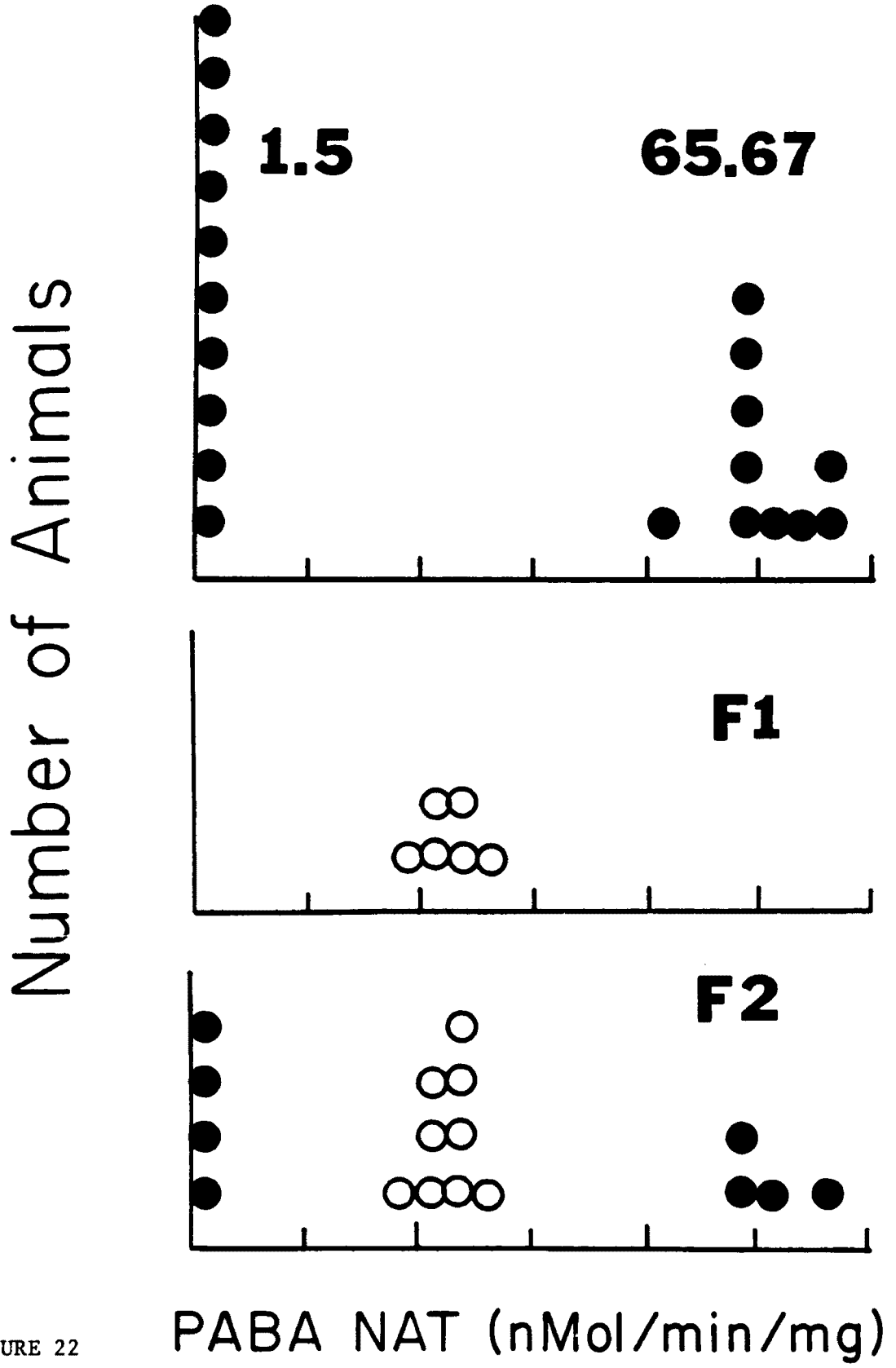


FIGURE 22

TABLE 18. Inheritance patterns of PABA NAT activity in inbred hamsters.

HAMSTER DESCRIPTION	N	AVERAGE NAT ACTIVITY (nmoles/min/mg)
1.5	10	0.144 ± .039*
65.67	10	50.5 ± 4.1
Arithmetic mean of 1.5 & 65.67		25.3
1.5 X 65.67 F1	6	22.7 ± 2.2
1.5 X 65.67 F2 (low)	4	0.218 ± .072
1.5 X 65.67 F2 (intermediate)	9	22.7 ± 2.3
1.5 X 65.67 F2 (high)	4	51.5 ± 4.0
1.5	4	0.181 ± .032
41.56	4	47.4 ± 2.5
Arithmetic mean of 1.5 & 41.56		23.8
1.5 X 41.56 F1	4	23.5 ± 3.3
1.5 X 41.56 F2 (low)	7	0.289 ± .101
1.5 X 41.56 F2 (intermediate)	22	23.6 ± 3.3
1.5 X 41.56 F2 (high)	13	47.3 ± 4.9
1.5	5	0.172 ± .039
4.24	5	55.1 ± 4.9
Arithmetic mean of 1.5 & 4.24		27.6
1.5 X 4.24 F1	5	26.5 ± 3.7

* Standard deviation
F1 values and arithmetic mean of parentals are not significantly different ($p > .05$).

yielded trimodal distributions of PAS NAT activity (FIGURES 23-24) in exactly the same ratios found for liver PABA NAT activity (see above). In other words, PABA and PAS NAT activity levels for each individual F2 animal always fell into the same (low, intermediate, or high) mode.

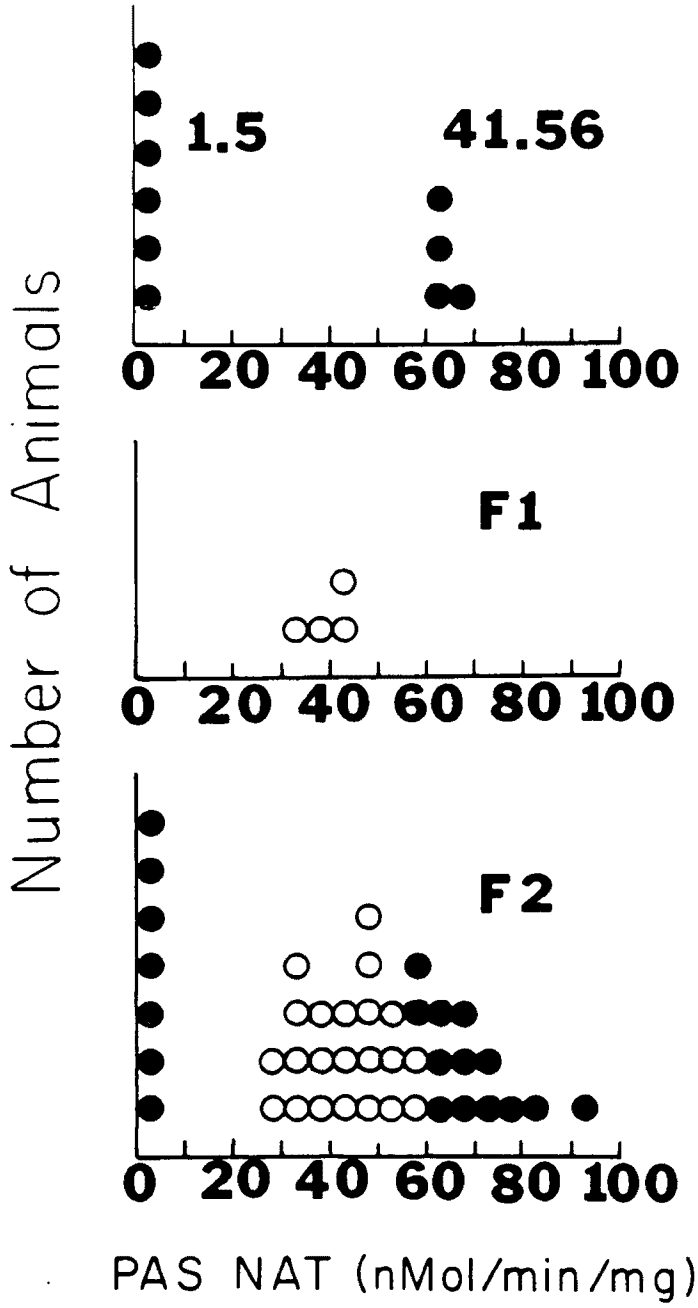


FIGURE 23. Inheritance patterns of liver PAS NAT activity in inbred hamsters Bio. 1.5 and Bio. 41.56

Each dot represents one animal. The average PAS NAT activity and total number of animals within each mode are shown in TABLE 19. Solid circles represent homozygous (RR) and (rr) acetylator genotypes. Open circles represent the heterozygous (Rr) rapid acetylator genotype. Animals were assigned as RR or Rr genotype based on PABA NAT activity, which yielded a clearer trimodal distribution (see FIGURE 21).

FIGURE 24. Inheritance patterns of liver PAS NAT activity in inbred hamsters Bio. 1.5 and Bio. 65.67.

Each dot represents one animal. The average NAT activity and number of animals within each mode is shown in TABLE 19.
Solid circles represent homozygous rapid (RR) and slow (rr) acetylators. Open circles represent heterozygous rapid acetylator (Rr) animals.

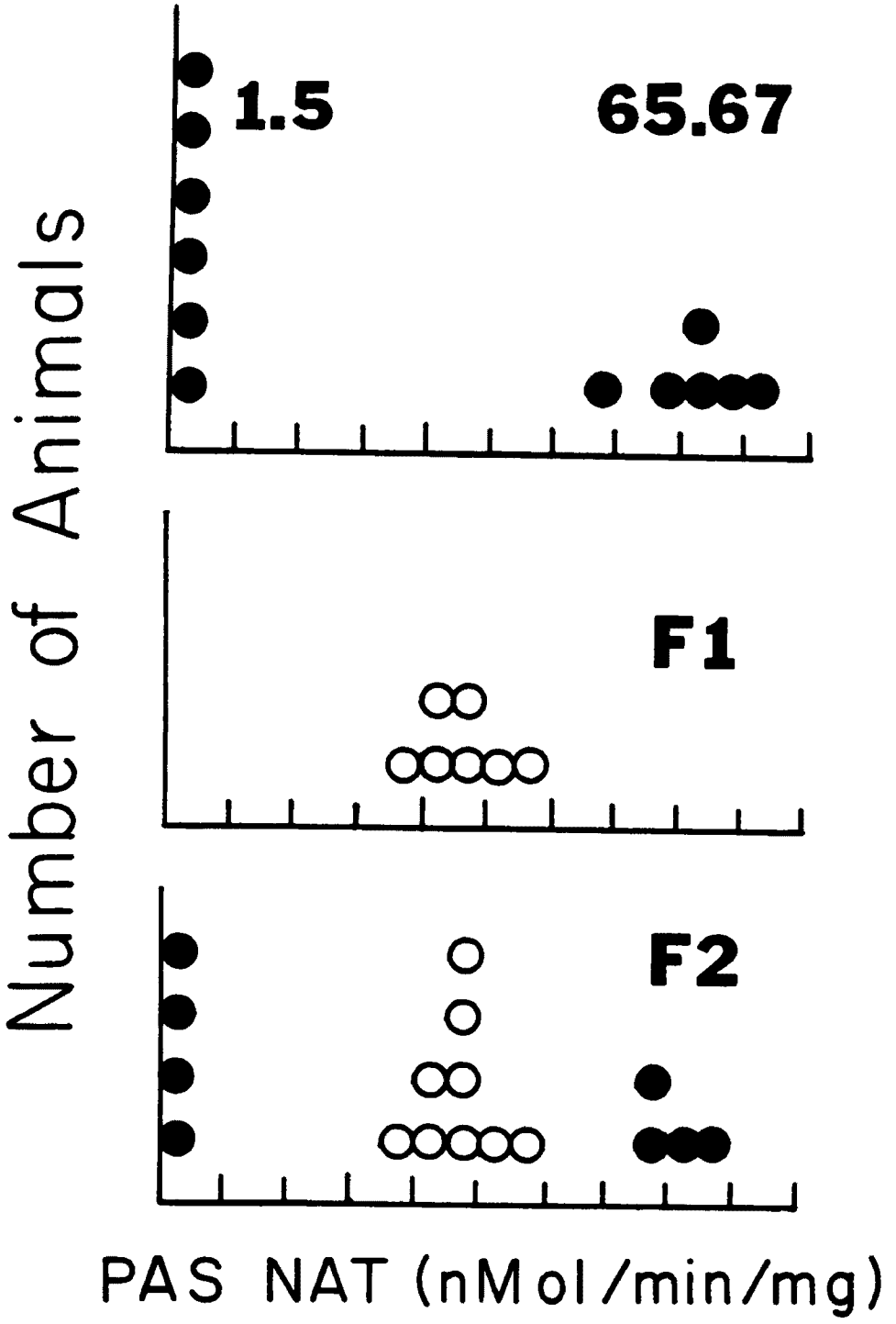


FIGURE 24

TABLE 19. Inheritance patterns of PAS NAT activity in inbred hamsters.

HAMSTER DESCRIPTION	N	AVERAGE NAT ACTIVITY (nmoles/min/mg)
1.5	6	3.40 ± .39*
65.67	6	81.7 ± 8.5
Arithmetic mean of 1.5 & 65.67		42.6
1.5 X 65.67 F1	6	45.5 ± 7.6
1.5 X 65.67 F2 (low)	4	3.61 ± .18
1.5 X 65.67 F2 (intermediate)	9	46.5 ± 6.5
1.5 X 65.67 F2 (high)	4	81.9 ± 5.9
1.5	6	3.40 ± .39
41.56	4	62.5 ± 3.6
Arithmetic mean of 1.5 & 41.56		33.0
1.5 X 41.56 F1	4	38.2 ± 3.9
1.5 X 41.56 F2 (low)	7	3.94 ± .73
1.5 X 41.56 F2 (intermediate)	22	42.9 ± 9.3
1.5 X 41.56 F2 (high)	13	69.7 ± 11.0

* Standard deviation
Values for F1 and arithmetic mean of parentals are not significantly different ($p > .05$).

Discussion

In man and rabbit, a widely diverse number of arylamine drugs and carcinogens are N-acetylated by liver NAT. Examples include arylamine and hydrazine drugs such as INH, SMZ, PA, PAS, PABA, and hydralazine (Weber, 1973). In addition, several of the carcinogenic arylamines such as 2AF, benzidine, and BNA (Glowinski et al., 1978) and biogenic amines such as serotonin (Schloot et al., 1969; Steinberg et al., 1969; Weisbach et al., 1960; White et al., 1969) are also substrates. In these two species, the genetically determined acetylator phenotype corresponds to large differences between rapid and slow acetylators in the N-acetylation rate of polymorphic substrates such as INH, SMZ, PA and 2AF, but only small differences in the N-acetylation rate of monomorphic substrates such as PABA and PAS (Weber, 1973; Glowinski et al., 1978; see CHAPTERS II, III, and IX).

Recent evidence from studies of rabbit liver NAT have shown that a single NAT enzyme is responsible for the monomorphic and polymorphic N-acetylation of this diverse set of arylamines and hydrazines (see CHAPTER III B). Furthermore, substrates such as PAS and PABA do not show a rigid monomorphic expression, rather, they show substantially less variation in acetylation rate between rapid and slow acetylators than polymorphic (e.g., SMZ, INH) substrates (see CHAPTERS II, III, and IX). These findings suggest that there is a progression from highly polymorphic to slightly polymorphic N-acetylation activity in man and rabbit. Since it is apparent that a single NAT protein probably catalyzes both types of activities (see CHAPTER III B), it is also reasonable to hypothesize that the chemical structure of the substrate is an important determinant affecting the degree of

polymorphic or monomorphic expression. Of the NAT substrates which have been evaluated with rabbit liver preparations, a progression can be described ranging from high (INH, SMZ) to lower (PAS) to least (PABA) degree of polymorphic expression (see TABLE 8).

In contrast to the qualitative similarity in expression of the acetylation polymorphism in man and rabbit, the substrate expression found in the hamster is strikingly opposite. Large differences (400-fold) were observed between rapid and slow acetylator hamsters for PABA; intermediate differences were found for PAS NAT (20-fold); and only small differences (2-fold) were demonstrated for SMZ, INH, PA, and 2AF. Mixing experiments argue against the presence of an inhibitor or activator in the slow or rapid acetylator liver preparation, respectively. It is interesting to speculate about the mechanism whereby the substrate structural determinants that result in polymorphic expression with hamster NAT also result in monomorphic expression by rabbit NAT, and vice versa. In both rabbit and hamster, ortho substitution of a hydroxyl group on the aromatic ring of PABA, to form PAS, results in a significant alteration in the degree of polymorphic expression. Thus, in the rabbit, PAS is significantly more polymorphic than PABA, whereas the opposite occurs in hamster.

These contrasts between the rabbit and hamster in the acetylation polymorphisms were also demonstrated in vivo. In the rabbit, Weber et al. (1976) showed a monomorphic acetylation of PABA in vivo compared to a polymorphic acetylation of sulfadiazine and INH. In the hamster, the in vivo acetylation of PABA was significantly ($p < .001$) higher in the rapid acetylator hamsters than in the slow, whereas SMZ acetylation was not higher in the rapid acetylators (TABLE 17). It is

pertinent to note that the in vivo differences in PABA N-acetylation between the rapid and slow acetylator hamsters were considerably smaller than those observed in vitro. A similar result has been shown for example, in the rabbit with the polymorphic substrate INH. The N-acetylation half-life of INH in vivo averaged only 2.6-fold higher in slow versus rapid acetylator rabbits (see CHAPTER VII), whereas liver INH NAT activity in vitro averaged 150-fold higher in rapid versus slow acetylator rabbits (see TABLE 8). In both hamster and rabbit, other renal and metabolic factors undoubtedly contribute to drug elimination in vivo and may account for the discrepancy between the in vivo and in vitro data. For example, Hearse and Weber (1973) have shown that the contribution of extrahepatic tissues toward the total N-acetylating capacity of the rabbit is relatively higher in slow acetylators which at least partially accounts for the discrepancy between the magnitude of in vivo and in vitro differences in N-acetylation.

The inheritance patterns of liver PABA and PAS NAT activity (FIGURES 20-24) were identical, and showed characteristics suggestive of simple autosomal Mendelian inheritance with additive expression of two codominant alleles at a single locus. In two different test crosses, the trimodal distribution of F2 progeny was not significantly ($p < .05$) different from a 1:2:1 ratio. Mean PABA and PAS NAT activities of F1 animals and the intermediate mode of F2 animals were usually very similar to the arithmetic mean of the rapid and slow acetylator parental strains (TABLES 18-19). In addition, mean PABA and PAS NAT activities in the low and high modes of F2 progeny were very similar to mean levels determined in the slow and rapid

acetylator parental strains, respectively (TABLES 18-19).

Recently, polymorphic expression of arylamine N-acetylation has also been identified among inbred mouse (A/J and C57B/J) strains (Tannen and Weber, 1980a; Glowinski and Weber, 1982a), although the expression for different substrates is different from that in man, rabbit, or hamster. Approximately 8-10 fold differences in liver NAT activity are observed for 2AF and benzidine (Glowinski and Weber, 1982a), whereas INH (D. W. Hein and W. W. Weber, unpublished observations), 1-napthylamine, PA and PABA (Tannen, 1978) show a monomorphic expression. SMZ NAT activity was undetectable in vitro in mouse liver preparations and PAS was not evaluated. Thus, although genetically determined rapid and slow acetylators can be readily identified in several mammalian species, further studies reveal qualitative differences in their expression.

In summary, the pharmacogenetic expression of the N-acetylation polymorphism in the hamster is qualitatively a mirror image of that in man and rabbit. This finding complements other studies of the N-acetylation polymorphism in rabbit (Glowinski et al., 1978; see CHAPTERS II and III), mouse (Tannen and Weber, 1980a,b; Glowinski and Weber, 1982a,b), and in other rodents (Tannen and Weber, 1979). Each of these species has particular attributes as an animal model to aid in understanding the INH acetylation polymorphism in man. Selection of the most appropriate model is dependent on several factors. In our laboratory, inbred and recombinant inbred mice are particularly appropriate for our genetic studies to map the gene locus for NAT in the mouse genome. In addition, both rabbit (see CHAPTER VII) and mouse (Tannen and Weber, 1980b) have been used in investigations to

determine the significance of the N-acetylation polymorphism with respect to drug toxicity. Studies of rabbit and hamster NAT are in progress to understand the biochemical nature of the substrate-dependent N-acetylation polymorphism more precisely. These studies may lead to a priori predictions of the magnitude of the genetically determined difference in acetylation rate between rapid and slow acetylators for new or novel arylamine drugs and carcinogens of known chemical structure. Preliminary information of this type in inbred rabbit liver is discussed in CHAPTER III C.

CHAPTER V

BIOCHEMICAL BASIS OF THE N-ACETYLATION POLYMORPHISM IN THE HAMSTER

Introduction

Genetically determined differences in N-acetylation rate have recently been reported in hamster (see CHAPTER IV) and were found to be both similar in some respects and dissimilar in others to those previously described in man (Evans et al., 1960), rabbit (Frymoyer and Jacox, 1963a,b) and mouse (Tannen and Weber, 1980a; Glowinski and Weber, 1982a). For example, the N-acetylation polymorphism in hamster is similar to human, rabbit, and mouse in that inheritance patterns of liver NAT activity are controlled by simple autosomal Mendelian genetics with two codominant alleles at a single locus (see CHAPTER IV). Thus, rapid and slow acetylator hamsters are either homozygous (RR) or heterozygous (Rr) for a rapid acetylator allele (R) whereas slow acetylators are homozygous (rr) for a slow acetylator allele, as previously described in man (Evans et al., 1960), rabbit (Frymoyer and Jacox, 1963a,b), and mouse (Glowinski and Weber, 1982a). The N-acetylation polymorphism in the hamster is unique, however, in that rapid and slow acetylation is strikingly apparent with substrates such as PABA and PAS (polymorphic in hamster) but not appreciably so with 2AF, PA, SMZ, and INH (monomorphic in hamster) (see CHAPTER IV), a finding in direct contrast to the other mammalian species listed above.

Although the substrate expression of the N-acetylation polymorphism and its mode of inheritance have previously been reported (see CHAPTER IV), the biochemical basis of this unique pharmacogenetic trait has yet to be elucidated. Thus, the purpose of the experiments described in this chapter was primarily to answer two questions analogous to studies of the rabbit in CHAPTER III: 1) Do rapid and slow acetylator hamsters have quantitative differences in amount of NAT enzyme, or, alternatively, have qualitative, structural differences between rapid and slow acetylator NAT, and 2) are monomorphic and polymorphic expressions of hamster liver NAT activity catalyzed by a common NAT or separate NATs.

Methods

Chemicals and reagents:

(see TABLE 33)

Animals:

Male and female Syrian inbred hamsters were obtained from stocks maintained in our animal care facilities at the University of Michigan. A full description of their characteristics (Altman and Katz, 1979) and animal care (see CHAPTER IV) have previously been reported. The rapid acetylator hamster (Bio. 65.67) selected for liver NAT purification was a 134-day old male. The slow acetylator hamster (Bio. 1.5) selected for liver NAT purification was a 192-day old female.

Liver NAT purification:

Hamsters were sacrificed by carbon dioxide intoxication. The livers were rapidly removed, minced, and homogenized (25% w/v) in 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM DTT and EDTA (starting buffer) in a Kontes-Dual ground glass homogenizer. The homogenate was centrifuged at 10,000 g for 20 min followed by 105,000 g for 60 min. The cytosolic supernatant was removed (about 20 ml) and carefully applied to a DEAE-cellulose (medium mesh) column (2.5 x 10 cm) which had been throughly washed with starting buffer. The column was eluted with a linear 24-hour gradient of 20 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, to 20 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, 0.5 M KCl. Five ml fractions were collected and assayed for NAT activity as described below. Concentrations of KCl in each fraction were determined through construction of a standard curve of conductivity

versus known KCl concentration. Conductivity readings were measured with a Yellow Springs Instrument Co., Inc., Model 31 Conductivity Bridge.

PABA and SMZ NAT activity determinations:

Except where noted in the Lineweaver-Burk analyses of PABA NAT activity (TABLE 20), PABA and SMZ NAT activities were determined at 37°C by the micromodification of the Bratton-Marshall assay as described in CHAPTER II.

INH NAT activity determinations:

INH NAT activity was determined at 37°C as described in CHAPTER II.

PAS, PA, 2AF, and BNA NAT activity determinations:

PAS, PA, and 2AF NAT activities were determined at 37°C by the DMAB assay as described in CHAPTER II. BNA NAT activity was also measured at 37°C using the DMAB assay. BNA hydrochloride was dissolved in water; in all other respects, the BNA NAT and 2AF NAT activity assays are identical. Under the conditions of this assay, a decrease of one sod absorbance unit corresponds to the acetylation of 27.0 pmoles of BNA.

Protein determinations:

Protein concentrations in peak NAT activity Sephacryl S-200 fractions was determined by the method of Warburg and Christian (1941).

Results

Kinetic properties for PABA NAT activity:

Liver NAT preparations (105,000 g) from four rapid acetylator hamsters (Bio 4.24) and four slow acetylator hamsters (Bio. 82.73) were used for determination of the Michaelis-Menten and Vmax constants of PABA NAT activity. As shown in TABLE 20, apparent Km values for PABA ranged from 0.0381 to 0.104 mM in the rapid acetylators for an average of 0.0607 mM. In contrast, apparent Km values for PABA were 20-fold higher in the slow acetylators, ranging from 0.624 to 1.72 mM for an average of 1.21 mM. The apparent Vmax for PABA NAT activity ranged from 68.6 to 144 nmoles/min/mg for an average of 104.3 in the rapid acetylators (TABLE 20). In contrast, the apparent Vmax for PABA NAT activity was about 15-fold lower in the slow acetylators, ranging from 4.68 to 12.5 nmoles/min/mg for an average of 7.15 (TABLE 20).

Rapid acetylator liver NAT purification:

Rapid acetylator hamster liver cytosol was found to contain two NATs which could be separated by ion-exchange chromatography on DEAE-cellulose. The protein purification pattern, as determined by absorbance at 280 nm, and the KCl concentration in each fraction is shown in FIGURE 25. The polymorphic NAT enzyme eluted from the column at fraction 21 (0.105 M KCl) whereas the monomorphic NAT enzyme eluted from the column at fraction 28 (0.180 M KCl). PABA and PAS acetylation was rapidly catalyzed by the polymorphic NAT , but to only minimally detectable levels by the monomorphic NAT (FIGURE 26 and TABLE 21). PA and INH acetylation were rapidly catalyzed by the monomorphic NAT and to an appreciable, but much lower extent by the

TABLE 20. Kinetic constants of rapid and slow acetylator inbred hamster liver PABA NAT activity.[a]

Rapid Acetylators[b]		Slow Acetylators[c]	
Km (mM)	Vmax (nMoles/min/mg)	Km (mM)	Vmax (nMoles/min/mg)
0.0596	80.8	0.624	4.68
0.0658	68.8	0.666	5.55
0.104	91.8	1.61	10.0
0.0381	96.5	1.72	12.5
0.0444	104	1.46	6.78
0.0599	144	1.26	5.47
0.0613	137	1.12	5.72
0.0528	112	1.20	6.53
0.0607 ± .0070[d]	104.3 ± 9.2	1.21 ± .14	7.15 ± .95

[a]Plots were done under assay conditions which yielded linear Lineweaver-Burk plots. PABA concentrations ranged from 20-100 μM in the rapid acetylators and 100-400 μM in the slow acetylators. AcCoA concentration was 2.22 mM for all assays. Protein concentration was approximately 10 mg/ml in slow acetylator NAT assays and 0.1 mg/ml in rapid acetylator assays.

[b]Duplicate determinations in 4 hamsters (Bio 4.24).

[c]Duplicate determinations in 4 hamsters (Bio 82.73).

[d]Mean ± S.E.

polymorphic NAT (FIGURE 27 and TABLE 21). 2AF and BNA acetylation were rapidly catalyzed by both the monomorphic and polymorphic NAT (FIGURE 28 and TABLE 21).

Slow acetylator liver NAT purification:

Slow acetylator hamster liver cytosol was found to contain only one NAT after ion-exchange chromatography on DEAE-cellulose chromatography. The protein purification pattern, as determined by absorbance at 280 nm, and the KCl concentration in each fraction is shown in FIGURE 29. The monomorphic NAT enzyme eluted from the column at fraction 40 (0.180 M KCl). No other NAT enzyme activity was detectable in any other fraction, including those near 0.105 M KCl. The acetylation of PABA was catalyzed very slowly by the monomorphic NAT (FIGURE 30 and TABLE 21) and the acetylation of PAS by this enzyme was also much slower than PAS acetylation in the rapid acetylator preparations (FIGURE 30 and TABLE 21). In contrast, the acetylation of 2AF (FIGURE 30 and TABLE 21), INH, BNA, and PA (FIGURE 31 and TABLE 21) were each rapidly catalyzed by the monomorphic NAT.

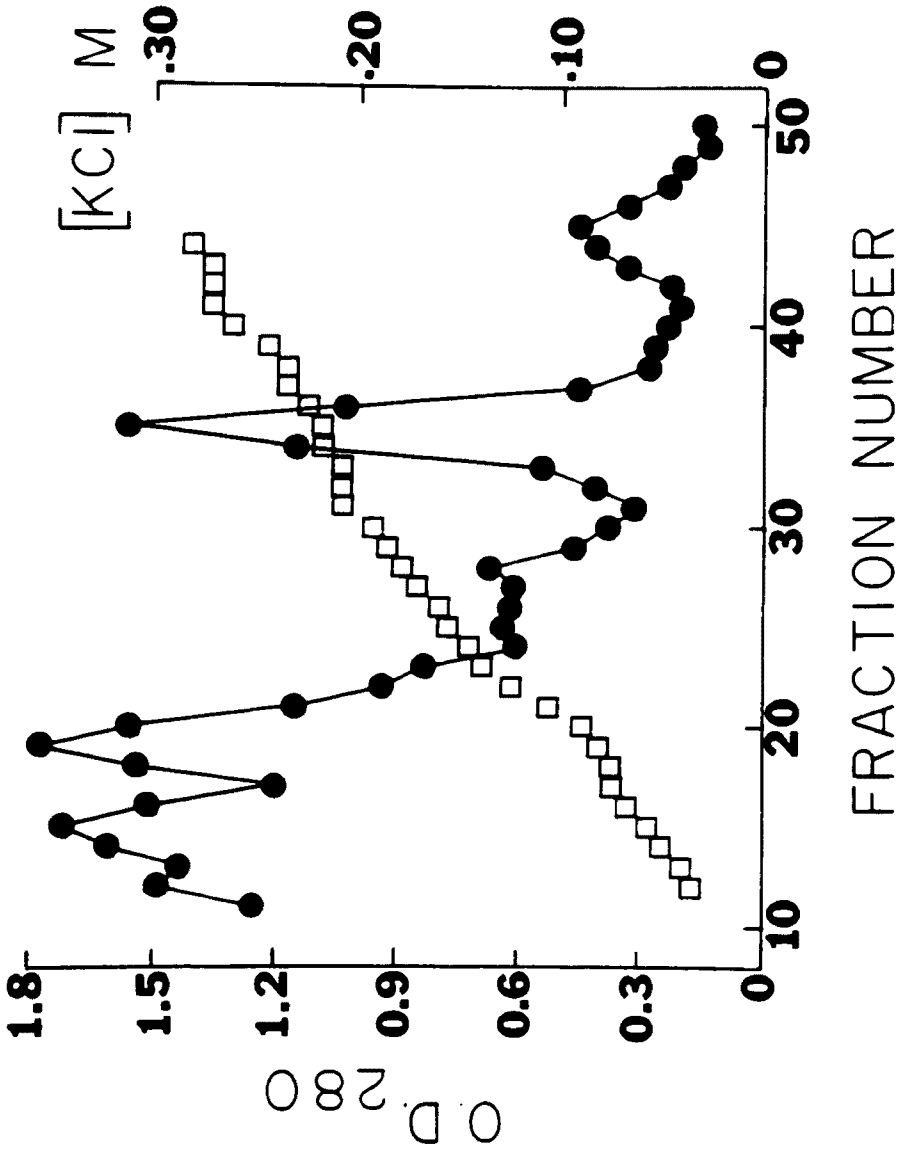


FIGURE 25. DEAE-cellulose purification pattern of rapid acetylator inbred hamster liver cytosolic protein.

The DEAE-cellulose fraction number on the abscissa is plotted versus protein concentration (circles) and molar concentration of KCl (squares) on the ordinates. Each data point represents an individual fraction. Peak polymorphic NAT activity is found in fraction 21. Peak monomorphic activity is found in fraction 28.

TABLE 21. Purification summary of rapid and slow acetylator inbred hamster liver monomorphic and polymorphic NATs.

	Rapid acetylator (Bio. 65.67)		Slow acetylator (Bio. 1.5)	
	Monomorphic activity	Polymorphic activity	Monomorphic activity	Polymorphic activity
KCl**	.180	.105	.180	----
PABA NAT	13.5*	446	4.18	N.D.
PAS NAT	116	1673	114	N.D.
PA NAT	118	3.29	152	N.D.
INH NAT	1069	59.2	993	N.D.
2AF NAT	247	129	392	N.D.
BNA NAT	398	195	466	N.D.

*NAT activity is nmoles/min/mg for peak fraction.

** Molar concentration of peak fraction.

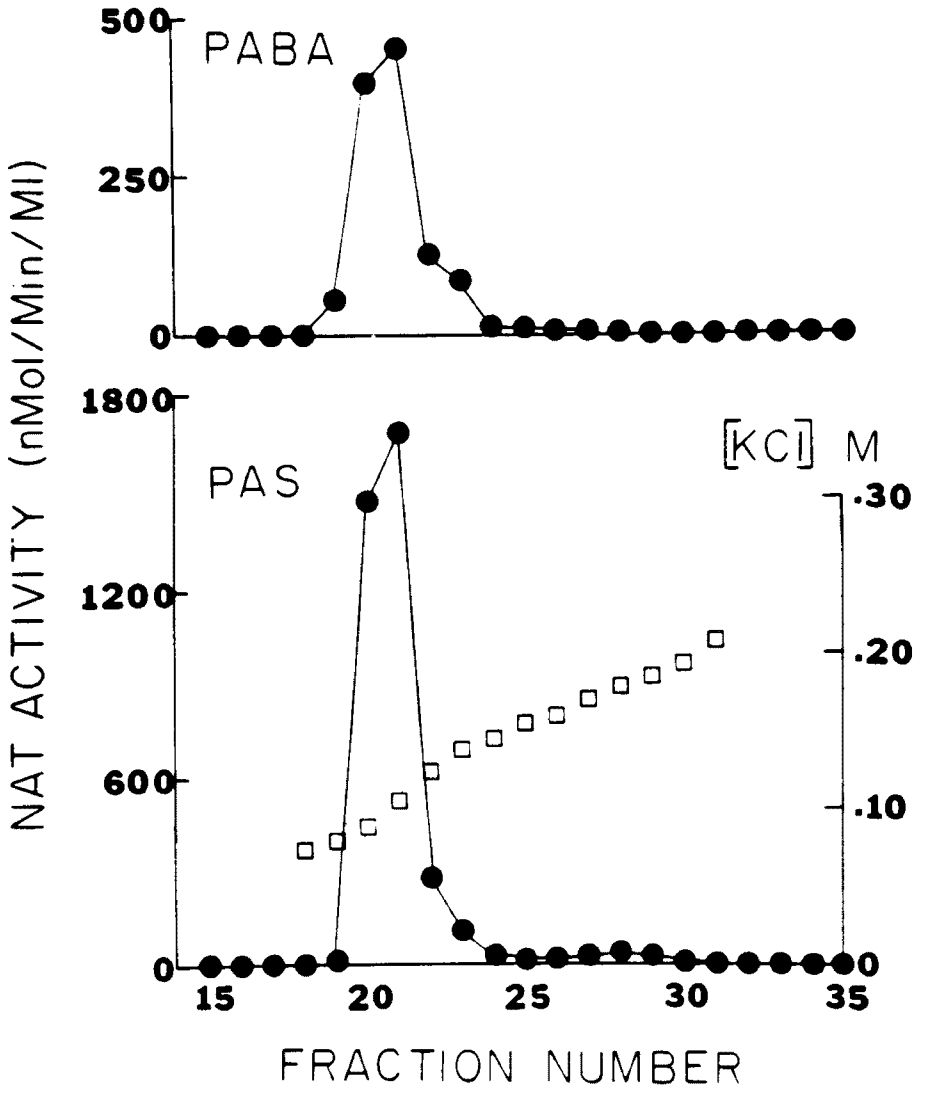


FIGURE 26. DEAE-cellulose purification pattern of rapid acetylator inbred hamster liver PABA and PAS NAT activities.

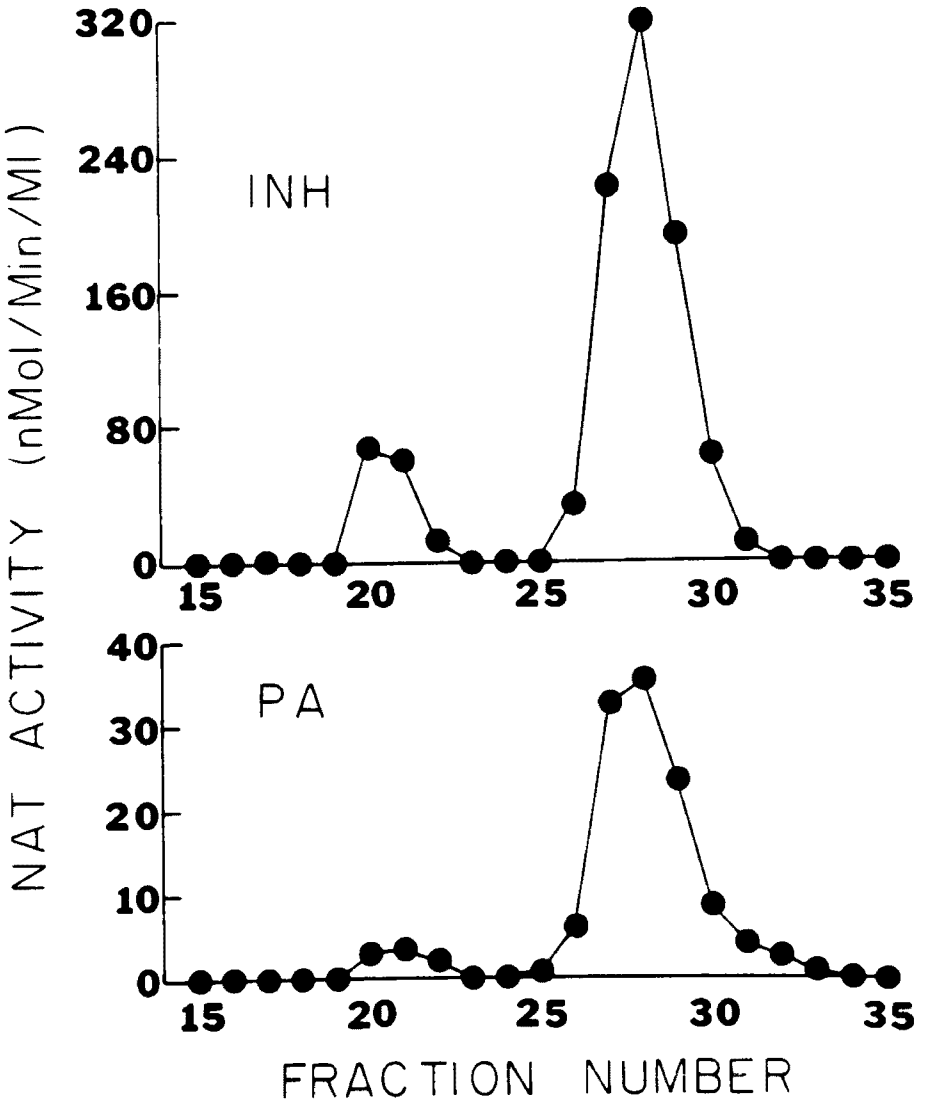


FIGURE 27. DEAE-cellulose purification pattern of rapid acetylator inbred hamster liver INH and PA NAT activities.

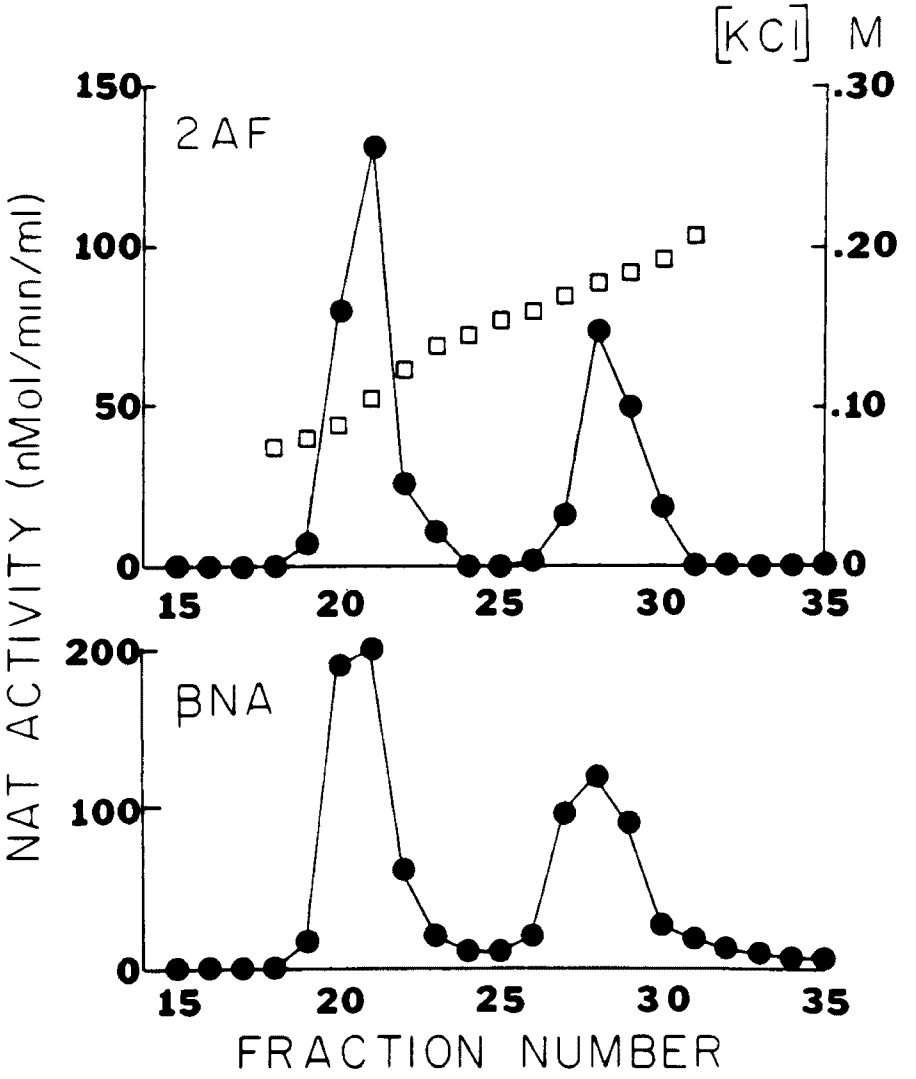


FIGURE 28. DEAE-cellulose purification pattern of rapid acetylator inbred hamster liver 2AF and BNA NAT activities.

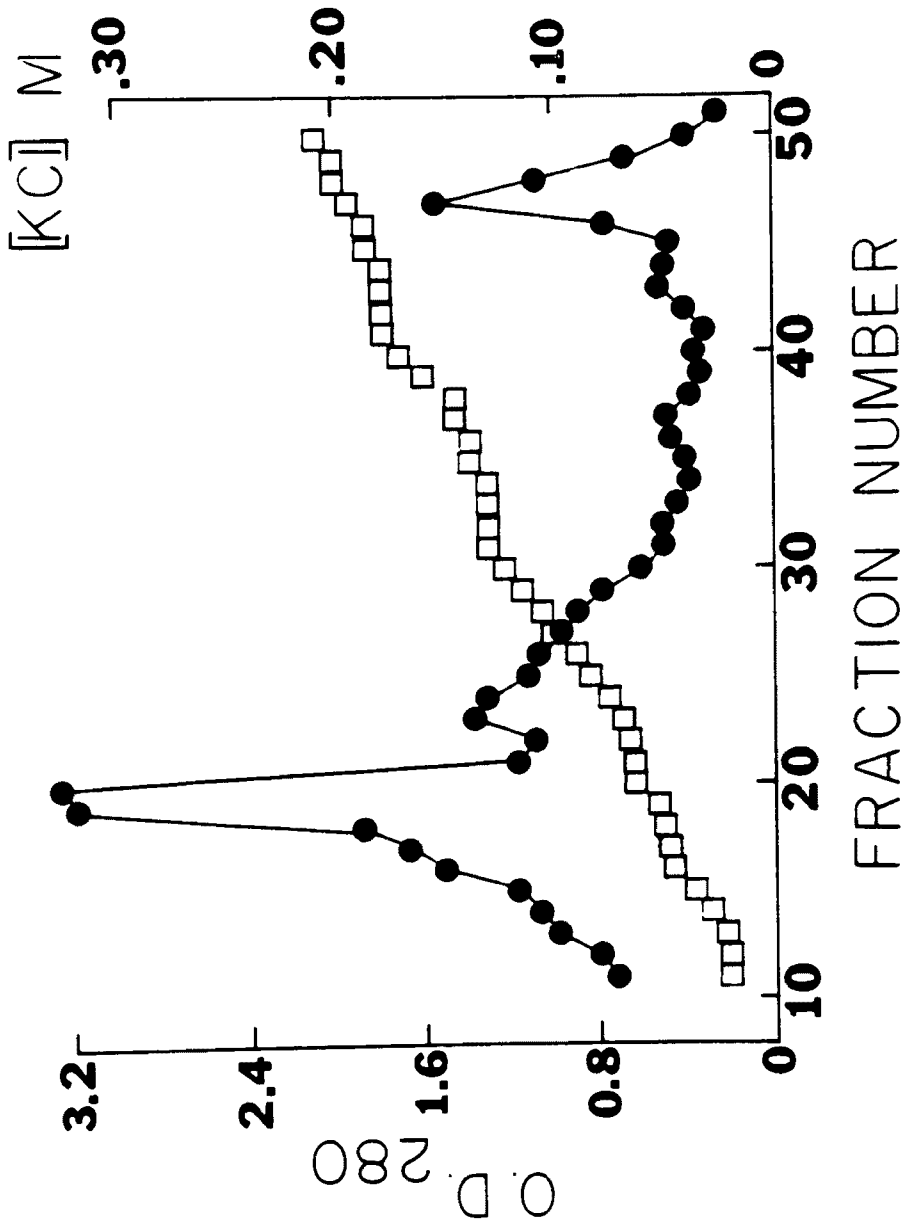


FIGURE 29. DEAE-cellulose purification pattern of slow acetylator inbred hamster liver cytosolic protein.

The DEAE-cellulose fraction number on the abscissa is plotted versus protein concentration (circles) and molar concentration of KCl (squares) on the ordinates. Peak monomorphic NAT activity is found in fraction 40. No polymorphic NAT activity was detectable in any fraction.

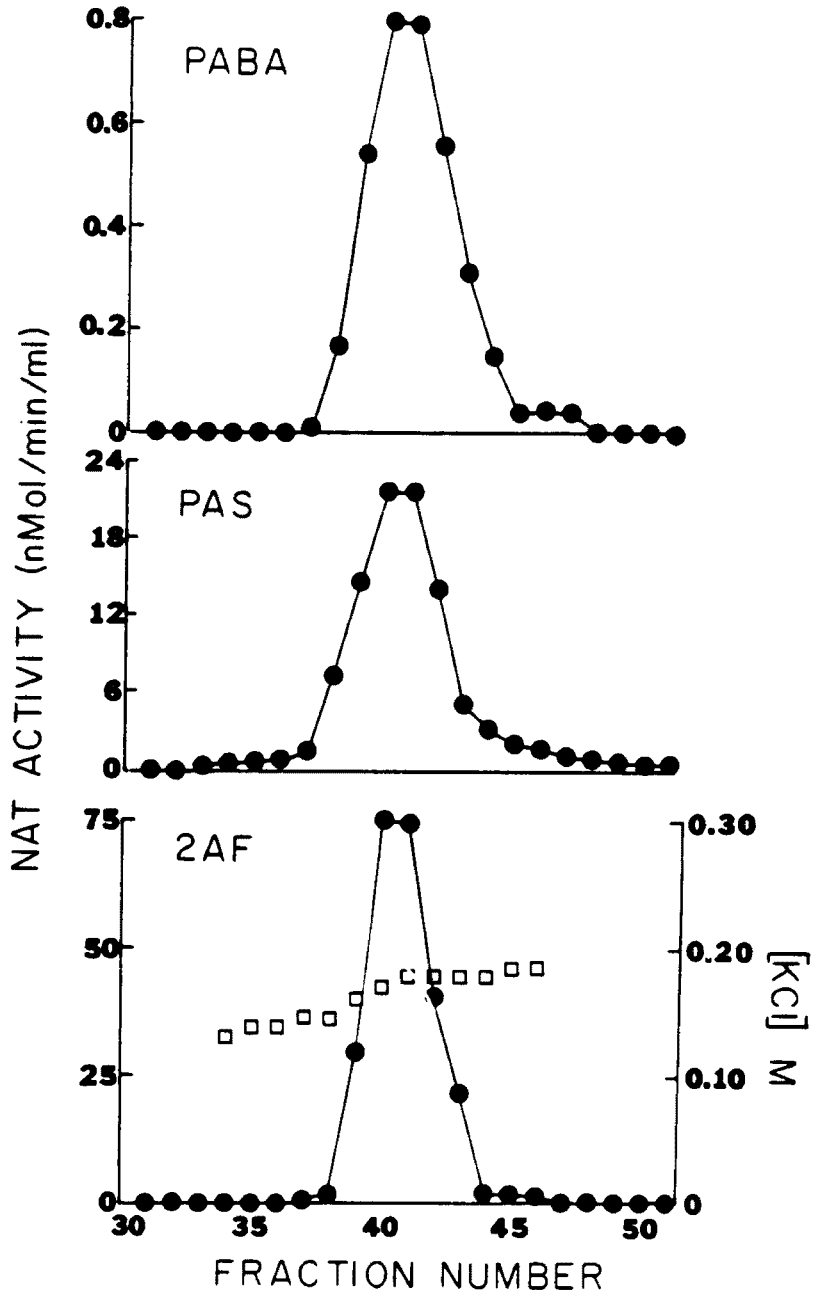


FIGURE 30. DEAE-cellulose purification pattern of slow acetylator inbred hamster liver PABA, PAS, and 2AF NAT activities.

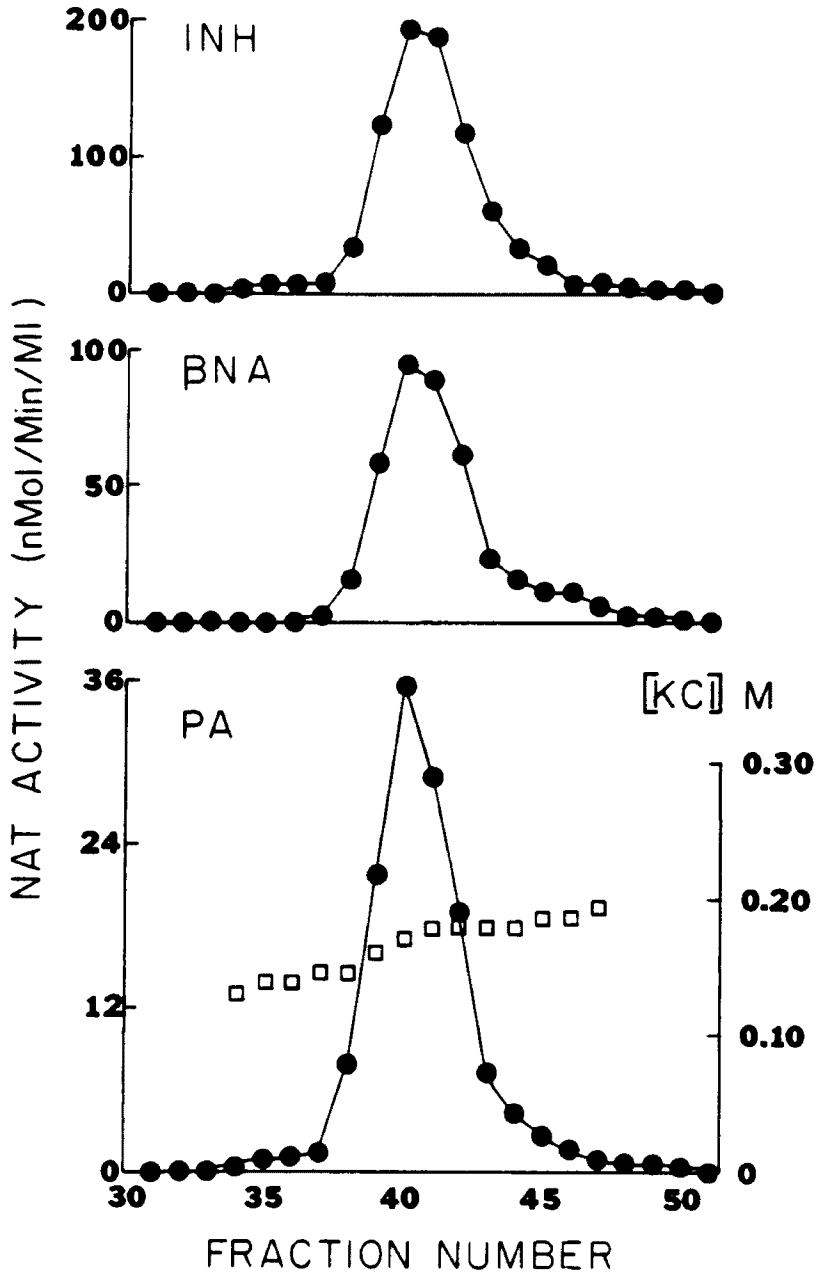


FIGURE 31. DEAE-cellulose purification pattern of slow acetylator inbred hamster liver INH, BNA, and PA NAT activities.

Discussion

The biochemical basis of the N-acetylation polymorphism in man in unknown, although Jenne (1965) initially suggested that rapid and slow acetylators have the same liver NAT enzyme, with greater quantities of it in rapid acetylators. Studies of the biochemical basis of rapid and slow acetylator NAT in rabbits (see CHAPTER III) and mouse (Glowinski and Weber, 1982b) have been much more extensive. In the rabbit, rapid and slow acetylators contain similar amounts of qualitatively different NATs (Patterson et al., 1980; see CHAPTER III A). In the mouse, Glowinski and Weber (1982b) have shown differences in NAT Michaelis-Menten constants indicative of qualitative differences between rapid and slow acetylator NAT.

With respect to monomorphic and polymorphic expression of NAT activity, both types of activities appear to be catalyzed by a common NAT enzyme in both rabbit and mouse. In rabbit, liver PABA (monomorphic) and SMZ (polymorphic) NAT activities could not be separated by two extensive purification procedures, one of which was carried out to electrophoretic homogeneity (see CHAPTER III B). In addition, the same study showed that they exhibited indistinguishable heat inactivation properties at two different temperatures. Similarly, in the mouse, liver PABA (monomorphic) and 2AF (polymorphic) NAT activities also were not separable by purification techniques which exploit differences in protein size and charge, and both of these activities migrated as a single symmetrical protein band after polyacrylamide disc gel electrophoresis.

As discussed in CHAPTER IV, however, the N-acetylation polymorphism in the hamster is unique in some respects, differing

markedly from man, rabbit, or mouse. For example, PABA and PAS are polymorphic, and INH, SMZ, 2AF, and PA are monomorphic in the hamster (see CHAPTER IV). Thus it is perhaps not surprising that in contrast to the other mammalian species listed above, hamster liver contains two distinctly different and separable forms of NAT. In rapid acetylator hamsters, the acetylation of PABA and PAS are rapidly catalyzed by polymorphic NAT, and only minimally by monomorphic NAT. In slow acetylator hamsters, the acetylation of PABA and PAS is again only minimally catalyzed by monomorphic NAT. The polymorphic NAT, however, is not expressed in the slow acetylator hamster liver, which accounts for the much lower levels of PABA and PAS NAT activity in slow acetylator cytosol.

These findings also provide an explanation for the apparent Km differences between rapid and slow acetylator NAT for PABA (TABLE 20). Although 105,000 g cytosol was used in these experiments, the apparent Km for PABA in the rapid acetylators (0.0607 mM) was primarily that of the polymorphic NAT, whereas the apparent Km for PABA in the slow acetylators (1.21 mM) was that of monomorphic NAT, a a distinctly different NAT enzyme. Thus, although these results are consistent with those of rabbit (see CHAPTER III A) and mouse (Glowinski and Weber, 1982b) which have also demonstrated apparent Km differences between rapid and slow acetylator NAT, the hamster represents the first species where two qualitatively distinct NATs have been separated and identified.

INH and PA acetylation were primarily catalyzed by monomorphic NAT, a finding consistent with the overall monomorphic expression of INH and PA NAT acetylation in 105,000 g liver cytosol (see CHAPTER

IV). Interestingly, however, both substrates are acetylated to an appreciable extent by the polymorphic NAT. It was with perhaps greater interest that we noted 2AF and BNA exhibited approximately equal levels of activity for monomorphic and polymorphic NAT. Perhaps future studies will yield selective NAT inhibitors which will inactivate monomorphic 2AF NAT activity and leave polymorphic 2AF NAT activity unaffected. The recent studies of Hanna and coworkers (Hanna et al., 1982) are of importance in this regard. They were able to selectively protect hamster liver PABA NAT activity from suicide inactivation by N-hydroxy-acetylaminofluorene by pretreatment with cysteine, while SMZ NAT activity (also monomorphic in hamster; copurifies with 2AF NAT activity) was not protected. Thus, it is likely that liver polymorphic BNA and 2AF NAT activities will be protected from N-hydroxy-acetylaminofluorene suicide inactivation by pretreatment with cysteine while monomorphic BNA and 2AF NAT activities will not. Of greater importance, however, is the fact that monomorphic and polymorphic BNA and 2AF NAT activities can be separated by ion-exchange chromatography as described in the present study.

King and coworkers (Olive and King, 1975; King and Allaben, 1978) were the first to suggest that hamster liver and small intestine contained multiple forms of AHAT activity. While NAT and AHAT have been shown to be enzyme activities catalyzed by a common protein in rabbit liver (Glowinski et al., 1980), Hanna et al. (1982) have reported that only the SMZ NAT activity (monomorphic NAT) also has AHAT activity, while the PABA NAT activity (polymorphic) lacks this property.

It will be interesting to determine the substrate specificity of purified hamster liver polymorphic NAT as has already been done in the rabbit (see CHAPTER III C). Preliminary results with the six substrates of this study indicate that polymorphic NAT has high levels of activity for PABA and PAS, intermediate for 2AF and BNA, and low for PA and INH. The monomorphic NAT, in contrast, has high levels of activity for PA and INH, intermediate for 2AF and BNA, and negligible for PABA and PAS. The consistency of this mirror image relationship with respect to many other arylamine and hydrazine drugs and environmental carcinogens awaits further investigation.

CHAPTER VI

RELATIONSHIP OF ACETYLATOR PHENOTYPE TO SPONTANEOUS SYSTEMIC LUPUS ERYTHEMATOSUS IN HUMANS

Introduction

Spontaneous systemic lupus erythematosus (SLE) is a multisystem, immune complex disease involving both cell- and antibody-mediated immune reactions to numerous cellular constituents, producing various types of tissue damage (Koffler, 1974). One of the early signs of SLE is an errosive inflammation of the skin, typically in the form of a butterfly rash over the face, that produced the name of lupus erythematosus, or "red wolf" (Koffler, 1974). It is appropriately characterized as a systemic disease because it affects not only the skin, but also the joints, blood vessels, lung, heart, and most significantly, the kidneys (Koffler, 1974). Current estimates indicate that there are 500,000 patients with SLE in the United States alone, which is about 1 in every 500 females and 1 in every 5000 males (Hess, 1981).

Both genetic and environmental factors have been implicated in the occurrence of SLE. The most striking evidence for genetic contributions is the much higher concordance of SLE among monozygotic twins (50-60%) than among dizygotic twins and other siblings (2-3%) (Block et al., 1975). The presence of autoantibodies in relatives of SLE patients has also been documented in several studies (e.g., Lappat

and Cawein, 1968; Cleland et al. 1978), although no clear pattern of SLE inheritance has been established.

Following the administration of certain drugs, including PA, hydralazine, and to a lesser extent, INH, a number of patients develop a drug-induced syndrome which presents many of the clinical and laboratory characteristics found in spontaneous SLE. For example, Perry and coworkers reported a 54% incidence of antinuclear antibodies, and a 12% incidence of the SLE syndrome in patients treated with hydralazine (Perry et al., 1970; Perry, 1972). Similarly, the incidence of antinuclear antibodies has been estimated to be almost 100% in long term PA therapy (Woosley et al., 1978) and the incidence of the SLE syndrome is at least 30% (Henningsen et al., 1975). Of particular interest is the finding that drug-induced antinuclear antibodies and SLE are more frequently encountered in slow acetylators (e.g., Uetrecht and Woosley, 1981). Since N-acetylation is a major pathway in PA, hydralazine, and INH metabolism (Weber, 1973), it has been suggested that N-acetylation via polymorphic liver NAT is a detoxification pathway for this toxicity, accounting for the higher frequency in slow acetylators.

Recently, as reviewed by Uetrecht and Woosley (1981), it has been reported by various laboratories around the world that the incidence of <u>spontaneous</u> SLE is also more frequently encountered in slow acetylators, although other laboratories from around the world have provided other evidence of the same type which disputes this claim. Consequently, with the availability of patients diagnosed with spontaneous SLE being treated at the University of Michigan Medical Center, and our facilities for determination of acetylator

phenotype, it was the purpose of this experiment to examine this disputed hypothesis by determining acetylator phenotype of the SLE patients, and, where possible, by determining the acetylator genotype of rapid acetylator SLE patients. Acetylator genotype determinations of rapid acetylators required the collection of family pedigree information of acetylator phenotype.

Methods

Determination of acetylator phenotype:

Randomly selected individuals in good health, patients diagnosed with SLE, and healthy family members of these patients were classified as rapid or slow acetylators by a modification of methods previously reported (Evans, 1969). Each individual was requested to abstain from eating the morning of the acetylator phenotype determination and to disclose all current medications. Any individual currently taking medication which might interfere with the test or the accuracy of the acetylator phenotype classification was omitted from the study. An initial blood and urine sample was collected followed by oral administration of 10 mg/kg SMZ in either capsule or suspension form. No water or food was permitted for two hours following administration of the drug. At 4.5 hours post drug administration, a final blood and urine sample was collected.

The experimental assay for free and acetyl-SMZ utilizes the Bratton-Marshall (1939) colorimetric assay for aromatic amines. 300 ul of blood or urine (diluted 1:10 with water) was added to 3 ml of water. To this mixture was added 1 ml of 20% trichloroacetic acid. After a 5 min wait, the tubes were centrifuged at 4500 g for 5 min in a Sorvall GLC II centrifuge. 1.5 ml of supernatant was carefully extracted and transferred to a clean tube. Color was developed by the sequential addition of 200 ul of 0.1% sodium nitrite (freshly prepared) followed 5 min later by 200 ul of 0.5% ammonium sulfamate followed 5 min later by 1 ml of 0.05% N-1-napthyethylene diamine. Absorbance was read at 540 nm in a Gilford 300N micro-sample spectophotometer. The amount of total drug (free and acetyl-SMZ) was

determined by adding 200 ul of 4 N HCl to the 1.5 ml sample and boiling for one hour in a water bath. This procedure hydrolyzes all acetyl-SMZ to free SMZ. The assay procedure described above then is carried out on the hydrolyzed samples to determine total SMZ in the sample. A simple subtraction of the amount of free SMZ (before hydrolysis) from total SMZ (after hydrolysis) yields the amount of acetyl-SMZ in the sample. All assays were done in duplicate. Individuals with percent acetyl-SMZ/SMZ values of 50 or greater in blood and 75 or greater in urine were classified as rapid acetylators. Individuals with percent acetyl-SMZ/SMZ values of 50 or lower in blood and 75 or lower in urine were classified as slow acetylators. All individuals tested (82) fit into one of these two classifications, and none were indeterminate.

Human use approval:

The protocol for use of human subjects in these studies was approved by the Committee to Review Grants for Clinical Research and Investigation Involving Human Beings of the University of Michigan Medical Center. A signed informed consent was obtained from each subject prior to the study (see Appendix).

Results

Acetylator phenotype determinations:

The acetylator phenotype was determined on a total of 82 individuals. They can be classified into three groups: 1) randomly selected, healthy volunteers, 2) patients diagnosed with spontaneous SLE, and 3) family members of patients with SLE.

Group 1 consisted of 28 randomly selected, healthy individuals (12 males, 16 females) residing in Southeast Michigan. Twelve of these individuals were rapid acetylators (42.9%) and 16 (57.1%) were slow acetylators. All were Caucasian except for two rapid acetylators who were a Chinese male and female. The percent acetyl-SMZ/SMZ values for these individuals are illustrated by the solid circles in FIGURE 32.

Group 2 consisted of 13 individuals (all female) diagnosed with spontaneous SLE. None had received prior administration of any drug known to produce drug-induced SLE. In this group, six (46.2%) were rapid and seven (53.8%) were slow acetylator phenotype. Each of the slow acetylators is an obligate homozygous slow acetylator genotype (rr). Acetylator genotype in the rapid acetylator SLE patients was only determined in two individuals because of the family pedigree sample size. Both of these rapid acetylator SLE patients were obligate heterozygous rapid acetylator (Rr) genotype. Eleven of these individuals were Caucasian and two (one rapid and one slow acetylator) were black. Within this group, two of the rapid acetylators are sisters, and two of the slow acetylators are first cousins. Thus, a totally random distribution of acetylator phenotype within the SLE patients would be five (45.5%) rapid acetylators and six (54.5%) slow

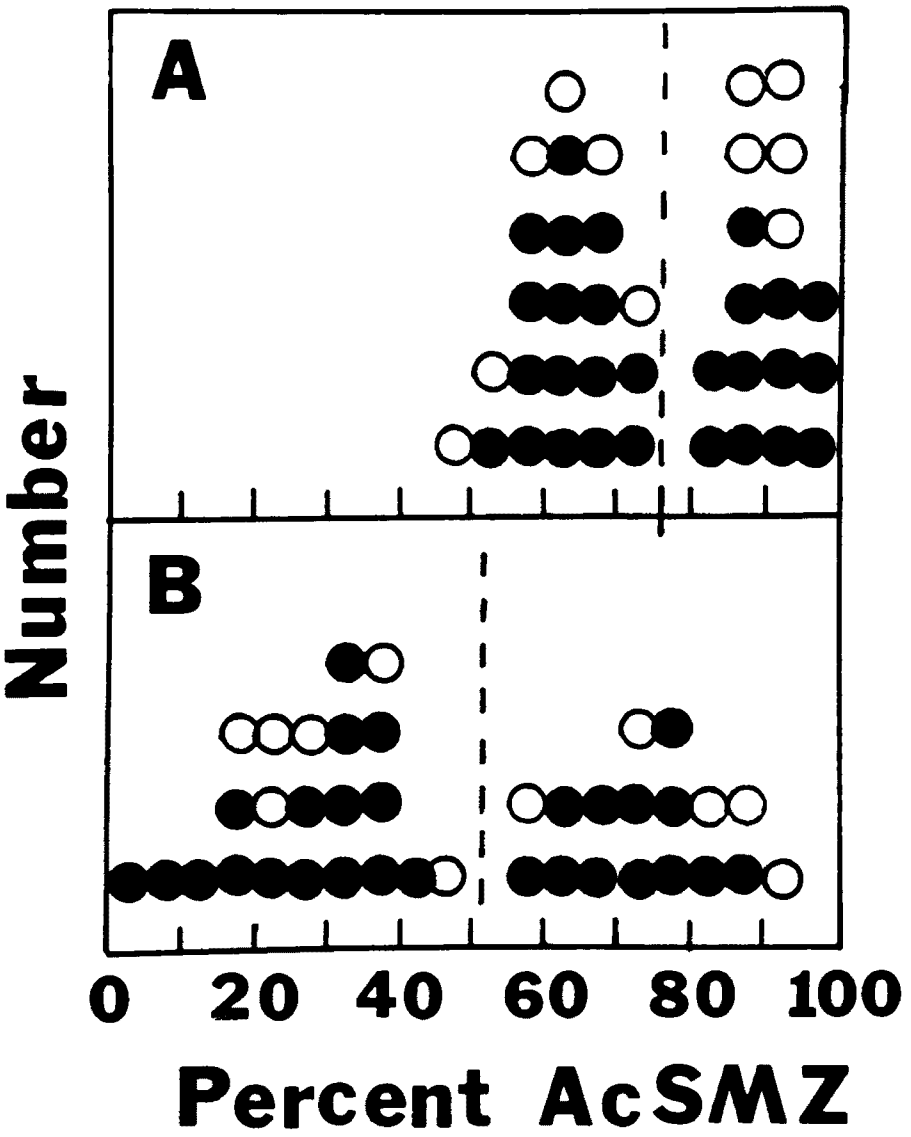


FIGURE 32. Acetylator phenotype distribution of random, normal individuals and patients with SLE.

The percent acetyl-SMZ/SMZ in urine (panel A) and blood (panel B) is plotted on the abscissa versus number of individuals on the ordinate. Each dot represents one individual. Open circles are patients with SLE. Closed circles are randomly selected, healthy individuals. The left modes represent slow acetylators, the right modes represent rapid acetylators.

acetylators. The percent acetyl-SMZ/SMZ values for these individuals are illustrated by the open circles in FIGURE 32.

Group 3 consisted of 41 individuals from four families of some of the SLE patients in group 2. The four family pedigrees of acetylator phenotype are illustrated in FIGURES 33-34. The acetylator phenotype pedigree of the KPC family is shown in FIGURE 33 (upper half). Within this Caucasian family, two female first cousins have been diagnosed with SLE. Both were found to be slow acetylators. One of them had a heterozygous rapid acetylator mother and a homozygous slow acetylator father, while the other's parents were both homozygous slow acetylators. The acetylator phenotype pedigree of the EBK family is shown in FIGURE 33 (lower half). This Caucasian family has one female diagnosed with SLE. Both she and her parents are homozygous slow acetylators. The acetylator phenotype pedigree of the ZBT family is shown in FIGURE 34 (upper half). This Caucasian family has two sisters diagnosed with SLE. Although they are both rapid acetylators, the pedigree information indicates that they are obligate heterozygous rapid acetylators and carry the slow acetylator gene. Their mother is also an obligate heterozygous rapid acetylator and their father is a homozygous slow acetylator. The acetylator phenotype pedigree of the DKE family is shown in FIGURE 34 (lower half). This Caucasian family has dizygotic female twins, one of which is healthy and one of which has been diagnosed with SLE. In this instance, both of the twins are homozygous slow acetylators; their mother is an obligate heterozygote rapid acetylator and their father is a homozygous slow acetylator.

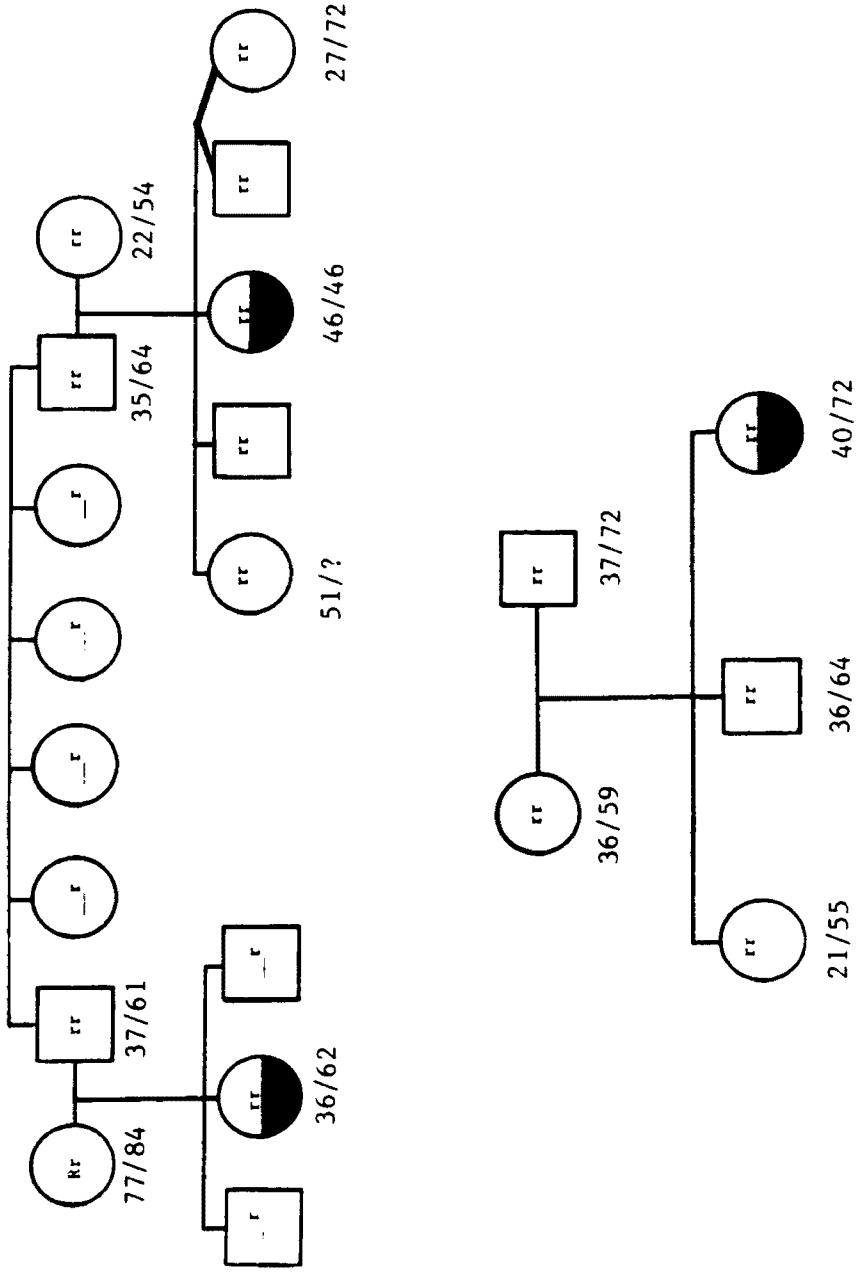


FIGURE 33. SLE family pedigrees of acetylator phenotype.

The KPC family pedigree is shown on the upper half of the figure and the EBK family pedigree is shown on the lower half. Half-shaded circles represent females with spontaneous SLE. Squares represent males. Letters within each symbol indicate acetylator genotype. Symbols with numbers underneath represent individuals who were tested for acetylator phenotype. The first number represents the percentage of acetyl-SMZ in blood, the second number represents the percentage of acetyl-SMZ in urine.

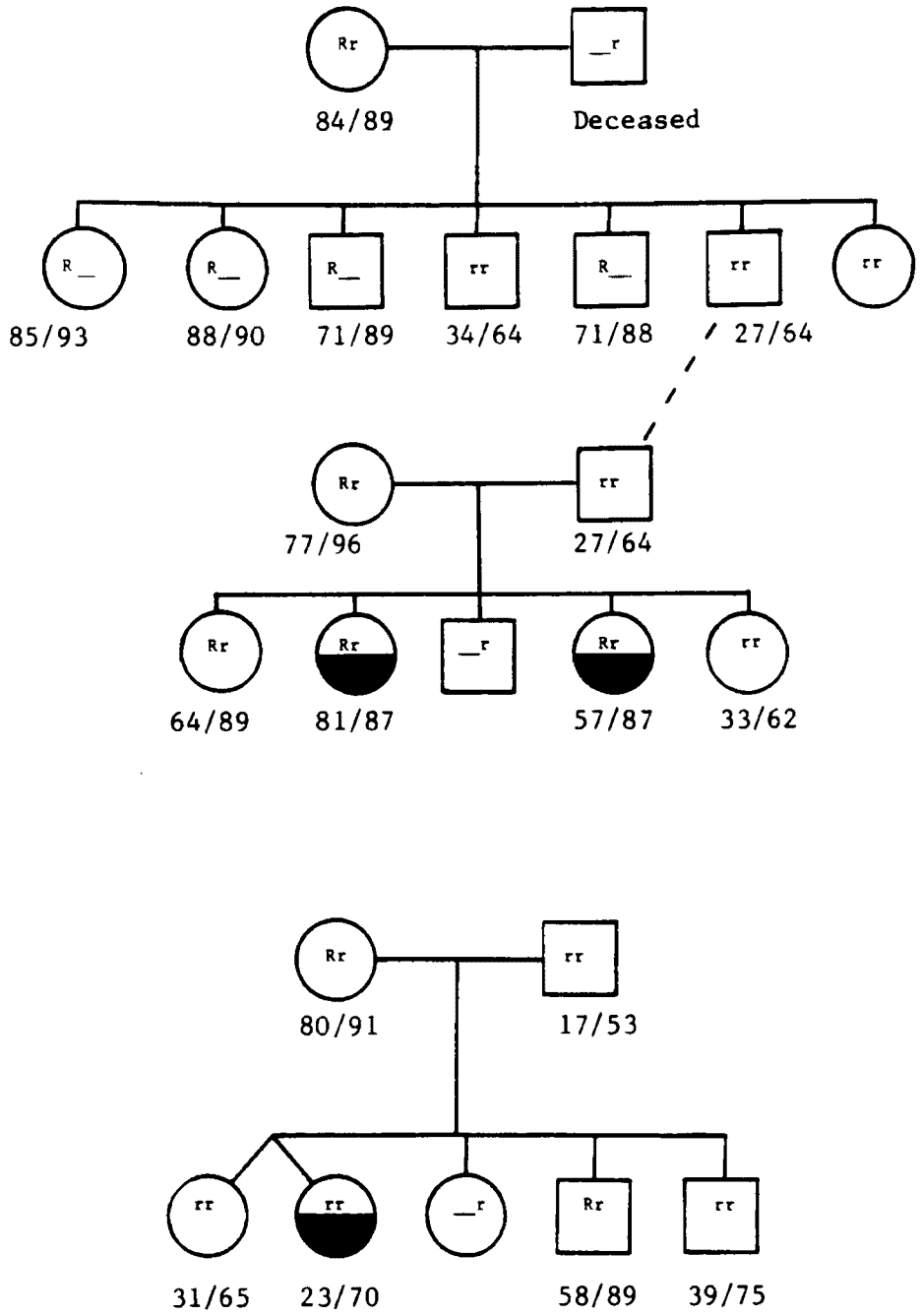


FIGURE 34. SLE family pedigrees of acetylator phenotype.

The ZBT family pedigree is shown on the upper half of the figure and the DKE family pedigree is shown on the lower half. Half-shaded circles represent females with spontaneous SLE. Squares represent males. Letters within each symbol indicate acetylator genotype. Symbols with numbers underneath represent individuals who were tested for acetylator phenotype. The first number represents the percentage of acetyl-SMZ in blood, the second number represents the percentage of acetyl-SMZ in urine.

Discussion

It appears to be well-accepted that drug-induced SLE produced by PA (Henningsen et al., 1975; Woosley et al., 1977) or hydralazine (Perry et al., 1970; Strandberg et al., 1976) is more frequently encountered in slow acetylators. Since both of these drugs are substrates for polymorphic NAT (e.g., Weber, 1973), it has been suggested that the slow acetylator phenotype is a predisposing factor in drug-induced SLE toxicity (e.g., Uetrecht and Woosley, 1981).

Although a relationship between slow acetylator phenotype and drug-induced SLE might appear to be of no significance with respect to the relative susceptibility of slow acetylators to spontaneous SLE, nevertheless, several investigators have reported that spontaneous SLE also occurs more frequently in the slow acetylator phenotype. Reidenberg and Martin (1974) found that 71% of their spontaneous SLE patients were slow acetylators versus only 52% of historical, healthy controls. Subsequently, this finding was confirmed by five additional studies (Johansson et al., 1976; Foad et al., 1977; Larsson et al., 1977; Fishbein and Alarcon-Segovia, 1979; and Reidenberg et al., 1980) carried out in different populations of the world. However, Vansant et al. (1978) were unable to confirm these findings, as they found similar frequencies of slow acetylators among spontaneous SLE patients and controls. More recent studies by Lawson et al. (1979), Morris et al. (1979) and Ishizaki et al. (1981) also failed to find higher frequencies of slow acetylators among patients with spontaneous SLE. For example, the study of Ishizaki and coworkers was carried out in Japanese patients, a population that is primarily rapid acetylators (see TABLE 1). In their study of 19

Japanese patients with spontaneous SLE, only one was found to be a slow acetylator. Reidenberg et al (1980) performed a statistical analysis on all the data which had been published in the literature at that time, including the negative studies, and found that, overall, the frequency of slow acetylators in spontaneous SLE patients was significantly higher ($p < .001$) than controls. This analysis did not include the Japanese study or our study, however. Based on this significance, Reidenberg and coworkers (1980) proposed that environmental amine chemicals, such as hair and food dyes, which would be expected to be polymorphically acetylated by liver NAT, could play a major role in the induction of "spontaneous" SLE. Since the distribution of these environmental chemicals is not uniform throughout the world, they suggest that certain populations of "spontaneous" SLE patients may not show higher frequencies of slow acetylators, which would, in part, explain the conflicting data.

It is important to note that several of the studies previously reported suffer from two problems which are not encountered in our results. First, many of these studies occasionally are unable to make definitive classifications of acetylator phenotype in some individuals, but rather list them as indeterminate. This suggests that they may not be using the most optimal, definitive method of phenotype classification. In our classification of 82 individuals, no uncertainty in phenotype classifications was encountered. Second, many of the previous studies base the frequency of slow acetylators in a particular population on <u>historical</u> (i.e., previously published) controls rather than collecting their own data on randomly selected, healthy controls with the same assay for acetylator phenotype

classification that is used in the SLE patients. A recent paper by Sacks et al. (1982) demonstrates that historical controls differ from experimental groups both more frequently and in greater magnitude than do randomly selected controls collected as part of the current study under investigation. In addition, studies which base comparisons on historical controls also may lack internal consistency in their phenotype classification since different methods and investigators are used to collect the data in the two groups. We eliminated these two problems, and though our sample size is small, we found no difference between the random frequency of slow acetylator phenotype in randomly selected, healthy adults of Southeast Michigan (57.1%) and randomly selected, spontaneous SLE patients being treated at the University of Michigan Medical Center (54.5%).

Family pedigrees of acetylator phenotype on some of the patients with SLE may provide some clues to a possible relationship between acetylator status and spontaneous SLE. As shown in FIGURE 34, both of the rapid acetylator SLE patients for which we could determine acetylator genotype through family pedigree information were obligate heterozygous individuals carrying the slow allele (r). The acetylator genotypes of the remaining rapid acetylator SLE patients could not be determined. This information is quite limited, but is consistent with the hypothesis that the slow acetylator allele may act as a genetic marker of some other genetically determined predisposition to spontaneous SLE. For example, Batchelor et al. (1980) have proposed that the frequency of the genetically determined HLA-DR4 antigen is much higher in drug- induced SLE patients than in controls and suggest that it acts as a genetic marker of drug-induced SLE. In addition, the

much higher frequency of both drug-induced and spontaneous SLE in females is well known (e.g., Kaslow and Masi, 1978) and 100% of our sample of SLE patients were females. Studies in an animal model by Tannen and Weber (1980b) are of interest in this regard. These investigators found a higher frequency of spontaneous and PA-induced antinuclear antibodies in slow acetylator (A/J) than rapid acetylator (C57B/J) mice despite the fact that the two strains of mice do not show differences in PA N-acetylation in vivo (including at doses used to produce the toxicity) or in vitro in liver, where most PA metabolism occurs. In addition, through a genetic analysis, they were able to show that development of drug-induced antinuclear antibodies and spontaneous antinuclear antibodies are dissociable processes. Thus, although slow acetylator phenotype may directly predispose to drug-induced SLE for reasons explained above, it appears that it does not necessarily follow for spontaneous SLE. Consequently, these findings, together with the results of the present report, are consistent with the concept that slow acetylator phenotype may be only one factor, in addition to HLA-DR4 phenotype and female sex, which may predispose to and/or act as a genetic marker for spontaneous SLE.

CHAPTER VII

TOXICOLOGICAL CONSEQUENCES OF ACETYLATOR PHENOTYPE IN HYDRAZINE-INDUCED TOXICITY

Introduction

One of the most important consequences of the N-acetylation polymorphism is the greater occurrence of certain arylamine- and hydrazine-induced toxicities associated with acetylator status. Recent papers by Drayer and Reidenberg (1977) and Lunde et al. (1977) have reviewed instances in humans where greater susceptibility to such toxicities is postulated to occur in the rapid or slow acetylator phenotype. For example, slow acetylators have been shown to be more susceptibile to drug-induced SLE-like syndromes and development of antinuclear antibodies from drugs such as PA, hydralazine, and INH. As discussed in CHAPTER VI, slow acetylators may also show higher frequencies of spontaneous SLE, and as discussed in CHAPTER VIII, slow acetylators may experience greater incidences of severe adverse effects from PHZ and lower incidences of hepatotoxicity from INH, although each of these findings reported in humans is currently disputed. In addition, Lower et al. (1979) have provided human epidemiological data suggesting that slow acetylators have higher incidences of arylamine-induced bladder cancer, but these findings also have yet to be confirmed. Thus many human studies associating acetylator phenotype with greater incidences of toxicity are often

open to debate, perhaps because such toxicological conclusions are not derived from well-designed experimental studies, but rather from human epidemiological data of selected patient groups. Consequently, it is particularly important to develop animal models that exhibit biochemical and physiological properties similar to the human trait under investigation to best assess its toxicological significance with respect to drug toxicity.

There has been only one instance in which an animal model of the N-acetylation polymorphism was used to study associations between acetylator status and drug toxicity. Tannen and Weber (1980b) examined the incidence of spontaneous and PA-induced antinuclear antibodies in rapid and slow acetylator mice. Through the use of this model, they were able to carry out F1, F2, and backcross genetic analyses to answer important experimental questions. Obviously, such genetic manipulations would not have been possible in human studies. Unfortunately, rapid and slow acetylator mice are not a suitable animal model for studies of hydrazine-induced toxicity because differences in hydrazine liver NAT activity between rapid (C57B/J) and slow (A/J) acetylator mice were not demonstrated in vivo (Tannen and Weber, 1980b) nor in vitro (Tannen, 1978; D. W. Hein and W. W. Weber, unpublished observations). Both INH and MAH (two hydrazines of clinical and toxicological importance), however, have recently been shown to be polymorphically acetylated in the rapid and slow acetylator rabbit model (see CHAPTER VIII). This finding is similar to those in humans where both INH (Evans et al., 1960; Weber, 1973) and MAH (Ellard and Gammon, 1976) have also been shown to be polymorphically acetylated. The significance of acetylator phenotype

with respect to the CNS toxicity of these hydrazines in man remains unknown, however, because it is unethical to administer these agents at such doses to patients, and also because pyridoxine is often administered in combination with INH to antagonize occurences of the toxicity.

There are several reasons to expect that the frequency of CNS toxicity induced by hydrazines could be related to acetylator status. For example, studies in humans (Hughes et al., 1955; Devadatta et al., 1960) have shown that the incidence of INH-induced peripheral neuropathy is higher in slow acetylators than rapid acetylators. In addition, CNS toxicity studies in rats with hydrazine, monomethylhydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine have shown that the first three hydrazines listed above cause lethal instances of CNS toxicity at 100 mg/kg or less, whereas 1,2-dimethylhydrazine is non-toxic at doses as high as 500 mg/kg, indicating that the free amine group is critical for the CNS toxicity (O'Brien et al., 1964). Since N-acetylation via polymorphic liver NAT can be an important step in hydrazine metabolism (Juchau and Horita, 1972; see CHAPTER VIII), it is possible that genetically-determined differences in N-acetylation capacity may be of consequence in relative susceptibility to hydrazine-induced CNS toxicity. This study was designed in the rapid and slow acetylator rabbit model to examine this possibility.

Methods

Chemicals and reagents:

(see TABLE 33)

Determination of acetylator phenotype:

Rabbits were classified as rapid or slow acetylators by determination of the N-acetylation half-life of SDZ (20 mg/kg intravenous injection) using a Bratton-Marshall (1939) colorimetric procedure as previously described (Weber et al., 1976).

Determination of INH serum levels:

Serum levels of INH after an intramuscular 30 mg/kg injection were determined by means of a fluorometric procedure as previously described (Miceli et al., 1975).

INH and MAH toxicity experiments:

The specific dosage regimens used for each of the toxicity experiments in this chapter varied from one experiment to another and are included as part of the results section to provide greater clarity and to avoid duplication.

SGPT determinations:

SGPT (E.C. 2.6.1.2) levels were measured in fresh serum derived from blood samples taken from the peripheral ear vein of each rabbit. The colorimetric assay procedure has previously been described (Reitman and Frankel, 1957) and all reagents for the analysis are commercially available in a kit from Sigma (see TABLE 33). The calibration curve and assay conditions were carried out as described in Sigma Technical Bulletin, No. 505. Samples were read at 505 nm against water as a blank in a Gilford 300N microsample spectrophotometer. SGPT enzyme activity is reported in Sigma-Frankel

units which are units of GPT that will form 0.482 nmoles of glutamate/min at pH 7.5 and 25°C.

Results

Determination of acetylator phenotype in rabbits used for toxicity experiments:

Acetylator phenotype was determined in all 64 rabbits used in the toxicity experiments. As shown in TABLE 22 and FIGURE 35, thirty of the rabbits were classified as rapid acetylators, with SDZ N-acetylation half-lives ranging from 22 to 44 min, and first order rate constants ranging from 0.0156 to 0.0312 min^{-1}. Thirty-four of the rabbits were classified as slow acetylators, with SDZ N-acetylation half-lives ranging from 71 to 297 min and first order rate constants ranging from 0.00234 to 0.00980 min^{-1} (TABLE 23 and FIGURE 35). All acetylator phenotype classifications determined in this manner were consistent with pedigree information.

Serum levels of INH in rapid and slow acetylator rabbits:

Serum levels of INH were measured over time in a rapid and slow acetylator rabbit after the intramuscular injection of 30 mg/kg of INH. As shown in FIGURE 36, peak serum levels of INH were achieved in both the rapid and slow acetylator phenotype 20 min after injection. The genetically determined differences were expressed at this high dose of INH, as the N-acetylation half-life of INH was 132 min in the slow acetylator and 58 min in the rapid acetylator.

Toxicity of daily injections of 15 mg/kg INH:

Intramuscular injections of 15 mg/kg INH were administered once each weekday (5 days/week) to an experimental group of seven rapid and seven slow acetylator rabbits. In addition, a control group of three rapid and three slow acetylators rabbits received sterile water injections. The rabbits were each monitored for SGPT level elevations

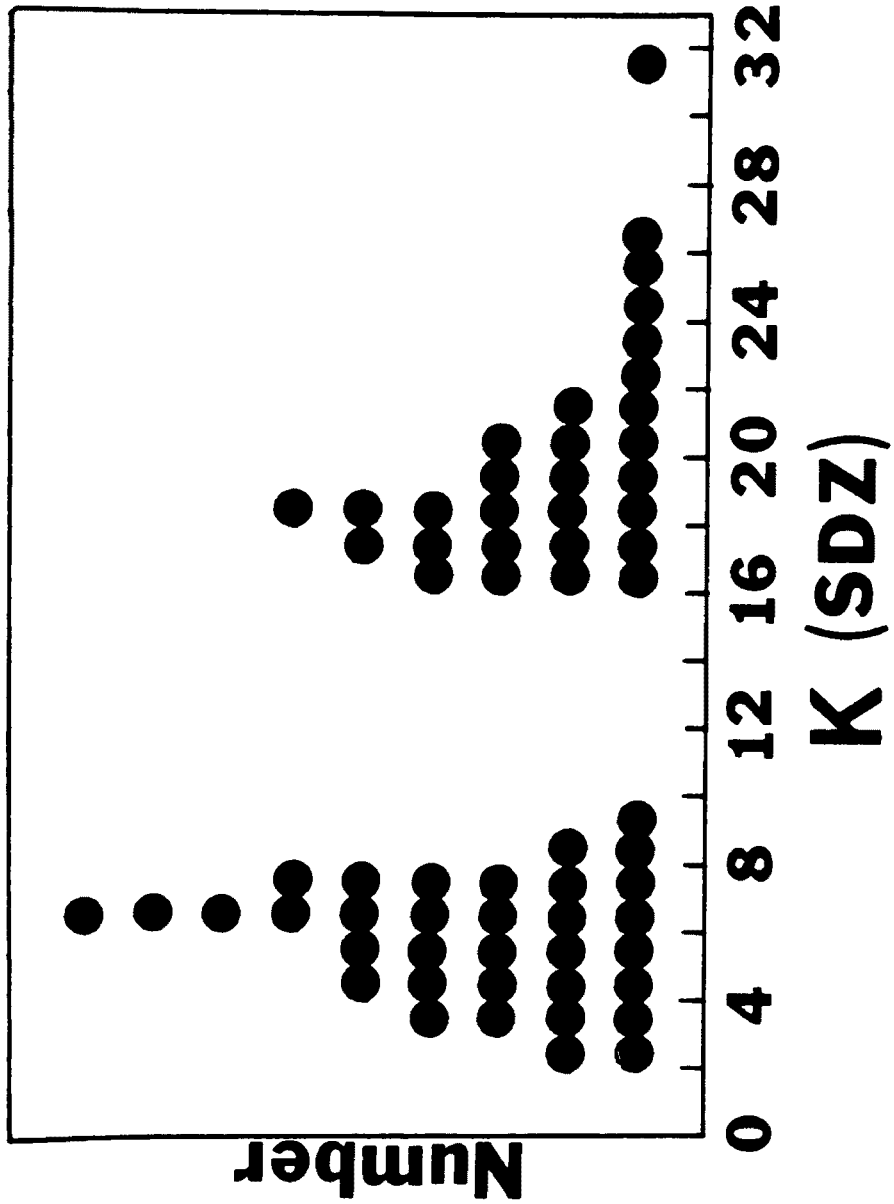


FIGURE 35. Pharmacokinetic rate constants of SDZ N-acetylation in rapid and slow acetylator rabbits.

Each dot represnts an individual rabbit used in the toxicity experiments described in CHAPTER VII. Values on the abscissa are $(min)^{-1}$ x 1000. For more specific information, see TABLES 22-23.

TABLE 22. Pharmacokinetic constants of SDZ N-acetylation in rapid acetylator rabbits.

NUMBER	SEX	T 1/2 (min)	K (min^{-1})	r
167	M	26	.0265	.9913
185	F	37	.0190	.9879
186	M	28	.0245	.9914
187	M	34	.0206	.9955
189	F	33	.0209	.9875
194	M	33	.0208	.9960
195	F	32	.0217	.9944
196	M	43	.0160	.9838
197	M	43	.0162	.9547
198	M	37	.0189	.9945
199	M	43	.0163	.9713
200	F	44	.0156	.9978
201	F	35	.0195	.9936
202	M	38	.0180	.9870
203	F	38	.0183	.9804
204	F	37	.0187	.9642
205	F	38	.0184	.9542
206	F	22	.0312	.9977
233	M	38	.0182	.9773
234	F	27	.0259	.9810
235	F	39	.0178	.9908
240	M	36	.0191	.9860
251	F	39	.0176	.9907
253	F	30	.0232	.9757
264	F	43	.0161	.9786
267	F	32	.0219	.9892
268	F	37	.0190	.9933
269	F	40	.0172	.9972
272	F	40	.0172	.9948
274	M	30	.0230	.9971

TABLE 23. Pharmacokinetic constants of SDZ N-acetylation in slow acetylator rabbits.

NUMBER	SEX	T 1/2 (min)	K (min^{-1})	r
180	F	93	.00323	.9776
181	F	87	.00348	.9815
183	F	71	.00980	.9908
184	F	73	.00412	.9678
208	M	297	.00234	.8525
209	M	122	.00568	.9708
210	F	102	.00678	.9865
211	F	99	.00698	.9737
212	M	87	.00797	.9825
213	M	109	.00636	.9797
214	F	101	.00685	.9878
215	F	122	.00567	.9901
216	F	115	.00601	.9788
217	F	84	.00822	.9873
218	M	97	.00714	.9859
219	F	112	.00620	.9977
220	F	216	.00310	.9925
221	F	272	.00255	.9433
228	M	150	.00460	.9953
238	M	98	.00707	.9422
242	M	184	.00377	.9877
244	F	163	.00424	.9206
246	F	106	.00654	.9877
247	M	97	.00714	.9859
261	F	168	.00413	.9227
279	F	138	.00503	.9731
281	F	99	.00701	.9873
282	M	166	.00417	.9791
284	F	130	.00535	.9867
290	F	127	.00540	.9818
293	F	113	.00616	.9890
295	M	109	.00637	.9399
309	M	89	.00781	.9785
310	F	84	.00830	.9930

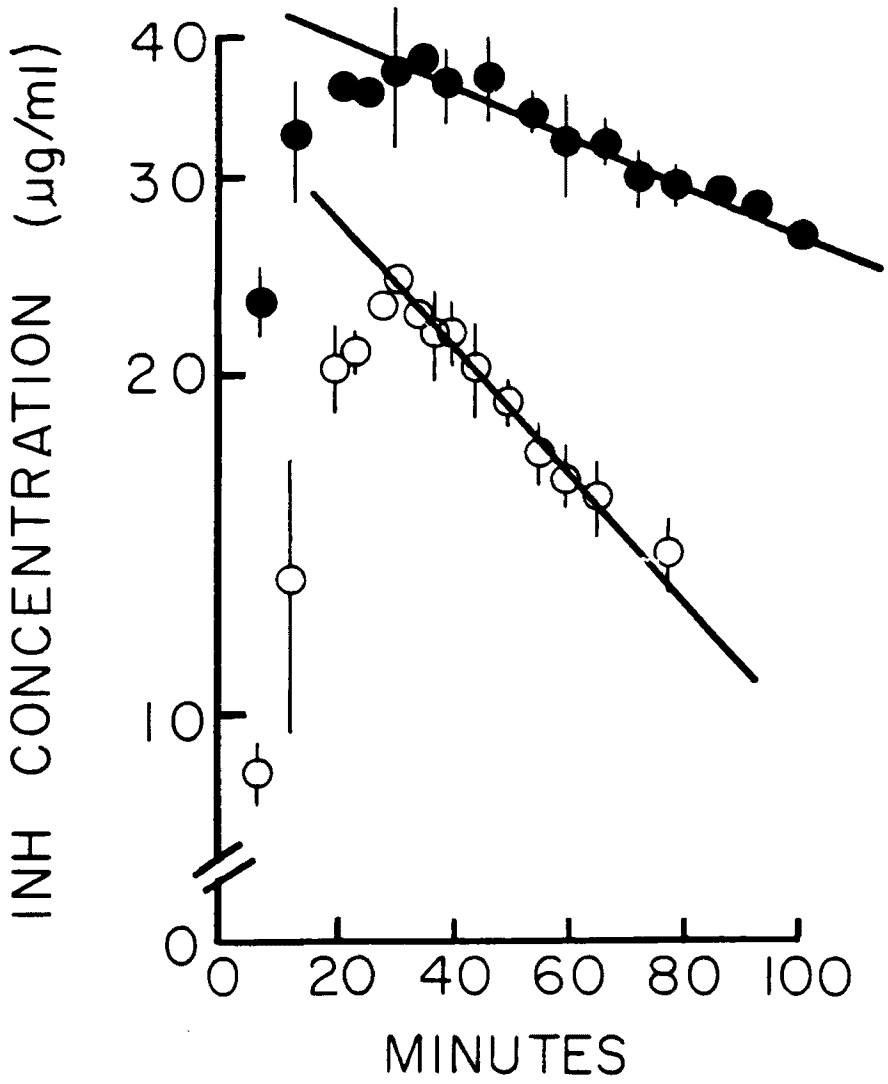


FIGURE 36. In vivo N-acetylation of INH in rapid and slow acetylator rabbits.

Serum levels of INH in a rapid acetylator (open circles) and slow acetylator (closed circles) rabbit following the intramuscular injection of 30 mg/kg INH. The abscissa represents minutes post-injection. Each data point is the average of 3 serum determinations and lines above and below each dot indicate S.D. In instances where lines are not shown, the S.D. lies within the dot. The N-acetylation half-life was 58 min in the rapid and 132 min in the slow acetylator.

and signs of CNS toxicity. None of the rabbits showed appreciable SGPT level elevations or any signs of CNS toxicity for the duration of the experiment which lasted 12 weeks (data not shown).

Toxicity of daily injections of 30 mg/kg INH:

Intramuscular injections of 30 mg/kg INH were administered once each weekday (5 days/week) to an experimental group of eight rapid and seven slow acetylator rabbits. In addition, three rapid and three slow acetylator rabbits (the same group as described in the previous paragraph) received sterile water injections as a control group. The rabbits were monitored for SGPT level elevations and signs of CNS toxicity. None of the rabbits showed appreciable SGPT level elevations; the control and rapid acetylators for the total duration of the experiment (12 weeks) and the slow acetylators for the duration of their survival in the study (1-10 weeks). As shown in TABLE 24, all rabbits in the control group, and all of the rapid acetylator rabbits in the experimental group survived the entire experiment of 12 weeks without signs of CNS toxicity. In contrast, the slow acetylators of the experimental group all died from CNS-related toxicity during the course of the experiment. The range of survival time for these rabbits was 1-10 weeks for an average of 3.4 weeks. Thus, the difference in CNS toxicity between rapid and slow acetylator rabbits on this dosage regimen was highly significant ($p < .001$). In addition, within the slow acetylator experimental group, an inverse correlation was observed between the length of the SDZ N-acetylation half-life determined in vivo and the survival time on this dosage regimen (TABLE 24).

TABLE 24. CNS toxicity of subacute* INH administration in rapid and slow acetylator rabbits.

RABBIT	SDZ HALF-LIFE (min)	PHENOTYPE	SEX	SURVIVAL TIME (weeks)
206	22	Rapid	F	> 12
186	28	Rapid	M	> 12
195	32	Rapid	F	> 12
194	33	Rapid	M	> 12
201	35	Rapid	F	> 12
198	37	Rapid	M	> 12
204	37	Rapid	F	> 12
200	44	Rapid	F	> 12
				> 12
212	87	Slow	M	10
218	95	Slow	M	7
214	101	Slow	F	1
210	102	Slow	F	1
219	112	Slow	F	1
215	122	Slow	F	3
208	297	Slow	M	1
				3.4 ± 1.4

*Each of the rabbits was 7-8 months of age at the beginning of the experiment. The survival time of the rapid acetylator rabbits is significantly higher ($p < .001$) than the slow acetylator rabbits. The total duration of the experiment was 12 weeks.

Toxicity of daily 30 mg/kg INH in phenobarbital-induced rabbits:

Seven rapid and seven slow acetylator rabbits in an experimental group, and three rapid and three slow acetylator rabbits in a control group, were allowed free access to a 0.1% solution of phenobarbital instead of water for the duration of this experiment. The animals were monitored for weight changes, signs of CNS toxicity, and SGPT elevations. On the fifth day of phenobarbital induction, the experimental group of rapid and slow acetylator rabbits began daily intramuscular injections of 30 mg/kg INH for 6 total days. The control group of rapid and slow acetylator rabbits received daily intramuscular injections of sterile water over the same time period. SGPT levels were measured prior to phenobarbital induction and at the conclusion of the experiment. As shown in TABLE 25, the average weight loss in the experimental group of slow acetylator rabbits was 27.7% which was significantly higher ($p < .001$) than both the slow acetylator control group (5.1%) and the experimental rapid acetylator group (4.0%). The average values of weight lost in rapid acetylator experimental and control groups (4.0 and 2.9% respectively) were not significantly ($p > .05$) different. SGPT levels in the rapid and slow acetylator experimental groups were each significantly higher ($p < .025$) at the conclusion of the study compared to measurements prior to drug administration. However, the final SGPT levels in the rapid and slow acetylator experimental groups were not significantly ($p < .025$) higher than in the control group. Final SGPT levels in the slow acetylator experimental group (average of 141.5 units) were significantly ($p < .025$) higher than final SGPT levels in the rapid acetylator experimental group (average of 53.1 units). At the

TABLE 25. Weight loss and SGPT levels in phenobarbital-induced rapid and slow acetylator rabbits treated with INH.

RABBIT NUMBER	PHENOTYPE	TREATMENT	WEIGHT LOSS (%)	CONTROL SGPT*	POST-DRUG SGPT*
167	Rapid	INH	8.0	34	65
187	Rapid	INH	0	33	75
189	Rapid	INH	4.3	25	82
197	Rapid	INH	6.7	47	51
202	Rapid	INH	2.7	21	30
203	Rapid	INH	3.4	25	30
205	Rapid	INH	2.7	27	39
			3.97 ± 1.01^a	30.3 ± 3.3^b	53.1 ± 8.0^c
185	Rapid	control	3.4	39	95
196	Rapid	control	3.8	40	100
199	Rapid	control	1.4	49	125
			$2.87 \pm .74^d$	42.7 ± 3.2^e	106.7 ± 9.3^f
180	Slow	INH	38.3	53	65
184	Slow	INH	25.0	43	74
209	Slow	INH	33.9	53	155
211	Slow	INH	20.6	46	125
213	Slow	INH	43.1	44	285
216	Slow	INH	12.2	116	145
219	Slow	INH	21.0	41	----
			27.7 ± 4.2^g	56.6 ± 10^h	141.5 ± 32^i
181	Slow	control	3.8	---	63
183	Slow	control	7.9	38	140
217	Slow	control	3.6	86	--
			5.1 ± 1.4^j	62.0 ± 24	101.5 ± 39

* SGPT units are described in Methods. In humans, SGPT values are usually less than 40 in healthy individuals. SGPT values may rise as high as 4000 units in human liver necrosis. SGPT values in normal, healthy rabbits also are usually less than 40 units.

g significantly higher than a and j ($p < .001$).
a not significantly higher than d ($p > .05$).
c significantly higher than b ($p < .025$)
i significantly higher than c ($p < .025$).
i significantly higher than h ($p < .025$).

conclusion of the experiment, each of the animals in both groups were sacrificed and liver specimens were removed and analyzed for histological evidence of hepatotoxicity in a single blind study in collaboration with Dr. Gerald Abrams of the Dept. of Pathology at the University of Michigan Medical School. Vacuolar changes were observed in hepatocytes from some of the animals, but no correlations between acetylator phenotype and type or extent of hepatotoxicity were noted, and the liver histology of the experimental group was not appreciably different from that of the control group.

Acute toxicity of INH and antagonism by pyridoxine:

One group of three rapid and three slow acetylator rabbits was administered an intramuscular injection of 30 mg/kg INH on day 1 of the experiment, and a 10 mg/kg higher dose of INH on each succeeding day, up to a total dose of 110 mg/kg, at which time the experiment ended. A second group of three rapid and three slow acetylator rabbits received the same dosage schedule of INH, but also received at the same time, by separate intramuscular injection, an equal mg/kg dose of pyridoxine hydrochloride. As shown in TABLE 26, all six (both rapid and slow acetylators) of the first group on INH only died of CNS toxicity during the course of the experiment. In contrast, four of the six rabbits in the second group which also received pyridoxine survived the experiment. The two rabbits of this group (one rapid and one slow) which died of CNS toxicity did so only at the highest dose (110 mg/kg).

Subacute toxicity of INH and antagonism by pyridoxine:

One group of three rapid and three slow acetylator rabbits received a daily (7 days/week) intramuscular injection of 0.4

TABLE 26. CNS toxicity of acute INH administration and effects of pyridoxine in rapid and slow acetylator rabbits.

Rabbit	Phenotype	Drug Regimen	Lethal Dose (mg/kg)	Cumulative Dose (mg/kg)
206	Rapid	INH	110	630
186	Rapid	INH	110	630
194	Rapid	INH	100	520
195	Rapid	INH & pyridoxine	Survived	630
201	Rapid	INH & pyridoxine	Survived	630
204	Rapid	INH & pyridoxine	110	630
228	Slow	INH	70	250
242	Slow	INH	100	520
221	Slow	INH	70	250
247	Slow	INH & pyridoxine	Survived	630
238	Slow	INH & pyridoxine	Survived	630
220	Slow	INH & pyridoxine	110	630

mMoles/kg INH (approximately 55 mg/kg). A second group of three rapid and three slow acetylator rabbits received a similar dosage regimen of INH, but were also pretreated (about 15 min before) with an intramuscular injection of 0.4 mMoles/kg pyridoxine. The animals were monitored for weight changes and signs of CNS toxicity. As shown in TABLE 27, pretreatment with pyridoxine increased the survival period from CNS toxicity significantly ($p < .005$) in both rapid and slow acetylator rabbits. Two of the three slow acetylator rabbits and all three of the rapid acetylator rabbits that were pretreated with pyridoxine survived the entire experiment of 16 consecutive days. The average survival time of the rapid acetylator rabbits receiving INH alone (8.0 days) was higher, but not significantly higher ($p > .05$) than the average survival time in the slow acetylators on INH alone (5.7 days).

Although rabbits pretreated with pyridoxine avoided lethal incidences of CNS toxicity, nevertheless, they suffered considerable anorexia and weight loss (TABLE 28). No appreciable differences in weight loss were observed between the rapid and slow acetylator groups (TABLE 28).

Subacute toxicity of MAH and antagonism by pyridoxine:

One group of three rapid and three slow acetylator rabbits received a daily (7 days/week) intramuscular injection of 0.4 mMoles/kg MAH. A second group of three rapid and three slow acetylator rabbits received a similar dosage regimen of MAH, but were always pretreated (about 15 min before) with an intramuscular injection of 0.4 mMoles/kg of pyridoxine. The animals were monitored for weight changes and signs of CNS toxicity. As shown in TABLE 29,

TABLE 27. CNS toxicity of subacute high dose INH with and without pyridoxine in rapid and slow acetylator rabbits.

NUMBER	PHENOTYPE	TREATMENT	T1/2 (min)	SEX	AGE (months)	SURVIVAL TIME (days)
264	Rapid	INH	43	F	26	10
267	Rapid	INH	32	F	26	9
268	Rapid	INH	37	F	26	5
						8.0 ± 1.5[a]
269	Rapid	INH + PDX*	40	F	26	> 16
272	Rapid	INH + PDX	40	F	26	> 16
274	Rapid	INH + PDX	30	M	26	> 16
						> 16.0[b]
281	Slow	INH	99	F	20	5
290	Slow	INH	127	F	19	7
310	Slow	INH	84	F	16	5
						$5.7 \pm .7$[c]
246	Slow	INH + PDX	106	F	28	16
293	Slow	INH + PDX	113	F	19	16
309	Slow	INH + PDX	89	M	16	11
						14.3 ± 1.7[d]

Injections were 0.4 mMoles/kg intramuscular for both INH and PDX
* Pyridoxine
b significantly higher than a ($p < .005$)
d significantly higher than c ($p < .005$)

TABLE 28. Weight loss produced by subacute INH administration with and without pyridoxine in rapid and slow acetylator rabbits*.

RABBIT NUMBER	2	4	6	8	10	12	14
	(days of treatment)						
264	4.5	7.9	11.2	15.7	--	--	--
267	3.3	7.8	10.0	13.3	--	--	--
268	2.3	5.8	--	--	--	--	--
269	6.9	8.0	9.2	12.6	12.6	12.6	12.6
272	0	6.3	8.8	15.0	21.3	30.0	33.8
274	0	2.7	2.7	2.7	4.0	6.7	13.3
281	3.6	2.4	--	--	--	--	--
290	1.1	8.0	10.3	--	--	--	--
310	3.5	7.1	--	--	--	--	--
246	3.2	6.5	11.3	11.3	17.7	21.0	24.2
293	3.8	3.8	10.0	15.0	11.3	18.8	23.8
309	0	2.5	6.3	6.3	13.8	--	--

* Weight loss is expressed as percent of original weight. Rabbits of the first and third groups did not receive pyridoxine, while rabbits of the second and fourth groups did receive pyridoxine. For further details, see TABLE 27. Dashed lines indicate that the animal had died.

nine of the twelve animals died of CNS toxicity during the course of the experiment. Each of the three rapid acetylators in the second group receiving pretreatments of pyridoxine survived the entire experiment of 24 consecutive days, which is a significantly higher ($p < .001$) survival time than that found in the rapid acetylator group receiving MAH alone (average of 7.3 days), the slow acetylator group receiving MAH alone (average of 5.3 days), and the slow acetylator group pretreated with pyridoxine (average of 7.7 days). Although pretreatment with pyridoxine also increased survival times within the slow acetylator groups (5.3 versus 7.7 days), this increase was not significant ($p > .05$). The rapid acetylators on MAH alone survived longer than the corresponding slow acetylators did (averages of 7.3 versus 5.3 days), although this difference was only marginally significant ($p < .10$).

As shown in TABLE 30, all of the rabbits experienced considerable anorexia and weight loss, even those rabbits that were protected from CNS toxicity by pyridoxine pretreatment.

TABLE 29. CNS toxicity of subacute high dose MAH with and without pyridoxine in rapid and slow acetylator rabbits.

NUMBER	PHENOTYPE	TREATMENT	T1/2 (min)	SEX	AGE (months)	SURVIVAL TIME (days)
233	Rapid	MAH	37	M	30	9
234	Rapid	MAH	27	F	30	7
251	Rapid	MAH	39	F	28	6
						$7.3 \pm .8$ [a]
240	Rapid	MAH + PDX*	36	M	30	> 24
235	Rapid	MAH + PDX	39	F	30	> 24
253	Rapid	MAH + PDX	30	F	28	> 24
						> 24.0 [b]
244	Slow	MAH	163	F	28	5
279	Slow	MAH	138	F	20	6
282	Slow	MAH	166	M	20	5
						$5.3 \pm .3$ [c]
261	Slow	MAH + PDX	168	F	28	5
284	Slow	MAH + PDX	130	F	20	12
295	Slow	MAH + PDX	109	M	19	6
						7.7 ± 2.2 [d]

Injections were 0.4 mMoles/kg intramuscular for both MAH and PDX
* Pyridoxine
b significantly higher than a ($p < .001$)
d is not significantly higher than c ($p > .05$)
a significantly higher than c ($p < .10$)
b significantly higher than d ($p < .001$)

TABLE 30. Weight loss produced by subacute MAH administration with and without pyridoxine in rapid and slow acetylator rabbits*.

RABBIT NUMBER	2	4	6	8	10	18	24
			(days of treatment)				
233	0	2.7	6.7	13.3	--	--	--
234	3.3	6.5	9.8	--	--	--	--
251	3.3	7.5	--	--	--	--	--
240	0	2.7	6.8	11.0	12.3	15.1	17.8
235	0	1.2	2.3	11.6	10.5	18.6	23.3
253	2.4	5.9	5.9	11.8	15.3	24.7	29.4
244	4.7	11.8	--	--	--	--	--
279	5.2	10.4	--	--	--	--	--
282	5.5	9.6	--	--	--	--	--
261	0	8.2	--	--	--	--	--
284	3.8	7.5	8.8	15.0	15.0	--	--
295	2.9	4.4	--	--	--	--	--

* Weight loss is expressed as percent of original weight. Rabbits of the first and third groups did not receive pyridoxine, while rabbits of the second and fourth groups did receive pyridoxine. For further details, see TABLE 29. Dashed lines indicate that the animal had died.

Discussion

INH overdosage in humans and the subsequent lethal incidences of CNS toxicity are currently a major problem due to the frequent use of INH in the treatment and prophylaxis of tuberculosis. For example, among Apache Indians of the Southwestern United States, INH is involved in 7% of all suicide attempts and 19% of all suicide deaths (Sievers and Herrier, 1975). Consistent with the human toxicity, we have shown that INH (and MAH) produced lethal occurrences of CNS toxicity in rabbits. In most instances there was a lag period following administration of at least one hour before the onset of irritability and seizures. The toxicity usually began with intermittent seizures and marked sensitivity to audiogenic stimuli. This was usually followed by a frantic running episode, with considerable banging of the metal cage walls, followed by a tonic extensor phase during which the animals often died of respiratory arrest. In some cases, the animals would survive this episode, but would die during the next 24 hours. Similar findings of hydrazine-induced CNS toxicity have previously been reported in many species including mice, rats, rabbits, dogs, cats, monkeys, birds, frogs, and humans (e.g., Williams and Bain, 1961). Interestingly, the marked sensitivity of the dog to this toxicity was unexplainable at that time, but now it is known that the dog is unique in its virtual absence of N-acetylation capacity (Weisburger et al., 1964; Lower and Bryan, 1973).

The mechanism of the hydrazine-induced CNS toxicity is not completely known, but there is general agreement that hydrazines produce the toxicity by causing a functional pyridoxine

(Vitamin B_6) deficiency by formation of hydrazine-pyridoxal hydrazones as illustrated in FIGURE 37 (e.g., Williams and Bain, 1961; Vilter, 1964). Cornish (1969) showed that only hydrazines containing free amine groups would react with pyridoxal phosphate, an important finding since only these types of hydrazines will produce the CNS toxicity (O'Brien et al., 1964). In addition, high doses of INH in animals have been shown to produce marked decreases in brain levels of pyridoxal phosphate, and increased brain levels of pyridoxal-hydrazones (e.g., Williams and Bain, 1961). Furthermore, administration of INH has been shown to produce increased urinary excretion of pyridoxal-hydrazones in man (Biehl and Vilter, 1954).

Depletion of pyridoxal phosphate is implicated as a critical event in the induction of this CNS toxicity because it serves as the coenzyme for glutamic acid decarboxylase, the brain enzyme responsible for the synthesis of GABA (e.g., Holtz and Palm, 1964), an amino acid which acts as an inhibitor of synaptic transmission in the CNS (e.g., Cooper et al., 1978). Thus, Killam and Bain initially proposed that hydrazine-induced CNS toxicity was accompanied by decreases in brain GABA levels that result in disinhibition of synaptic transmission (Killam, 1957; Killam and Bain, 1957). Subsequently, Wood and Peesker (1972a,b) also showed that the onset of hydrazine-induced seizures was closely associated with decreases in CNS levels of GABA, and in addition, also showed that administration of pyridoxine prevented both the decreases in brain GABA levels and the incidence of seizures. Consistent with this hypothesis, pyridoxine administration has been shown to protect animals and man from the CNS toxicity of hydrazines (e.g., Reilly et al., 1953; Holtz and Palm, 1964; Cornish, 1969;

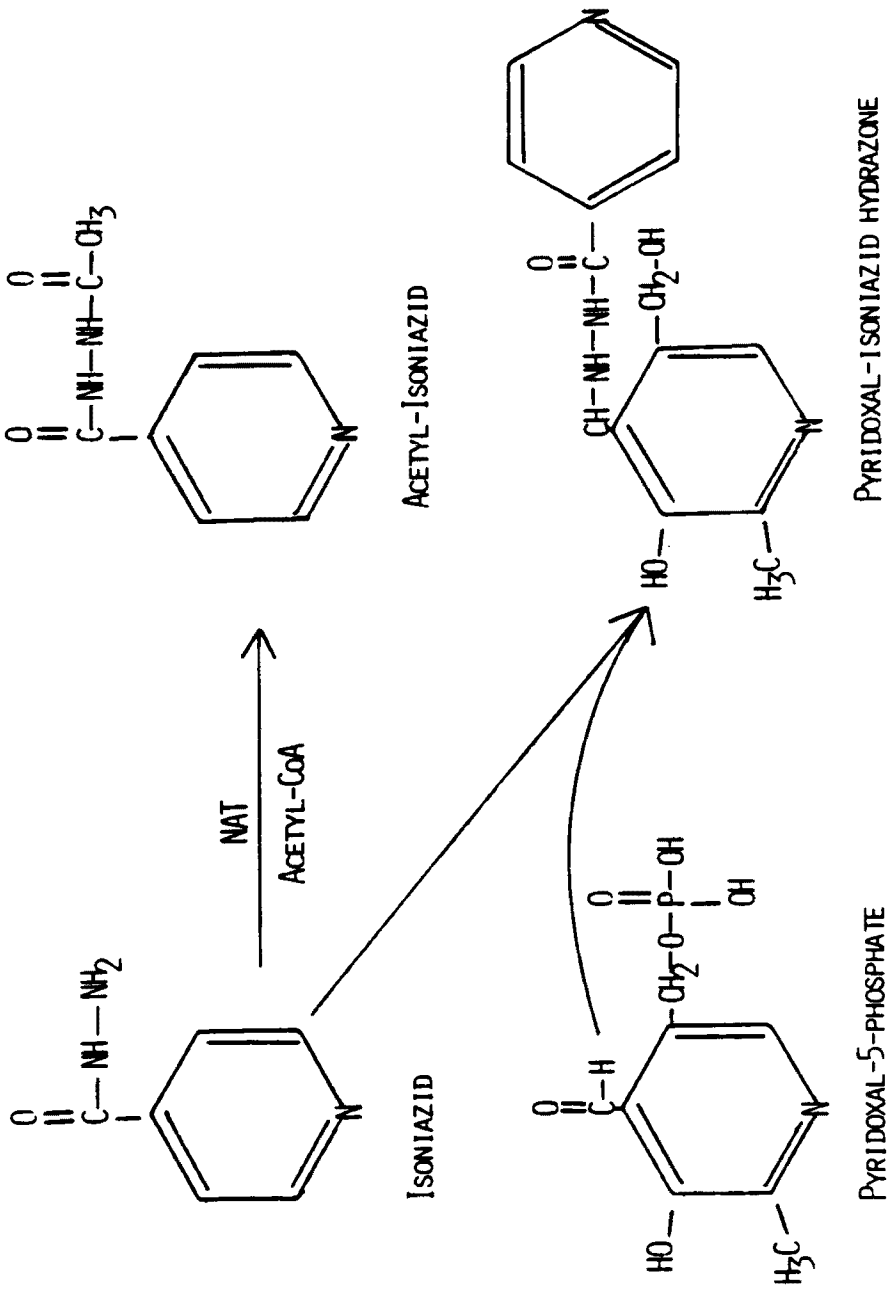


FIGURE 37. Formation of INH-pyridoxine hydrazones.

Sievers and Herrier, 1975; Coyer and Nicholson, 1976).

The results of our experiments confirm that pyridoxine is useful in protecting against hydrazine-induced CNS convulsions. In high dose acute toxicity studies of INH (TABLE 26), co-administration of pyridoxine decreased mortality in both rapid and slow acetylators, although it did not completely eliminate it. In INH subacute toxicity experiments (TABLES 27 and 28), co-administration of equal molar doses of INH and pyridoxine resulted in significantly higher ($p<.005$) survival times regardless of phenotype than in rabbits receiving INH alone. In MAH subacute toxicity experiments (TABLES 29 and 30), co-administration of equal molar doses of MAH and pyridoxine resulted in significantly higher ($p<.001$) survival times in the rapid acetylators, but the results were not nearly as dramatic in slow acetylators.

Phenobarbital has previously been shown to induce liver microsomal P-450 levels and enhance INH-induced hepatotoxicity (Nelson et al., 1976; Timbrell et al., 1980). In our experiments examining the effects of INH in phenobarbital-induced animals, the weight losses in the slow acetylator animals treated with INH were substantial, but SGPT levels, indicative of hepatotoxicity, and examinations of liver histology were not significantly different from controls (TABLE 25). Similarly, although slow acetylators had significantly higher weight loss and SGPT levels than rapid acetylators receiving INH, differences in liver histology between the two phenotypes were not found (TABLE 25). Thus, our findings concerning INH-induced toxicity in phenobarbital-induced animals are interesting, but not consistent enough to produce definitive conclusions regarding the effect of

acetylator status without further studies.

The significance of acetylator status on INH-induced CNS toxicity is best illustrated in TABLE 24. All rapid acetylator rabbits receiving daily injections of 30 mg/kg INH survived the entire duration of the experiment of 12 weeks, whereas all the slow acetylators died from this dosage regimen in an average of 3.4 weeks. Similarly, rapid acetylators were also less resistant to the lethal CNS toxicity of INH in each of the other experiments using other dosage regimens, although differences between rapid and slow acetylators in these instances were less pronounced. Since a free amine group is required for pyridoxal-hydrazone formation (Cornish, 1969) and subsequent CNS toxicity (O'Brien et al., 1964) we propose that N-acetylation via liver NAT can serve as an important detoxification step for hydrazine-induced CNS toxicity (FIGURE 37). The results of our experiments are consistent with this hypothesis, since they indicate that differences in N-acetylation capacity between rapid and slow acetylators are important in susceptibility to hydrazine-induced toxicity.

INH- and MAH-induced anorexia and weight loss were not reduced by co-administration of pyridoxine (TABLES 28 and 30). These results suggest that this toxicity is produced through a mechanism different from pyridoxal-hydrazone formation. Based upon our experiments, differences in acetylator status appear to be important in the intensity of hydrazine-induced anorexia and weight loss only under conditions of phenobarbital induction as shown in TABLE 25. In each of the other experiments, weight loss and anorexia did not appear to be affected by acetylator phenotype.

CHAPTER VIII

POLYMORPHIC N-ACETYLATION OF PHENELZINE AND MONOACETYLHYDRAZINE BY RABBIT LIVER N-ACETYLTRANSFERASE

Introduction

N-acetylation is a major metabolic step in the biotransformation of arylamines and hydrazines via the cytosolic liver enzyme NAT (E.C. 2.3.1.5) (Juchau and Horita, 1972; Weber, 1973). Liver NAT, and, consequently, the total N-acetylating capacity of an individual, is subject to genetic variation. Each person can be classified as either a phenotypically rapid or slow acetylator of a number of arylamine and hydrazine drugs and carcinogens such as INH, SMZ, PA, and 2AF (Evans et al., 1960; Peters et al., 1965; Weber, 1973; Glowinski et al., 1978; Weber and Hein, 1979). Person-to-person differences in N-acetylating capacity may have important consequences with respect to arylamine and hydrazine drug toxicity. For example, slow acetylators are more susceptible to the toxic central nervous system actions of INH and MAH (see CHAPTER VII), and to drug-induced lupus erythematosus from INH, hydralazine, and PA (Drayer and Reidenberg, 1977; Lunde et al., 1977; Batchelor et al., 1980). An additional report found a higher predominance of arylamine-induced bladder cancer among slow acetylators (Lower et al., 1979), but further studies are needed to confirm this observation. Rapid acetylators are reported to be more susceptible to the hepatotoxic effects of INH (Mitchell et al., 1975;

Bernstein, 1981) , although these findings remain highly controversial (Ellard et al., 1978, 1981; Bernstein, 1980, 1981).

Interestingly, INH itself is not hepatotoxic; it must first be metabolically activated to its hepatotoxic metabolite, MAH by a pathway which includes the polymorphic N-acetylation reaction catalyzed by NAT (Mitchell et al., 1975a, 1976; Nelson et al., 1976; Timbrell et al., 1977, 1980; Timbrell, 1979). Ellard and his colleagues have argued that MAH is also polymorphically acetylated, and, consequently, rapid acetylators metabolize a greater percentage of the hepatotoxic metabolite MAH to its inactive metabolite diacetylhydrazine than slow acetylators (Ellard and Gammon, 1976, 1977). MAH, however, is markedly different in structure from any known substrate of NAT, and its polymorphic N-acetylation by NAT in vitro has yet to be demonstrated.

In addition to MAH, questions also exist concerning the polymorphic N-acetylation of PHZ, another hydrazine drug known to be a powerful monoamine oxidase inhibitor (Johnstone and Marsh, 1973), currently employed as an effective treatment for depressive illnesses (Ravaris et al., 1980; Rowan et al., 1980). Although PHZ is similar in structure to INH, its N-acetylation has yet to be demonstrated conclusively, and whether or not it is subject to polymorphic N-acetylation has not yet been established at the present time (Marshall, 1976; Sanders and Rawlins, 1979).

The purpose of this study was to investigate 1) whether the N-acetylation of MAH and PHZ by NAT occurs in vitro, 2) whether their N-acetylation exhibits a polymorphic expression, or like PABA exhibits a monomorphic N-acetylation expression (see CHAPTERS II and III) , 3)

whether MAH and PHZ are N-acetylated by the same genetically polymorphic NAT that acetylates INH, SMZ, PA, 2AF, etc., and 4) the Michaelis-Menten constants for the N-acetylation of these substrates.

The rabbit was chosen as the animal model for this study because rapid and slow acetylators have been identified in this species (Frymoyer and Jacox, 1963a,b; Weber et al., 1976). Although genetic variation in the N-acetylation of arylamines has also been observed in mouse (Tannen and Weber, 1980a; Glowinski et al., 1982a) , hamster (see Chapter IV), and rat (D. A. Morgott, J. V. Torres, and W. W. Weber, unpublished observations) the qualitative nature of the acetylation polymorphism is different in these species from that in man, and only the rabbit shows polymorphic expression in the N-acetylation of hydrazine compounds such as INH (see CHAPTERS II and IV; Tannen, 1978). Furthermore, methods have recently been reported for the isolation of highly purified rabbit liver NAT (Glowinski et al., 1980).

Methods

Chemicals and Reagents:

(see Table 33)

Source of N-acetyltransferase:

Rapid and slow acetylator rabbit liver was obtained from selected lines of mature New Zealand White rabbits of known acetylator phenotypes as described in CHAPTER II.

Liver N-acetyltransferase purification:

Rabbits were killed by cardiac puncture. Immediately after death, the liver was homogenized (20% w/v) in 50 mM pyrophosphate sodium chloride buffer, 1 mM DTT, pH 7.0, followed by centrifugation at 10,000 g for 20 minutes and 105,000 g for 60 minutes. The supernatant fraction was partially purified by DEAE-cellulose chromatography, followed by Sephacryl S-200 chromatography as previously described (Glowinski et al., 1980; see Chapter III B). Peak activity fractions were combined and concentrated in a Millipore ultrafiltration apparatus. All purification steps were conducted at $4^{\circ}C$.

PABA and SMZ NAT activity determinations:

PABA and SMZ NAT activities were determined at $37^{\circ}C$ by a micromodification of the Bratton-Marshall procedure as described in CHAPTER II.

INH NAT activity determinations:

INH NAT activity was determined at $37^{\circ}C$ as described in Chapter II.

MAH and PHZ NAT activity determinations:

Assays were designed to measure the AcCoA-dependent acetylation

of MAH and PHZ and were conducted in 1.5 ml capped polypropylene microsample tubes. To 50 ul of suitably diluted Sephacryl S-200 purified enzyme preincubated at 37°C was added 20 ul of 4 mM MAH or PHZ. The reaction was started with the addition of 20 ul of 10 mM AcCoA. Except where noted, final concentrations in the reaction mixture were 0.89 mM for MAH or PHZ and 2.22 mM for AcCoA. The reaction was terminated by the addition of 50 ul of trichloroacetic acid (10% w/v) at specified time points to give linear plots of activity with time. After centrifugation in a Brinkman Eppendorf Centrifuge 3200, color development was achieved with the addition of 1 ml of 0.5 M potassium phosphate dibasic solution (KH_2PO_4) with mixing, followed by the addition of 100 ul of TNBS solution (0.5% w/v). After final mixing and recentrifugation, the tubes were allowed to stand for at least 30 minutes. Absorbance was measured at 500 nm against a water blank in a Beckman Model 35 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 of AcCoA. Standard curves generated for this TNBS assay of NAT activity are illustrated in FIGURE 38. All assays were carried out in duplicate or triplicate at 37°C. Under the conditions of this assay, a decrease of one sod absorbance unit corresponds to the acetylation of 85 pmoles of MAH or 90 pmoles of PHZ.

Protein Determinations:

Protein was determined in the Sephacryl S-200 fractions by the method of Lowry et al. (1951).

FIGURE 38. Standard curves for TNBS assay of NAT activity.

The number of nmoles of MAH or PHZ added under conditions described in the TNBS assay of NAT activity (Methods) is plotted on the abscissa versus sod at 500 nm on the ordinate. These standard curves yield constants of 0.085 nmoles/sod for MAH and 0.090 nmoles/sod for PHZ. See the text for further details.

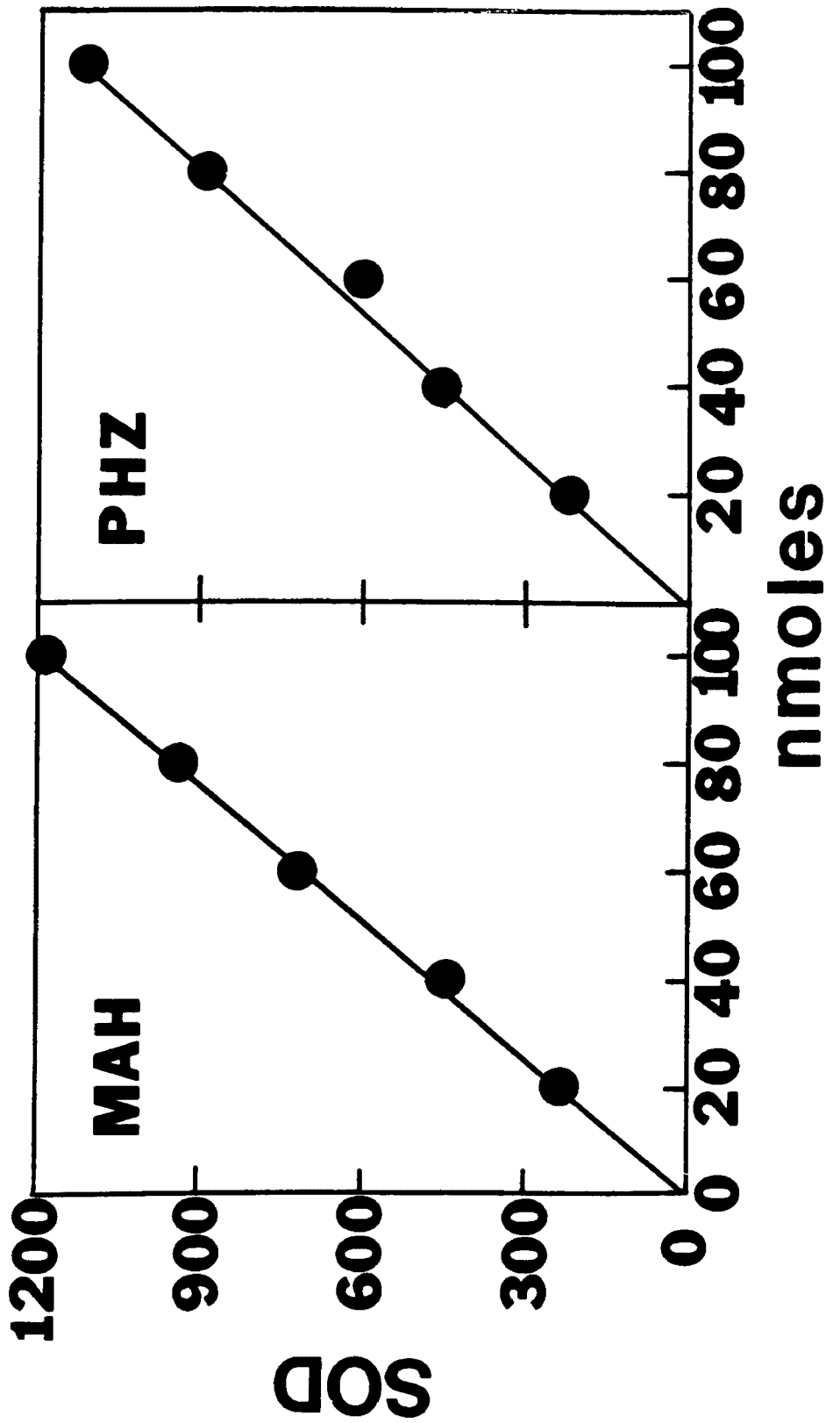

SOD
1200
900
600
300
0
MAH
PHZ
0 20 40 60 80 100
0 20 40 60 80 100
nmoles

FIGURE 38

Data analysis:

NAT activity determinations were conducted at protein concentrations and over time periods that yielded nearly linear plots of activity with time. Initial velocities were determined by extrapolation of the time-activity curves to the addition of AcCoA (time zero) and measuring the slope of the tangent to the curve. PABA, SMZ, MAH, and PHZ NAT heat inactivation rates were calculated using semi-log linear least squares regression analysis. Kinetic constants for PHZ and MAH NAT were calculated with a modified Hyper-program by Cleland (1963) which uses a least squares linear regression method.

Results

Copurification of INH, MAH, and PHZ NAT activities:

INH, MAH, and PHZ NAT activities could not be resolved by extensive purification steps through Sephacryl S-200 chromatography (FIGURE 39). Each of the three NAT activities were found only in fraction Nos. 37-44, with peak activity for each substrate found in fraction No. 40. Sephacryl fraction No. 40 also was the peak activity fraction for PABA, SMZ, PAS, and PA NAT activities (FIGURE 10).

Heat inactivation of NAT activities:

The heat inactivation rates of highly purified Sephacryl S-200 PABA, SMZ, PHZ, and MAH NAT activities at 55°C were highly similar (FIGURE 40). The NAT heat inactivation half-lives were 3.77 min for PABA, 3.63 min for SMZ, 3.78 min for PHZ, and 3.71 min for MAH.

Lineweaver-Burk analysis of PHZ and MAH NAT activities:

Lineweaver-Burk reciprocal plots were obtained for highly purified Sephacryl S-200 PHZ NAT (FIGURE 41) and MAH NAT (FIGURE 42) activities. The apparent Km was 4.18 $\pm$ 0.49 mM for PHZ NAT activity and 1.28 $\pm$ 0.48 mM for MAH NAT activity. The apparent Vmax was 199 $\pm$ 193 nmoles/min/mg for PHZ NAT activity and 38.1 $\pm$ 8.9 nmoles/min/mg for MAH NAT activity.

Polymorphic N-acetylation of PHZ and MAH:

While peak activity levels for highly purified Sephacryl S-200 enzyme from rapid acetylator rabbit were 5.92 nmoles/min/ml for MAH NAT activity and 3.33 nmoles/min/ml for PHZ NAT activity, the two activities were undetectable with Sephacryl S-200 enzyme from a slow acetylator rabbit. The viability of the slow acetylator enzyme was ensured by a finding of high (13.5 nmoles/min/ ml) PABA NAT activity

because in both rabbit and man, high PABA NAT activity levels ensure reliable determinations of polymorphic NAT activity (see CHAPTER IX). In addition, differences in PHZ and MAH NAT activity levels between rapid and slow acetylator inbred rabbits are listed as part of the complete structure-activity study reported in CHAPTER III C (see TABLE 16).

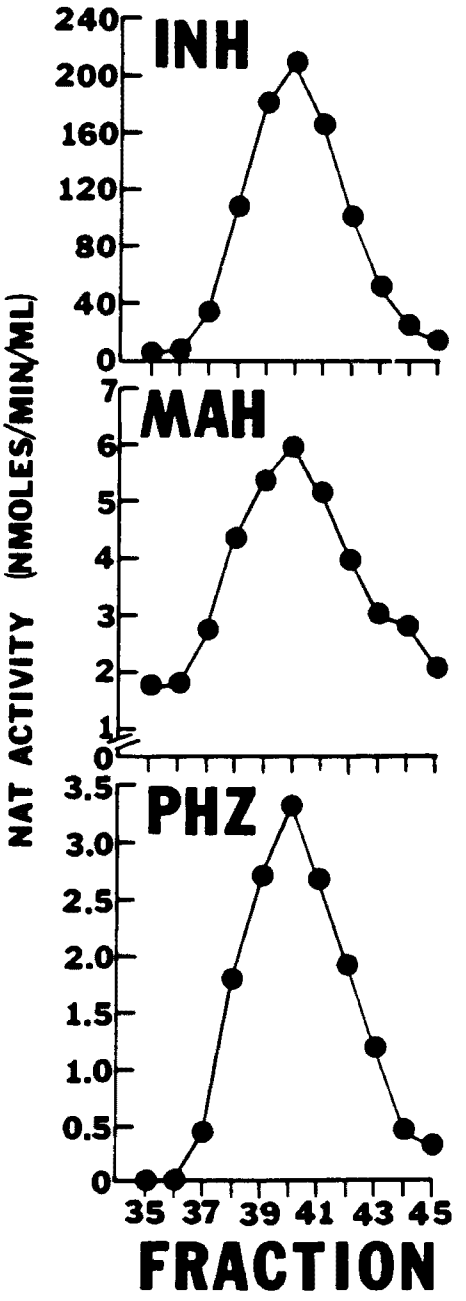


FIGURE 39. Co-purification of INH, MAH, and PHZ NAT activities of rabbit liver through Sephacryl S-200 chromatography.

NAT activity level in nmoles/min/ml is listed on the ordinate versus the Sephacryl S-200 fraction number on the abscissa. PHZ concentration in PHZ NAT was 0.222 mM. MAH concentration in MAH NAT assays was 0.556 mM. INH concentration in INH NAT assays was 11.1 mM. AcCoA concentration in each assay was 2.22 mM.

FIGURE 40. Heat inactivation of MAH, PHZ, PABA, and SMZ NAT activities.

Sephacryl S-200 purified enzyme was subjected to heat inactivation at 55°C. The heat inactivation half-lives of PHZ (□) and MAH (■) were compared to those of two standards, the prototypic monomorphic substrate, PABA (○), and a prototypic polymorphic substrate SMZ (●). NAT activities for each substrate were determined as described in Methods. The heat inactivation half-lives were as follows: PABA NAT 3.77 min (r = -.9961); SMZ NAT 3.63 min (r = -.9962); PHZ NAT 3.78 min (r = -.9931); MAH NAT 3.71 min (r = -.9866). The PABA and SMZ NAT heat inactivation rates for this preparation have been previously reported (see CHAPTER III B).

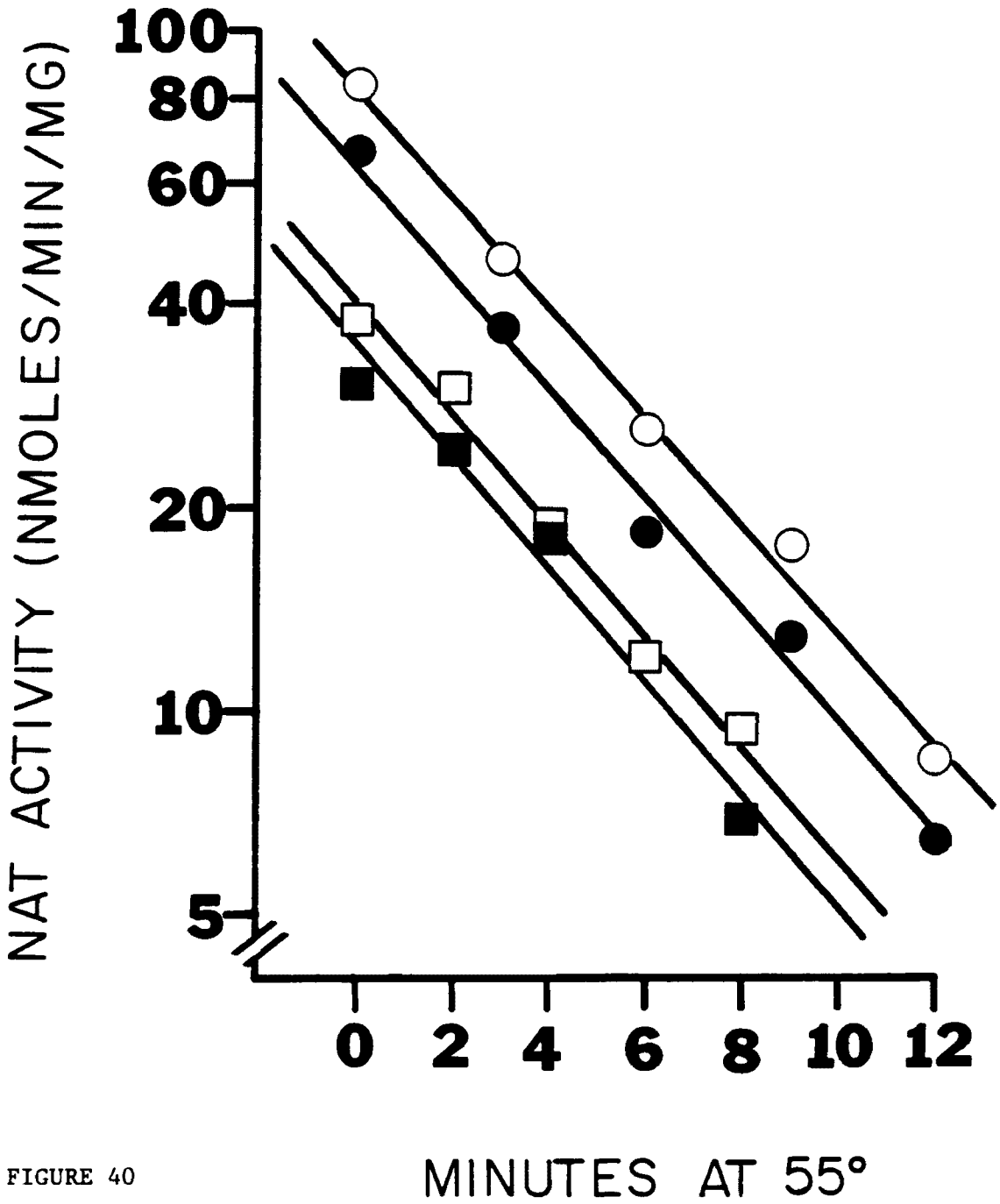

NAT ACTIVITY (NMOLES/MIN/MG)
100
80
60
40
20
10
5
0
2
4
6
8
10
12
MINUTES AT 55°

FIGURE 40

FIGURE 41. Lineweaver-Burk analysis of rapid acetylator rabbit liver PHZ NAT activity.

Liver NAT was purified through Sephacryl S-200 chromatography as described in Methods. The reciprocal NAT specific activity $(nmoles/min/mg)^{-1}$ is plotted on the ordinate versus the reciprocal PHZ concentration in the assay $(molar)^{-1}$. Kinetic constants were calculated as described in Methods. Km = 4.18 $\pm$ .49 mM. Vmax = 199 $\pm$ 193 nmoles/min/mg.

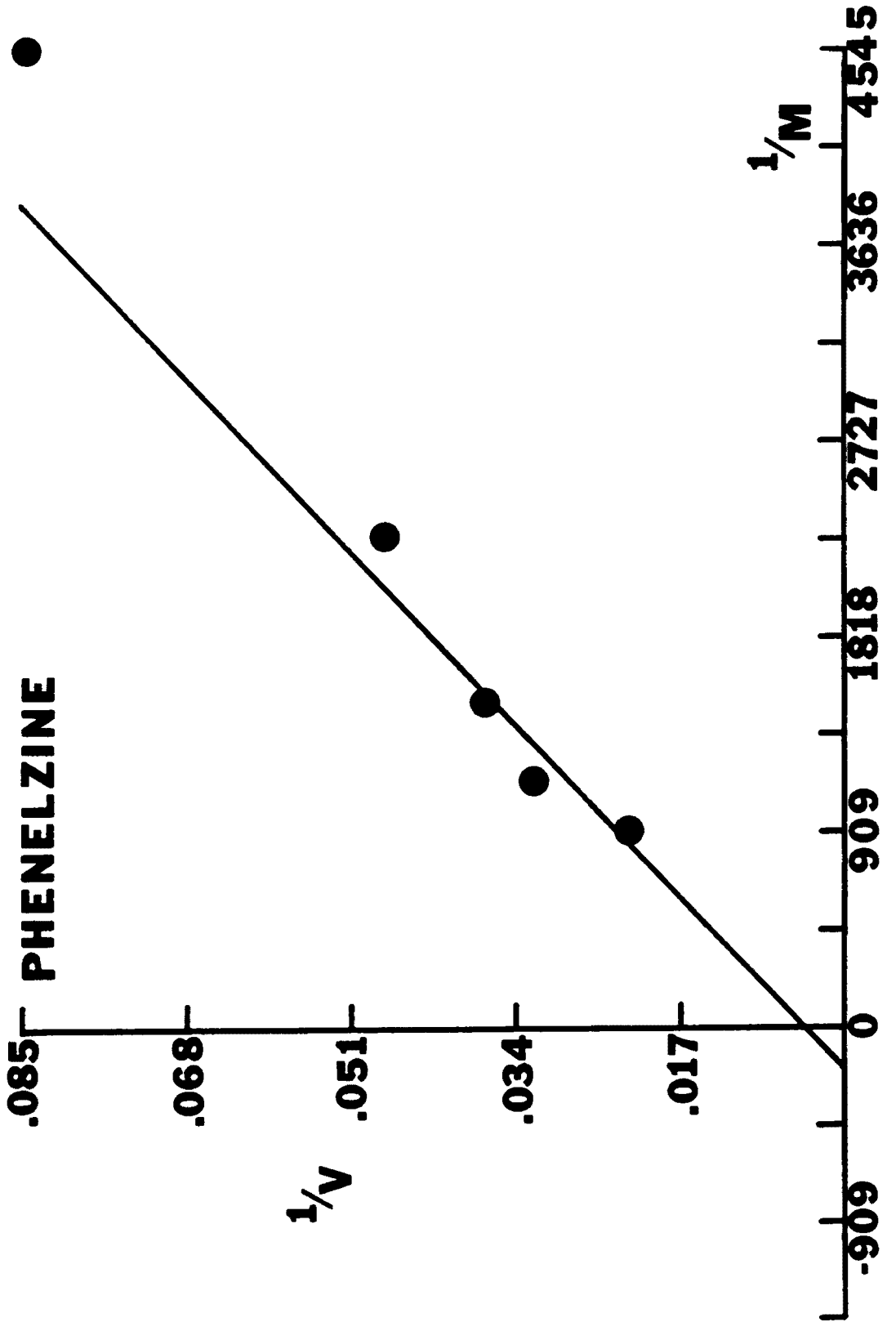

PHENELZINE
.085
.068
.051
.034
.017
1/V
-909
0
909
1818
2727
3636
4545
1/M

FIGURE 41

FIGURE 42. Lineweaver-Burk analysis of rapid acetylator rabbit liver MAH NAT activity.

Liver NAT was purified through Sephacryl S-200 chromatography as described in Methods. The reciprocal NAT specific activity $(\text{nmoles/min/mg})^{-1}$ is plotted on the ordinate versus the reciprocal MAH concentration in the assay $(\text{molar})^{-1}$. Kinetic constants were calculated as described in Methods. Km = 1.28 $\pm$ .48 mM. Vmax = 38.1 $\pm$ 8.9 nmoles/min/mg.

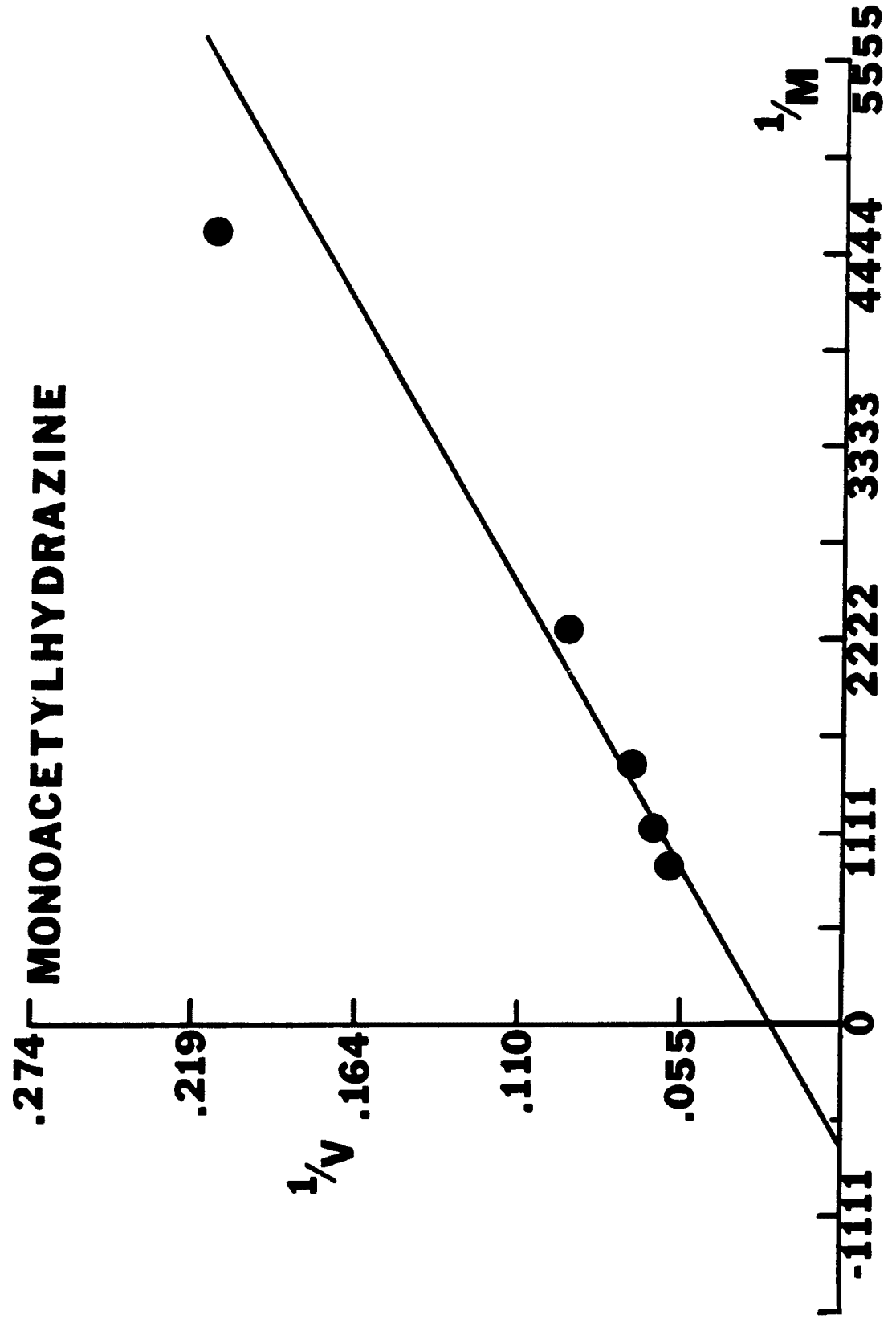

MONOACETYLHYDRAZINE
.274
.219
.164
.110
.055
1/V
-1111
0
1111
2222
3333
4444
5555
1/M

FIGURE 42

Discussion

Hydrazines have high chemical reactivity resulting in a wide spectrum of biological activity. Substituted hydrazine derivatives are currently used as therapeutic agents for the treatment of such notably dissimilar conditions as hypertension, depression, cancer, and tuberculosis. Hydrazines also produce a host of toxicities including methemoglobinemia, cell necrosis, central nervous system toxicity, peripheral neuritis, drug-induced lupus erythematosis, mutagenesis, and carcinogenesis (Juchau and Horita, 1972; Toth, 1975, 1980; Drayer and Reidenberg, 1977; Lunde et al., 1977). With respect to MAH and PHZ, for example, MAH is a potent hepatotoxin (Mitchell et al., 1975a; Nelson et al., 1976; Timbrell et al., 1980). while PHZ has been shown to be mutagenic in the Ames assay (Parodi et al., 1981) and tumorigenic in the mouse (Toth, 1976). Information concerning genetically determined differences in the N-acetylation of hydrazine compounds is needed in light of the wide variety of therapeutic and toxic effects of hydrazines, and the well-established associations between acetylator phenotype and drug toxicity (Drayer and Reidenberg, 1977; Lunde et al., 1977; see CHAPTERS VI and VII) Unfortunately, to our knowledge, only two hydrazines (INH and hydralazine) have been reported to be polymorphic substrates for the liver enzyme NAT (Weber, 1973).

Our results show that PHZ and MAH are polymorphically N-acetylated in the rabbit by the same liver NAT that polymorphically acetylates INH, SMZ, PA, 2AF, etc. PHZ, MAH, and INH NAT activities were not resolved by extensive purification procedures that exploit differences in solubility, size, and charge. Previous studies have

shown the Sephacryl S-200 enzyme preparation contains two major electrophoretically homogeneous proteins, one of which contains all of the NAT activity (Glowinski et al., 1980; see CHAPTER III B and FIGURE 11). The heat inactivation patterns of MAH, PHZ, and SMZ NAT activity, which are indistinguishable, lend additional support for the identity of polymorphic SMZ, MAH, and PHZ NAT, since heat inactivation differences can be used as a sensitive method for identifying small, qualitative differences in enzyme structure, despite failure of other methods, such as extensive separation techniques (Paigen, 1971). It should be noted that heat inactivation studies of a similar nature have previously provided evidence for qualitative differences between rapid and slow acetylator NAT (see CHAPTER III A and TABLE 8).

It is also interesting that the apparent affinity of rabbit liver NAT for hydrazine compounds which have been studied is lower than for arylamines, and that among the hydrazines, the affinities for MAH and PHZ are the lowest (TABLE 31). The low affinities of rabbit liver NAT for hydrazines might be expected to diminish the metabolic significance of polymorphic N-acetylation of these hydrazine compounds in vivo. This conclusion, however, may be unwarranted because studies in rabbits of both phenotypes have shown that INH- and MAH-induced CNS toxicity is substantially greater in slow acetylators than rapid acetylators (see CHAPTER VII and TABLES 25-27, 29). While such phenotypically-related differences in toxicity may not be directly extrapolatable from rabbit to man, it is pertinent that human liver NAT has a higher affinity than rabbit liver NAT for both INH (9-fold) and hydralazine (7-fold) (Weber et al., 1968; Weber, 1973). Thus, it seems likely that the human liver NAT would also have relatively

TABLE 31. Apparent Km values of arylamines and hydrazines for rapid acetylator rabbit liver NAT[a].

Compound	Chemical Class	Apparent Km (mM)	Reference
Benzidine	Arylamine	0.0059	Glowinski et al., 1978
2-Aminofluorene	Arylamine	0.0075	Glowinski et al., 1978
p-Aminosalicylic acid	Arylamine	0.038	Weber et al., 1968
Beta-napthylamine	Arylamine	0.040	Glowinski et al., 1978
Sulfamethazine	Arylamine	0.042	Weber et al., 1968
p-Aminobenzoic acid	Arylamine	0.055	Weber et al., 1968
Alpha-napthylamine	Arylamine	0.081	Glowinski et al., 1978
Hydralazine	Hydrazine	0.36	Weber et al., 1968
Isoniazid	Hydrazine	0.89	Weber et al., 1968
Monoacetylhydrazine	Hydrazine	1.3	CHAPTER VIII
Phenelzine	Hydrazine	4.2	CHAPTER VIII

[a]AcCoA concentrations were 2.22 mM for the studies of Glowinski et al., 1978 and CHAPTER VIII. AcCoA concentrations were 0.2 mM for the studies of Weber et al., 1968.

higher affinities for MAH and PHZ, although this remains to be established. This generalization is not accurate with respect to arylamines, however, since human liver NAT has a lower affinity for arylamines than rabbit liver NAT does (Weber et al., 1968; Weber, 1973).

Our finding that PHZ undergoes polymorphic N-acetylation is of interest in connection with various clinical studies of the therapeutic effectiveness and toxicity of this drug, since none of the previous investigations have conclusively demonstrated polymorphic N-acetylation of PHZ. Evans et al. (1965) initially proposed that PHZ should be polymorphically acetylated by NAT, due to its close structural resemblance to INH, SMZ, and hydralazine. Their study showed no correlation between acetylator phenotype and therapeutic response to PHZ, but did report that adverse effects from PHZ were more common among slow acetylators (Evans et al., 1965). Subsequent investigators (Johnstone and Marsh, 1973; Sanders and Rawlins, 1979) also showed that clinical response and inhibition of monoamine oxidase by PHZ were greater in slow acetylators, while others , however, found no relationship between clinical response, or monoamine oxidase inhibition to acetylator phenotype (Davidson et al., 1978; Marshall et al., 1978; Yates and Loudon, 1979; Mountjoy et al., 1980; Tyrer et al., 1980). Biochemical studies which reported the urinary excretion levels of acetyl-PHZ in rapid and slow acetylators were similarly inconclusive. Johnstone (1976) and Caddy et al. (1976) reported that urinary excretion of free PHZ is significantly greater in slow acetylators. Tilstone et al. (1979) reported correlations between PHZ and SMZ urine levels in rapid and slow acetylators and also

demonstrated acetylation of PHZ in vitro by rat and human liver preparations. In contrast, other investigators recently developed a specific GC/MS assay for acetyl-PHZ and concluded that plasma and urinary levels of acetyl-PHZ are extremely low in patients receiving therapeutic doses (60 mg/day) of PHZ and that acetylation was not a major pathway in PHZ metabolism (Narasimhachari and Friedel, 1980; Narasimhachari et al., 1980).

Our direct demonstration of polymorphic N-acetylation of MAH in vitro provides additional information concerning the possible relationship between INH-induced hepatotoxicity and acetylator phenotype. Mitchell et al. (1975b; 1976) have reported that INH-induced hepatotoxicity is more common in the rapid acetylator phenotype and Bernstein (1981) has suggested that such toxicity may be seen preferentially in homozygous rapid acetylators; others have reported that INH-related hepatotoxicity is more common in slow acetylators (Lal et al., 1972; Smith et al., 1972; Beaudray et al., 1974; Gronhagen-Riska et al., 1978). Clear-cut evidence has been obtained by Mitchell, Timbrell, and others in rats that INH-induced hepatotoxicity is mediated by MAH and that MAH is formed directly from acetylated INH (Mitchell et al., 1975a, 1976; Nelson et al., 1976; Timbrell et al., 1977, 1980; Timbrell, 1979). Ellard and Gammon (1976) have shown that diacetylhydrazine excretion in man is 4-5 times higher in a rapid acetylator than in a slow acetylator, which led them to propose that MAH is biotransformed to the non-toxic diacetyl derivative via polymorphic acetylation. Consequently, during INH therapy, MAH formation would be more rapid in rapid acetylators, but this could be offset by more rapid detoxification of MAH to

diacetylhydrazine. Thus, rapid and slow acetylators receiving therapeutic doses of INH might not be exposed to dissimilar amounts of MAH. In support of this view, Ellard and coworkers have presented epidemiological evidence that individual susceptibility to hepatotoxicity associated with INH administration is unrelated to acetylator phenotype (Hong Kong Chest Service, 1977; Singapore Tuberculosis Service, 1977; Ellard et al., 1978; Ellard et al., 1981). Thus, our data that MAH is a substrate for polymorphic NAT tends to support Ellard's contention. On the other hand, the role of acetylator status in INH-related liver damage may be more complex than either of these views, as the data of Timbrell and coworkers suggest. These investigators have recently shown in vivo that one effect of INH in rats is to inhibit the rate of N-acetylation of MAH to diacetylhydrazine, and thereby to increase the amount of MAH metabolized through the microsomal pathway leading to hepatotoxicity (Wright and Timbrell, 1978). However, they have also shown in vitro that INH inhibits the microsomal-enzyme mediated covalent binding of MAH to liver macromolecules (Timbrell and Wright, 1979) and MAH-induced hepatic necrosis (Bahri et al., 1981).

In conclusion, we have shown polymorphic N-acetylation in vitro of PHZ and MAH and have provided evidence that these compounds are N-acetylated by the same genetically polymorphic NAT that acetylates INH. These findings suggest that this NAT catalyzes the N-acetylation of a greater diversity of substrates than previously known, and that many hydrazine compounds are also polymorphically N-acetylated. They are also of interest in connection with the importance of acetylator status in relation to the therapeutic and

toxic effects of PHZ and INH.

CHAPTER IX

AN ENZYME MARKER TO ENSURE RELIABLE DETERMINATIONS OF HUMAN ACETYLATOR PHENOTYPE IN VITRO

Introduction

The N-acetylation polymorphism is of therapeutic and toxicological importance for a variety of arylamine and hydrazine drugs. For example, slow acetylators have been reported to have a higher incidence of adverse effects to INH, PA, hydralazine, and perhaps PHZ (Drayer and Reidenberg, 1977; Lunde et al., 1977). It is also possible that the N-acetylation polymorphism may be of consequence in determining the relative susceptibility to carcinogenic arylamines and their metabolites (Weber, 1978; Lower et al., 1979), although such a role has yet to be elucidated with certainty.

The acetylator phenotype of each individual is genetically determined by simple autosomal Mendelian genetics. Slow acetylators are homozygous for a recessive allele (rr) and rapid acetylators are either homozygous (RR) or heterozygous (Rr) for a dominant allele (Evans et al., 1960).

Expression of the acetylation polymorphism is dependent on the level of NAT activity (E.C. 2.3.1.5) present in the liver (Evans and White, 1964; Jenne, 1965). Persons can be classified as rapid or slow acetylators in vivo by measuring blood levels of acetylated

metabolite at a specified period of time after the administration of a test drug such as INH or SMZ (Evans et al., 1960; Jenne, 1960; Evans, 1969). Alternatively, the acetylator phenotype can also be determined by measurement of the amount of liver NAT activity in vitro (Evans and White, 1964; Weber, 1973). No discrepancies in phenotype classification between the two methods have been found, and the in vitro method has been considered completely dependable for phenotype classification in the absence of in vivo information.

While the in vitro differences in N-acetylation capacity between rapid and slow acetylators are large for a variety of compounds such as INH, SMZ, and 2AF, other compounds such as PABA show substantially less variation between the two phenotypes (Evans, 1965; Jenne, 1965; Glowinski et al., 1978). As a consequence, compounds such as INH, SMZ, and 2AF are considered polymorphic substrates, while PABA is considered more monomorphic.

The N-acetylation polymorphism in the rabbit is similar to man, and the rabbit has been studied extensively as an animal model, both for elucidating the biochemical characteristics of hepatic and extra-hepatic NAT (Frymoyer and Jacox, 1963a,b; Weber et al., 1976; see CHAPTERS II and III), and the toxicological consequences of the acetylation polymorphism (see CHAPTER VII). The relative instability of rabbit hepatic NAT under in situ conditions has previously been reported (Weber et al., 1978). If such instability is also present in human liver, the possibility for misclassification of an individual by in vitro measurement of liver NAT activity must be considered. For example, a low level of SMZ NAT activity in human biopsy or autopsy liver sample may be due to the fact that the

individual was a slow acetylator. Alternatively, a rapid acetylator liver NAT may have been sufficiently inactivated under in situ conditions to exhibit a low level of activity, and misclassification as a slow acetylator could result.

The purpose of this experiment was to study the relative stabilities of monomorphic (PABA) and polymorphic (SMZ) NAT activities under in situ conditions in the rapid and slow acetylator rabbit, and under simulated in situ conditions in the rabbit and in fresh human liver biopsy samples, to investigate whether the monomorphic activity, which is high in all humans and rabbits regardless of acetylator phenotype, could serve as a marker to ensure a reliable assessment (either high or low) of the polymorphic liver NAT activity.

Methods

Chemicals and reagents:

(see TABLE 33)

PABA and SMZ NAT activity determinations:

PABA NAT activity was selected as the prototype monomorphic activity and SMZ NAT was selected as the prototype polymorphic activity which is consistent with the current literature. PABA and SMZ NAT activities were conducted at room temperature (22-23°C) by a micromodification of the Bratton-Marshall assay as described in CHAPTER II. In circumstances where the SMZ NAT activity was extremely low in a slow acetylator rabbit preparation, the specific activity was determined by a radiolabel assay using ^{3}H-AcCoA as previously described (Glowinski et al., 1978).

Protein determinations:

Total protein was determined by a microbiuret method (Gornall et al., 1949).

Rabbit NAT in situ inactivation procedure:

Rabbits were sacrificed by injection of approximately 50 ml of air into a marginal ear vein. Temperature monitoring of the abdominal cavity throughout the experiment (5 hours) showed a decline of less than 2°C from physiologic temperature (37°C). Immediately after death, approximately 1 gram of tissue was cut out, homogenized with 5 mM potassium hydrogen phosphate buffer (25% w/v), pH 7.4 at 4°C, and centrifuged at 30,000 g for 20 min. The supernatant fraction was assayed for PABA and SMZ NAT activity. This procedure was repeated each hour for 5 hours to determine the extent of inactivation in situ of PABA and SMZ NAT activity with time. The

stability of the supernatant fraction prepared at zero time and maintained at 37°C was also measured after 5 hours to ensure that all inactivation observed was occurring in situ.

Human NAT simulated in situ inactivation procedure:

Fresh human liver biopsy samples (0.5 to 1.0 grams) were obtained from 4 individuals undergoing exploratory laparotomy. Each sample was kept on ice for 10-15 min before the procedure as described below was initiated. At zero time, a small section of liver was excised from the biopsy, minced, and homogenized with 5 mM potassium hydrogen phosphate buffer (25% w/v), pH 7.4, at 4°C, and centrifuged at 30,000 g for 20 min. The supernatant fraction was assayed for PABA and SMZ NAT activity. The remaining portion of the biopsy sample at zero time was totally wrapped in cellophane and incubated at 37 or 15°C. At predetermined time points, a small section of liver was excised from the total incubated biopsy, purified as described above, and assayed for PABA and SMZ NAT activity.

Data treatment:

NAT activity determinations were carried out and analyzed as described in CHAPTER II. Half-lives for PABA and SMZ inactivation were calculated using semi-log linear least squares regression analysis.

Human use approval:

Approval for the use of the human liver biopsies was provided by the Committee to Review Grants for Clinical Research and Investigation Involving Human Beings of the University of Michigan Medical Center.

Results

Rabbit NAT in situ-inactivation:

The in situ inactivation of PABA and SMZ NAT activities in slow and rapid acetylator rabbits is shown in TABLE 32. The in vitro activity levels for PABA and SMZ NAT are listed for each rabbit, and illustrate the much higher variation which exists in SMZ NAT activity between rapid and slow acetylator animals, as compared to PABA NAT activity, which is similar in magnitude in both phenotypes. Both activities were unstable under these in situ conditions in both the rapid and slow acetylator phenotype, with 50% activity decrement occurring within 92-477 min. Although there is considerable variability between animals in the stability of these activities, nevertheless, in each case, regardless of phenotype, the SMZ NAT activity was considerably more stable than the PABA NAT.

Results comparable to those in TABLE 32 were obtained in a simulated in situ experiment in which the rabbit liver was treated in an identical manner as that described in Methods for the human liver biopsies. In rabbit No. 77, the time required for 50% PABA NAT activity inactivation was 230 min, while it was 507 min for 50% SMZ NAT activity inactivation.

In contrast to the in situ inactivation observed in the above experiments, the PABA and SMZ NAT activities of a 30,000 g preparation obtained at zero time were stable for at least 5 hours at 37°C in both phenotypes of rabbit, indicating that the inactivation observed in situ was due to degradative processes within the whole liver after death.

TABLE 32. In situ inactivation of liver PABA and SMZ NAT activities in rapid and slow acetylator rabbits.

Acetylator Phenotype	Rabbit Number	Initial PABA NAT Activity	Initial SMZ NAT Activity	Time in Minutes Required to 50% Inactivation	
		(nmoles acetylated/min/mg protein)		PABA NAT Activity	SMZ NAT Activity
Slow	64	1.38	0.00692	92	151
Rapid	77	3.27	1.76	202	343
Rapid	117	1.74	0.853	237	477
Rapid	119	1.55	0.441	146	283

Human NAT simulated in situ inactivation:

Each of the four biopsies demonstrated NAT activity for both the monomorphic substrate (PABA) and the polymorphic substrate (SMZ). The initial PABA NAT activities were 2.27, 1.00, 2.65, and 2.23 nmoles/min/mg and the initial SMZ NAT activities were 0.148, 0.040, 0.042, and 0.033 nmoles/min/mg for patients A,B,C,and D, respectively. Each of the patients is classified as a slow acetylator due to their low levels of SMZ NAT activity which are similar to shoe previously reported for this phenotype (Glowinski et al., 1978). Since PABA is a monomorphic substrate, the PABA NAT activities were high and also in the range of those reported previously (Glowinski et al., 1978).

Both PABA and SMZ NAT activities declined with time in each patient (FIGURES 43-46) under the simulated in situ procedure. In each human biopsy, the PABA NAT activity declined at a faster rate (3- to 10-fold) than the SMZ NAT activity. In patient A (FIGURE 43), the PABA NAT activity was no longer detectable after incubation at 37°C for 60 min. The SMZ NAT activity also declined over the experimental period, showing a half-life of 51 min. In patients B (FIGURE 44) and C (FIGURE 45), the PABA NAT activities declined over the 37°C incubation period, demonstrating half-lives of 19 and 11 min, respectively. The SMZ NAT activities from the two patients were more stable for each patient, showing half-lives of 131 and 36 min, respectively. Thus, although there is considerable variation in half-lives between individuals, nevertheless, the SMZ NAT activity is consistently more stable (7-fold for patient B; 3-fold for patient C) than the PABA NAT activity.

In a fourth patient (FIGURE 46) the incubation was conducted at 15°C. Both of the activities were more stable at this temperature; the half-life for PABA NAT inactivation was 214 min, for SMZ NAT it was 2120 min. It is again evident that the SMZ NAT activity was significantly more stable (10-fold) than the PABA NAT activity.

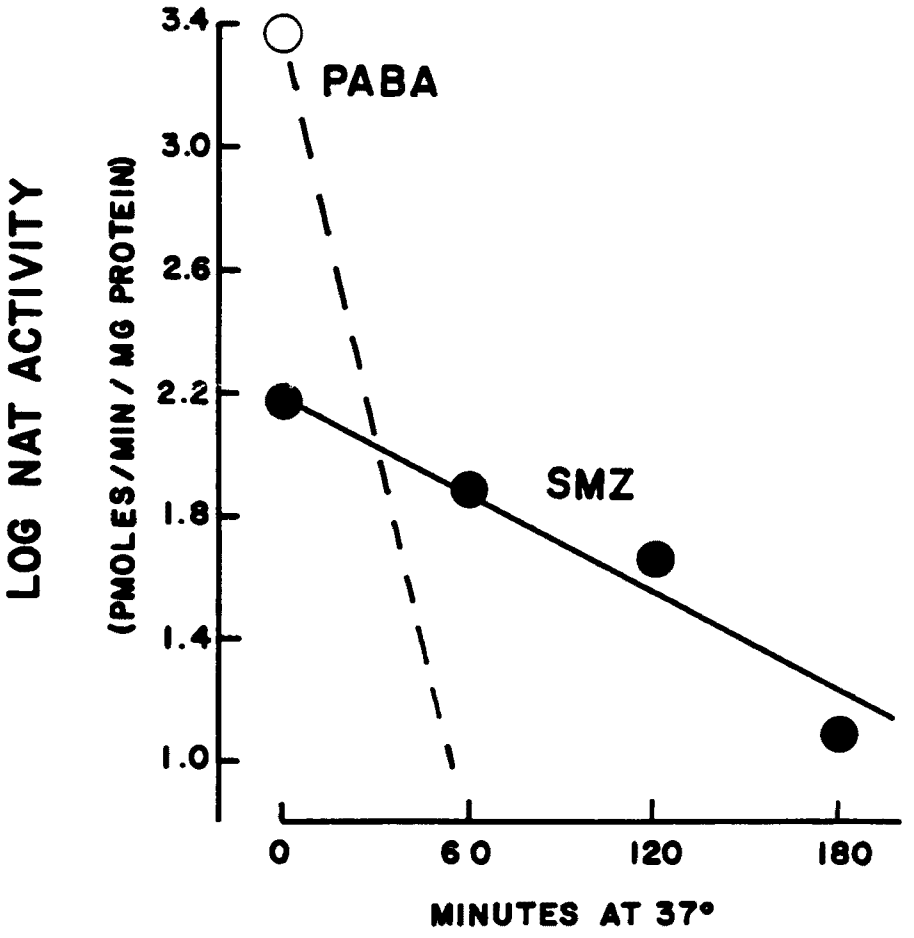


FIGURE 43. In situ inactivation of human liver PABA and SMZ NAT activity (patient A).

The ordinate indicates the logarithm of the NAT activity in pmoles/min/mg. The abscissa indicates the time of incubation at 37°C. The half-life of SMZ NAT activity inactivation was 51 min ($r = -0.978$). PABA NAT activity was undetectable at the 60 min time point.

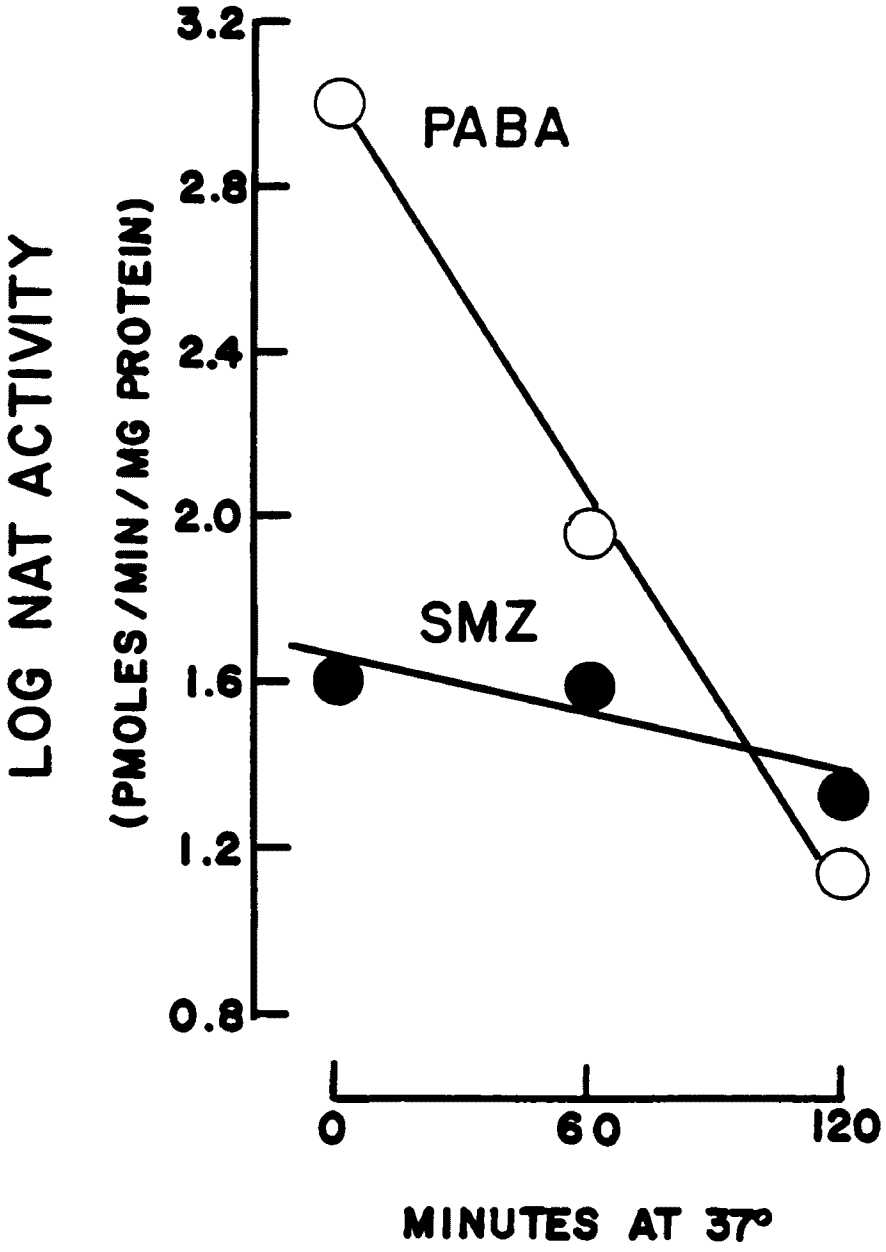


FIGURE 44. In situ inactivation of human liver PABA and SMZ NAT activity (patient B).

The ordinate indicates the logarithm of NAT activity in pmoles/min/mg. The abscissa indicates the time of incubation at 37°C. The half-life of SMZ NAT activity inactivation was 131 min (r = -0.892). The half-life of PABA NAT activity inactivation was 19 min (r = 0.997).

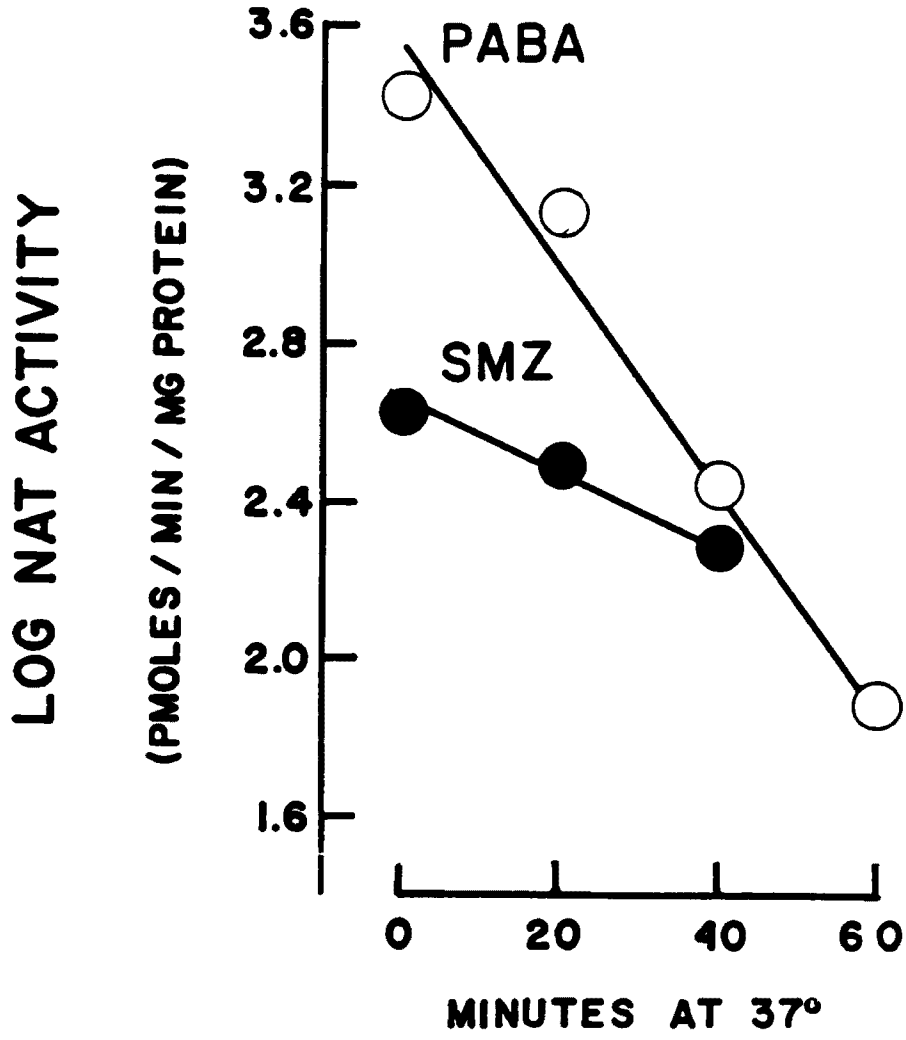


FIGURE 45. In situ inactivation of human liver PABA and SMZ NAT activity (patient C).

The ordinate indicates the logarithm of NAT activity in pmoles/min/mg. The abscissa indicates the time of incubation at 37°C. The half-life of SMZ NAT activity inactivation was 36 min (r = -0.993). The half-life of PABA NAT activity inactivation was 11 min (r = -0.989).

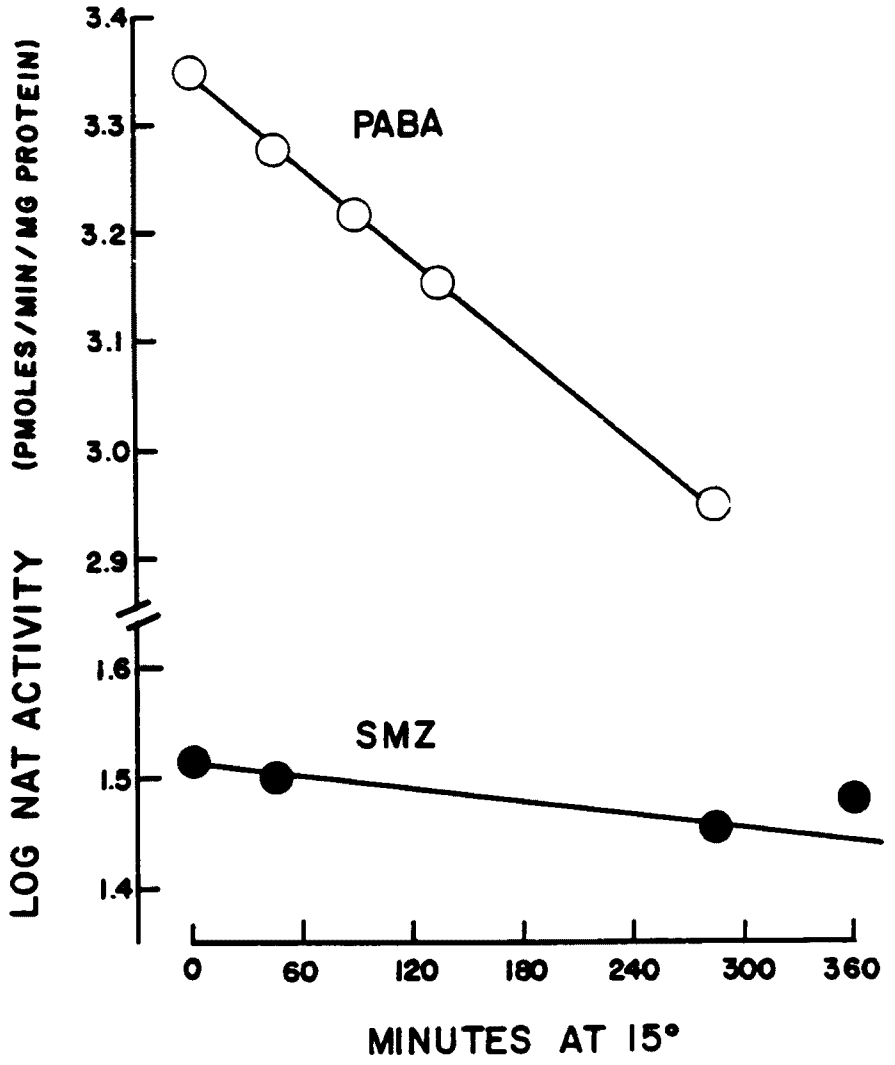


FIGURE 46. In situ inactivation of human liver PABA and SMZ NAT activity at 15°C. (patient D).

The ordinate indicates the logarithm of NAT activity in pmoles/min/mg. The abscissa indicates the time of incubation at 15°C. The half-life of SMZ NAT activity inactivation was 2120 min ($r = -.900$). The half-life of PABA NAT activity inactivation was 214 min ($r = -.9998$).

Discussion

Due to the marked instability of NAT from both rabbit and human under in situ conditions, it is readily apparent that misclassification of acetylator phenotype by in vitro enzyme methods is quite possible in autopsy samples. The results of this investigation, however, show that in both the rapid and slow acetylator rabbit and in the fresh human biopsies, the PABA NAT activity shows considerably greater instability than the polymorphic activity. Consequently, the PABA NAT activity can be used as an enzyme marker to ensure an accurate assessment of polymorphic NAT activity in vitro. PABA NAT activity will be high in all individuals, regardless of acetylator phenotype (i.e., it is monomorphic). Thus, a finding of low PABA NAT activity in vitro should be indicative of a liver sample which has undergone significant in situ inactivation of NAT. Moreover, a finding of high PABA NAT activity ensures a minimum of NAT inactivation, and a reliable in vitro measurement of polymorphic activity.

Recent studies in the rabbit have suggested that PABA and SMZ NAT activities are properties of a single enzyme species (see CHAPTER III B). In addition, further studies in the rabbit have indicated that NAT activity covaries with AHAT activity, an enzyme which has the capacity to catalyze an intramolecular transfer of the acetyl group (Glowinski et al., 1980; see CHAPTER II). Moreover, NAT and AHAT activities were found to be properties of a single protein species (Glowinski et al., 1980). AHAT activity may also be of toxicological significance, particularly in the susceptibility to carcinogenesis from certain arylamines such as 2AF (Bartsch et al.,

1972,1973; King, 1974). Thus, it would appear that PABA NAT activity could also serve as an enzyme marker to ensure a reliable phenotype classification of AHAT activity.

The acetylator phenotype of an individual may have clinical significance after death, particularly in the investigation of the toxicological significance of the acetylation polymorphism. Several of the proposed differential susceptibilities to toxicity between rapid and slow acetylators remain controversial, such as INH-induced hepatotoxicity and SLE. The only mechanism for acetylator phenotype determination after death is the in vitro determination of polymorphic liver NAT activity in autopsy specimens. It has been our experience, however, that NAT activity from human autopsy specimens is markedly unstable; usually the liver must be isolated and frozen within 2 hours after death. In the absence of additional information, one cannot assume that partial inactivation has not occurred. However, the results of this investigation find that the greater instability of the PABA NAT activity allows it to serve as an enzyme marker to ensure accurate classifications of acetylator phenotype based on measurement of liver polymorphic NAT activity in vitro.

CHAPTER X

FINAL CONCLUSIONS AND SUMMARY

Two animals models of the N-acetylation polymorphism were developed, characterized, and used in the examination of its biochemical basis. Genetically determined rapid and slow acetylation was demonstrated among inbred rabbit strains, and three distinct acetylator genotypes (RR, Rr, and rr) were identified by a gene dose-response relationship in NAT activity. The rapid and slow acetylator inbred rabbit model expresses an N-acetylation polymorphism that is qualitatively identical to that in humans. Thus, large phenotypic differences in N-acetylation rate are found for polymorphic substrates such as INH, SMZ, PA, and 2AF, and only very small differences for monomorphic substrates such as PABA and PAS. The inbred rabbit model also expresses that polymorphism for the AcCoA-independent acetyl transfer by AHAT, and we provided further evidence that NAT and AHAT activities are under common genetic control in this animal model.

In addition, genetically determined rapid and slow acetylation was also demonstrated among inbred hamster strains, and three distinct acetylator genotypes (RR, Rr, and rr) were identified. The N-acetylation polymorphism in the hamster was unique, however, as the substrate expression was directly opposite to that in man and rabbit. For example, PABA and PAS showed large N-acetylation differences between rapid and slow acetylators (polymorphic substrates) whereas

INH, PA, SMZ, and 2AF showed a monomorphic distribution (FIGURES 47-48). However, like those in man and rabbit, the hamster polymorphism is inherited as a simple Mendelian trait.

Comparative studies of the N-acetylation polymorphisms in these two animal models showed the biochemical basis to be similar in some respects and different in others. In rabbit, there are two qualitatively different NATs, identified for the purposes of this discussion as R1 and R2. Enzyme R1 is found primarily in the liver and small intestine of rapid acetylators only. It rapidly catalyzes the N-acetylation of all substrates, both monomorphic (PABA and PAS) and polymorphic (INH, SMZ, PA, and 2AF) and has a rather broad substrate specificity. The catalysis rate of arylamine N-acetylation by enzyme R1 is concordant with the extent of electron delocalization at the substituent para to the amine. Thus, relatively nonpolar substrates, such as p-isopropylaniline and p-phenetidine are rapidly acetylated, whereas acetylation of PABA and PAS is more slowly catalyzed. In addition, enzyme R1 also catalyzes the AHAT reaction rapidly. In contrast, enzyme R2 is found primarily in the liver and small intestine of slow acetylators only. It catalyzes the N-acetylation of monomorphic substrates (PABA and PAS) at relatively high rates, but catalyzes the N-acetylation of polymorphic substrates such as INH, PA, SMZ, and 2AF very slowly. Enzyme R2 appears to have more limited capabilities. It rapidly catalyzes the N-acetylation of only those substrates with high electron density at the substituent para to the amine function. To this point, only PABA and PAS, among the many substrates tested, were able to fulfill this requirement. In addition, as might be expected, enzyme R2 also catalyzes the AHAT

FIGURE 47. Genetic differences in liver 2AF and PABA NAT activity in man and inbred mice, hamsters, and rabbits.

Open squares represent rapid acetylators; closed squares represent slow acetylators. Human liver NAT activities were taken from Glowinski et al., 1978. Mouse liver NAT activities were taken from Glowinski and Weber, 1982a. Hamster liver NAT activities were taken from CHAPTER IV. Rabbit liver NAT activities were taken from CHAPTER II.

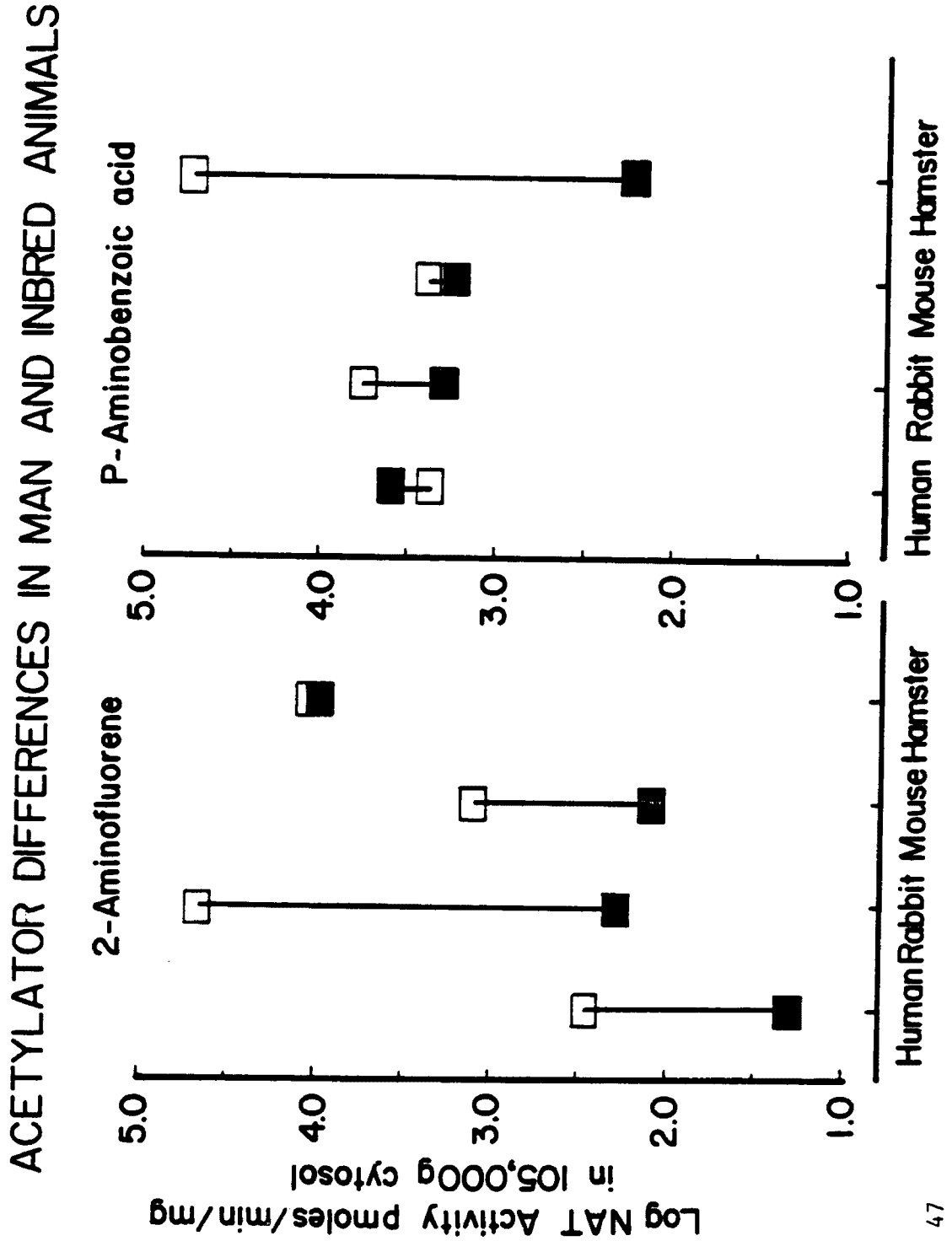

ACETYLATOR DIFFERENCES IN MAN AND INBRED ANIMALS
Log NAT Activity pmoles/min/mg in 105,000g cytosol
2-Aminofluorene
P-Aminobenzoic acid
5.0
4.0
3.0
2.0
1.0
Human Rabbit Mouse Hamster
Human Rabbit Mouse Hamster

FIGURE 47

ANIMAL	"MONOMORPHIC SUBSTRATES"	"POLYMORPHIC SUBSTRATES"
RABBIT	P-AMINOBENZOIC ACID P-AMINOSALICYCLIC ACID	ISONIAZID SULFAMETHAZINE 2-AMINOFLUORENE PROCAINAMIDE
HAMSTER	ISONIAZID SULFAMETHAZINE 2-AMINOFLUORENE PROCAINAMIDE	P-AMINOBENZOIC ACID P-AMINOSALICYCLIC ACID

FIGURE 48. Monomorphic and polymorphic substrates in rabbit and hamster.

reaction very slowly.

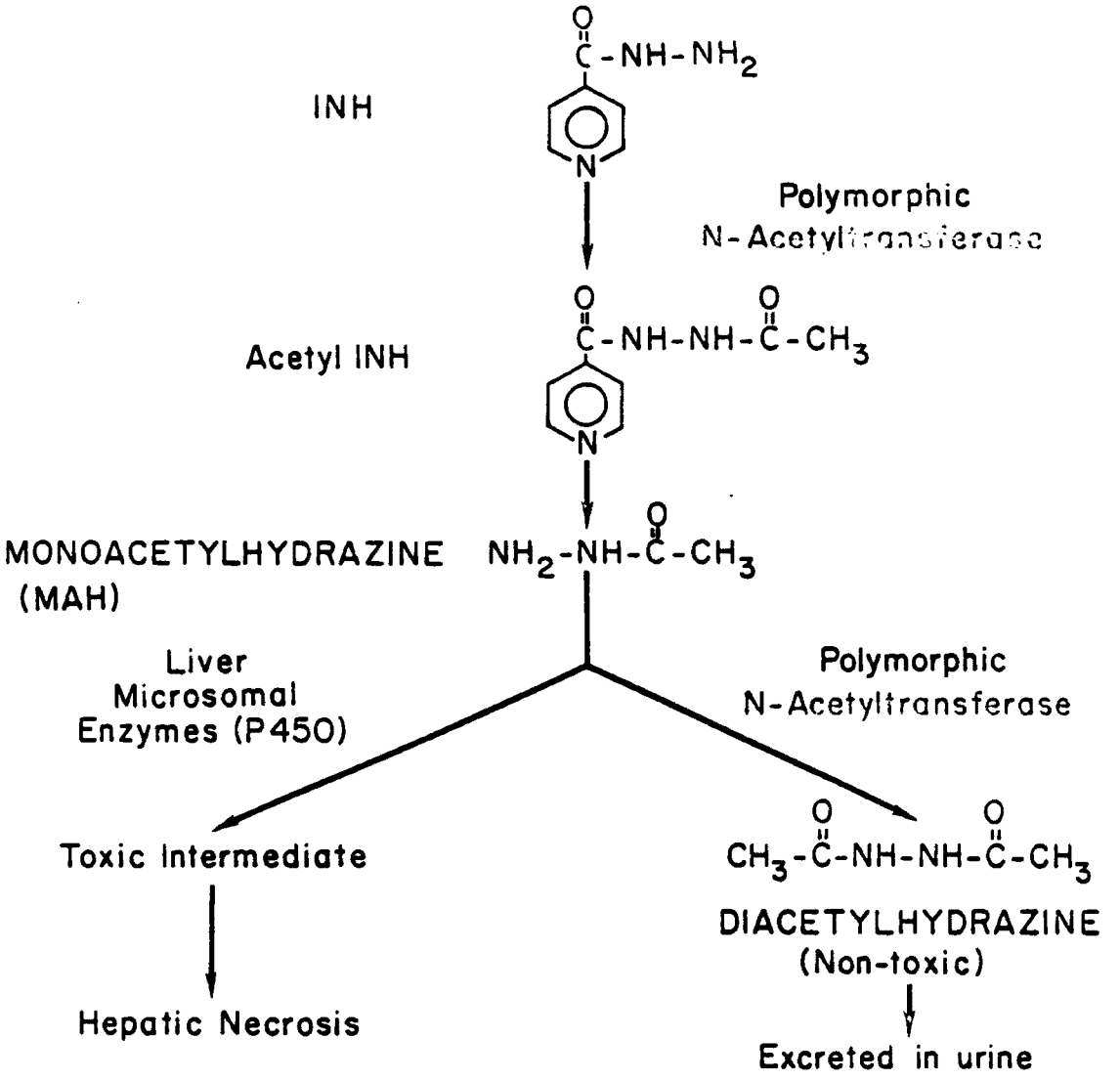
In the hamster, two qualitatively different NATs were also identified, and are termed H1 and H2 for the purposes of this discussion. H1 and H2 are both present in rapid acetylator liver. They can be separated by ion exchange chromatography. Enzyme H1 rapidly catalyzes the N-acetylation of substrates such as PABA and PAS, and multicyclic arylamines such as 2AF and BNA, but only slowly catalyzes the N-acetylation of substrates such as PA and INH. Enzyme H2, in contrast, rapidly catalyzes the N-acetylation of substrates such as INH and PA, and multicyclic arylamines such as 2AF and BNA, but only slowly catalyzes the N-acetylation of substrates such as PABA and PAS. In the slow acetylator hamster, enzyme H1 is located in the liver. It behaves in purification and substrate catalysis characteristics exactly as does the H1 enzyme in rapid acetylator liver and may be the same enzyme. However, slow acetylator hamster liver does not have detectable activity levels of enzyme H2. Thus, all the N-acetylation catalysis carried out by enzyme H2 in rapid acetylators is absent in slow acetylators, which accounts for the substantially lower activity levels of PABA and PAS NAT activity in the slow acetylator genotype. Although a systematic and complete structure activity study for these two enzymes in hamster has yet to be completed, it is already apparent that one of the chemical features of the substrate that is important for conversion from monomorphic to polymorphic N-acetylation expression in man and rabbit (high electron density para to the amine function) is also important in determining the catalysis rate by enzymes H1 and H2 in hamster. Thus, enzyme H1 greatly prefers that its substrate provide high electron density para

to the amine, whereas enzyme H2 greatly dislikes it, thus providing an important insight into the biochemical basis for the polymorphic expression of PABA and PAS, and the monomorphic expression of INH, 2AF, SMZ and PA in the hamster.

Results of this dissertation provide much needed information important to three very controversial research questions involving the N-acetylation polymorphism. We found similar proportions of slow acetylators among spontaneous SLE patients and controls, a finding which is in agreement with some investigators, but not with others. In addition, we were able to directly demonstrate polymorphic N-acetylation of two hydrazines, PHZ and MAH, in the inbred rabbit model. The in vitro demonstration of polymorphic N-acetylation of MAH, the hepatoxic metabolite of INH, by the same liver NAT that polymorphically acetylates INH, SMZ, 2AF, etc., has important clinical implications with respect to INH-induced hepatotoxicity . Thus, rapid acetylators produce MAH more rapidly and they also detoxify it more rapidly to diacetylhydrazine via the same polymorphic NAT, as shown in FIGURE 49. Consequently, it does not necessarily follow that rapid acetylators should experience increased susceptiblity to INH-induced hepatotoxicity as some have proposed.

Results of this dissertation clearly demonstrate the toxicological significance of the N-acetylation polymorphism by the use of hydrazine-induced CNS toxicity in the rapid and slow acetylator rabbit model. The method of toxicological measurement (death) is clear enough that it would be difficult to argue with this conclusion. Finally, we utilized our knowledge of the biochemical basis of the N-acetylation polymorphism learned in animal models to provide a

clinical application in humans for acetylator phenotype classification in vitro.



METABOLIC PATHWAYS
POSTULATED IN INH-INDUCED
LIVER DAMAGE

FIGURE 49

APPENDIX

TABLE 33. Sources of Chemicals. (Appendix A)

CHEMICAL	SOURCE
AcCoA lithium salt	P.L. Biochemicals, Milwaukee, WI
acrylamide	Bio-Rad Laboratories, Richmond, CA
2AF	ICN Pharmaceuticals, Plainview, NY
p-aminoacetophenone	Aldrich Chemicals, Milwaukee, WI
2-aminoanthracene	ICN
4-aminoantipyrine HCl	ICN
p-aminobenzoyl hydrazide	Aldrich
6-aminochrysene	ICN
p-aminophenylethylalcohol	Eastman Kodak, Rochester, NY
p-aminophenol	Aldrich
ammonium persulfate	Bio-Rad
ammonium sulfamate	J.T. Baker Chemicals, Phillipsburg, NJ
ammonium sulfate	Sigma Chemicals, St. Louis, MO
ampholines	LKB Instruments, Bromma, Sweden
aniline	Fisher Scientific, Fairlawn, NJ
p-anisidine	Aldrich
anthranilic acid	Aldrich
benzoylhydrazine	Aldrich
bisacrylamide	Bio-Rad
BNA hydrochloride	Eastern Chemicals, Pequannock, NJ
p-chloroaniline	Eastman Kodak
DEAE-cellulose	Sigma
diacetylhydrazine	Pfaltz and Bauer, Stamford, CT
DMAB	Sigma
DTT	Sigma
EDTA	Sigma
glycine	Matheson Coleman and Bell, Norwood, OH
INH	Sigma
isobutanol	J.T. Baker
p-isopropylaniline	Aldrich
MAH	Aldrich
2-mercaptoethanol	Matheson Coleman and Bell
NED	Matheson Coleman and Bell
nicotinic acid hydrazide	Aldrich
PA hydrochloride	E.R. Squibb, Princeton, NJ
PABA potassium salt	Sigma
PAS sodium salt	Sigma
p-phenetidine HCl	Eastman Kodak
phenobarbital	Sigma
PHZ sulfate	Warner Lambert, Ann Arbor, MI
pyridoxine HCl	Sigma
salicylaldehyde	Eastman Kodak
SDZ sodium salt	ICN
Sephadex G-100	Pharmacia Chemicals, Piscataway, NJ
Sephacryl S-200	Pharmacia
SGPT assay kit	Sigma
SMZ	Nutritional Biochemicals, Cleveland, OH
sodium nitrite	Fisher
sulfapyridine	Sigma
TEMED	Bio-Rad
TNBS	Sigma

Appendix B

INFORMED CONSENT

ACETYLATOR TYPING WITH SUFAMETHAZINE

The ability to inactivate and eliminate medically used drugs is in many cases genetically determined. Our laboratory has been evaluating the effects of heredity on the elimination of a group of widely used medical drugs, including isoniazid, (Nydrazid^{R}), hydrazaline, (Apresoline^{R}), procainamide (Pronestyl^{R}), and various antibacterial sulfa drugs.

The general population can be split genetically into two groups, those that rapidly inactivate these drugs, and those that inactivate them slowly. The procedure used to determine this genetic trait is a routine one and has been done for several years in our laboratory. It involves taking an initial blood and urine sample for a control, and a second blood and urine sample after the oral administration of a test drug, to see how rapidly it is eliminated.

The oral drug we use for this purpose is sulfamethazine, an antibacterial sulfa drug. This drug has been widely used for long periods of time in patient therapy and is considered safe. It has also been used for years in our typing procedure. On rare occasions, a slight headache has been reported as its only side effect. For proper absorption of the drug, we ask you not to eat or drink for about two hours after the administration of the drug.

Recently, it has been reported that genetically slow inactivators of these drugs are more likely than rapid inactivators to experience drug-induced Systemic Lupus Erythmatosus (SLE) and spontaneous SLE. Our laboratory is investigating this contribution of heredity to the onset of SLE thru studies of SLE patients and their families. The information obtained from whole family studies is very valuable in studying this question.

Our laboratory is also studying the stability of an inactivator enzyme found in human lymphocytes. Preliminary results show a large difference in the stability of the enzyme in SLE patients compared to the normal population. The initial sample of blood which is drawn is used to isolate your lymphocytes for that study.

We would like your cooperation in this project. Please feel free to ask us any questions you have concerning it. Knowledge of your acetylation phenotype and genotype could be valuable to you when placed on certain drug therapy and we would be happy to furnish you with the results upon request. Thank you.

I have read the above statement. I understand what is involved and wish to participate as a volunteer in this project. Also I understand that I can discontinue participation and that this will not adversely affect treatment.

_____ _____

Signed Witness

_____

Date

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