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Research Article

The biochemical basis underlying the genetic polymorphism of drug N-acetylation was investigated using a combination of in vivo and in vitro assays for arylamine N-acetyltransferase (NAT) activity and content in human liver. The acetylator phenotype of 26 surgical patients was determined using caffeine as an innocuous probe drug by measurement of the 5-acetyl-amino-6-formylamino-3-methyluracil to 1-methylxanthine molar ratio in urine. Liver wedge biopsies from these patients and livers from 24 organ donors were then used for measurement of N-acetyltransferase activity with the substrate sulfamethazine and for quantitation of immunoreactive N-acetyl-transferase protein. In vivo (caffeine metabolites in urine) and in vitro (sulfamethazine acetylation) measures of N-acetyl-transferase activity correlated very highly (r = 0.98). Moreover, in all subjects tested, slow acetylation both in vivo and in vitro was associated with a decrease in the quantity of immunodetectable N-acetyltransferase protein in liver cytosol relative to that seen in cytosols from rapid acetylator livers. Two kinetically distinct enzyme activities, designated NAT-1 and NAT-2, were partially purified from low- and high-activity livers and their relationship to acetylator status was determined. Low acetylation capacity was related to decreases in the liver content of both of these immunologically related proteins. The results demonstrate that genetically defective arylamine N-acetylation is due to a parallel decrease in the quantity of two structurally and functionally similar acetylating enzymes.

Acetylation Pharmacogenetics

The Slow Acetylator Phenotype Is Caused by Decreased or Absent Arylamine *N*-Acetyltransferase in Human Liver

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Abstract

The biochemical basis underlying the genetic polymorphism of drug *N*-acetylation was investigated using a combination of in vivo and in vitro assays for arylamine *N*-acetyltransferase (NAT) activity and content in human liver. The acetylator phenotype of 26 surgical patients was determined using caffeine as an innocuous probe drug by measurement of the 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine molar ratio in urine. Liver wedge biopsies from these patients and livers from 24 organ donors were then used for measurement of *N*-acetyltransferase activity with the substrate sulfamethazine and for quantitation of immunoreactive *N*-acetyltransferase protein. In vivo (caffeine metabolites in urine) and in vitro (sulfamethazine acetylation) measures of *N*-acetyltransferase activity correlated very highly (r = 0.98). Moreover, in all subjects tested, slow acetylation both in vivo and in vitro was associated with a decrease in the quantity of immunodetectable *N*-acetyltransferase protein in liver cytosol relative to that seen in cytosols from rapid acetylator livers. Two kinetically distinct enzyme activities, designated NAT-1 and NAT-2, were partially purified from low- and high-activity livers and their relationship to acetylator status was determined. Low acetylation capacity was related to decreases in the liver content of both of these immunologically related proteins. The results demonstrate that genetically defective arylamine *N*-acetylation is due to a parallel decrease in the quantity of two structurally and functionally similar acetylating enzymes. (*J. Clin. Invest.* 1990. 85:968–972.) acetylator phenotype • caffeine • *N*-acetyltransferase • polymorphism • sulfamethazine

Introduction

Genetic polymorphisms of human enzymes mediating the biotransformation of xenobiotics cause marked variability in the response to drugs and environmental chemicals (1). Among the first of such pharmacogenetic conditions to be discovered was that controlling the disposition of the tuberculostatic drug isoniazid, where a clearly bimodal population distribution of plasma elimination half-lives distinguished individuals as phenotypically "rapid" or "slow" eliminators of the drug (2). Numerous subsequent investigations (3, 4) revealed that this variation was genetically determined by the action of at least two alleles at a single autosomal gene locus, and that the metabolic fate of a large number of additional chemicals possessing a primary aromatic amine or hydrazine group was affected by the same defect. These include many therapeutically useful drugs such as sulfamethazine (SMZ),[1] procainamide, hydralazine, dapsone, nitrazepam, and caffeine, as well as several potentially carcinogenic arylamines of which benzidine, β-naphthylamine, and 2-aminofluorene are among the best-documented examples.

Furthermore, the observed variation was found to be related to differences in the rate of acetyl coenzyme A (CoASAc)-dependent acetylation of the primary amine or hydrazine nitrogen (5), which is catalyzed by liver cytosolic arylamine *N*-acetyltransferase (NAT; EC 2.3.1.5) according to the two-step, substituted-enzyme reaction mechanism shown in Fig. 1.

Since > 50% of Caucasian subjects are genetically slow acetylators, the clinical and toxicological consequences of this phenomenon have been studied in considerable detail (4). However, most of our present understanding of the underlying mechanism of the *N*-acetylation polymorphism relies on extrapolation from animal model systems (6). Nonetheless, Jenne (7) proposed from the results of kinetic and thermal stability experiments with human liver *N*-acetyltransferase that activity differences between rapid and slow acetylators were due to variable quantities of enzyme(s) with similar affinity for arylamine substrates. This interpretation was confounded by two further sets of observations: (*a*) some arylamines, such as *p*-aminobenzoate and *p*-aminosalicylate, are *N*-acetylated yet are unable to distinguish rapid and slow acetylators in vivo (7, 8) or in liver tissues in vitro (9); and (*b*) in the model provided by the New Zealand White rabbit, chosen because its in vivo and in vitro patterns of arylamine *N*-acetylation closely resemble those in man, kinetic (10) and immu-

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1. *Abbreviations used in this paper:* AFMU, 5-acetylamino-6-formylamino-3-methyluracil; CoASAc, acetyl coenzyme A; 1X, 1-methylxanthine; KDL, kidney donor liver; NAT, arylamine *N*-acetyltransferase; SMZ, sulfamethazine.

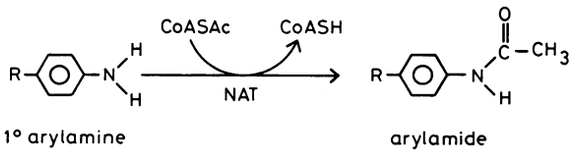

Figure 1. Summary of the two-step, substituted-enzyme reaction mechanism of arylamine *N*-acetyltransferase.

nological studies (11) suggested that livers from genetically slow and rapid acetylator animals contain equal quantities of *N*-acetyltransferase enzyme molecules with different kinetic behavior toward a variety of arylamine substrates.

We have recently used an enzyme assay of improved sensitivity to control the purification from human liver of two kinetically distinct *N*-acetyltransferase activities, NAT-1 and NAT-2 (12). Polyclonal rabbit antisera raised against purified NAT-1 recognize both of these proteins with an identical apparent M_r and 31 kD on Western blots.

In the present study we have investigated the relationship between liver *N*-acetyltransferase activity and the amount of immunologically detectable enzyme protein in a total of 50 human livers, 26 of which were obtained from living individuals whose acetylator phenotype could be determined. Our results indicate that slow acetylation is due to a coordinated decrease in the quantity of these two structurally and functionally similar acetylating enzymes.

Methods

Patients, urine collection, and liver samples. A total of 26 patients at the Robert-Bosch-Krankenhaus, Stuttgart, FRG (19 female, 7 male; mean age 48 yr, range 27–67 yr) provided a single urine sample 3–6 h after the intake of caffeine in the form of coffee, tea, or cola soft drink within 3 d before surgical removal of 0.2–2 g of liver tissue for diagnostic purposes. A further 24 liver samples from the human liver bank at the Biocenter in Basel, Switzerland (13) were obtained from kidney transplant donors at the time of circulatory arrest. Liver pieces were frozen in liquid nitrogen and stored at −80°C until the time of analysis. Experimental protocols for in vivo testing and for use of biopsy material were approved by the appropriate ethical review boards at the institutions involved.

Acetylator phenotyping with caffeine. Determination of acetylator phenotype by HPLC measurement of the urinary molar ratio of the caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylzanthine (1X) was performed essentially as described (14), except that urine aliquots were acidified to pH 3.5 immediately after collection and stored frozen to ensure chemical stability of AFMU, and urine was extracted using 30 vol of 100% chloroform. Authentic AFMU for assay calibration was isolated from human urine as previously described (15).

N-Acetyltransferase assay. Liver pieces were homogenized in TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) containing 0.02 mM leupeptin, 0.1 mM PMSF, and 0.05 mM butylated hydroxytoluene, and cytosols were prepared by differential centrifugation. *N*-Acetyltransferase activity was measured using a recent modification (12) of a published procedure (16), with final concentrations of 0.1 mM for the acetyl donor CoASAc and 0.5 mM for SMZ as acceptor amine except where required for determination of Michaelis constants. In blank incubations CoASAc was omitted. Kinetic constants (K_m and V_{max}) were determined using the linear transformation of Hofstee (17), and protein concentrations were measured with a dye-binding method (18).

Gel electrophoresis, Western blotting, and immunoquantitation. Cytosol aliquots (0.1 mg protein) were subjected to SDS-PAGE (19) on 12% gels and proteins were immobilized on nitrocellulose by an electrotransfer technique (20). Blots were reacted with a polyclonal rabbit antiserum raised against human NAT-1 (12) which was prepurified by binding to and elution from rabbit *N*-acetyltransferase (purified by a modification of a published method [21]) immobilized on nitrocellulose strips. Further reaction with ^{125}I-labeled protein A was followed by autoradiography at −80°C using X-omat AR film (Eastman Kodak Co., Rochester, NY) and an intensifying screen. Blot intensities on developed films were quantified using a Camag TLC Scanner II in transmission mode and results were expressed in arbitrary units of area density.

DEAE-Sephacel anion exchange chromatograpy. Cytosols isolated from 5 g of each of six different human kidney donor livers (KDL) were prepurified by ammonium sulfate precipitation and pH-shift precipitation as previously described (12), then applied to DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) columns (1.6 × 25 cm) that were pre-equilibrated with TEDK buffer. Columns were washed and then eluted at a flow rate of 20 ml/h with a linear 200-ml gradient from 50 to 200 mM KCl in TEDK buffer. Column fractions of 2 ml were collected and analyzed for *N*-acetyltransferase activity as described above. Peak activity fractions were pooled, concentrated on Centrikon PM-10 membranes, and analyzed for enzyme kinetic constants and immunoreactive NAT-1 and NAT-2 proteins.

Results and Discussion

Our studies strongly suggest that the genetic polymorphism of human liver *N*-acetyltransferase leading to defective arylamine *N*-acetylation is a consequence of a marked decrease or absence of two related acetylating enzymes. This conclusion is based on combined characterization of *N*-acetyltransferase activity, chromatographic behavior, and kinetic parameters, along with measurement of immunoreactive enzyme protein in 50 human livers, over half from individuals of clearly established in vivo acetylator phenotype.

In vivo/in vitro correlation of acetylation indices. The urinary AFMU/1X molar ratio after caffeine intake allowed for unambiguous classification of all of the surgical patients as phenotypically slow (< 0.55, $n = 17$) or rapid (> 0.55, $n = 9$) acetylators according to previously established population distributions (14, 22). Liver biopsy specimens from the same subjects expressed cytosolic *N*-acetyltransferase activities with SMZ as acceptor substrate, which were also distinctly bimodal and correlated nearly perfectly ($r = 0.98$, $P < 0.001$) with the in vivo acetylation index (Fig. 2). Two presumably homozygous rapid acetylators having AFMU/1X ratios > 1.8 (14) also showed particularly high levels of cytosolic enzyme activity (Fig. 2) and protein (see below). However, verification of the homozygous rapid genotype by family pedigree analysis has not yet been possible.

Empirical observations had previously shown (23) that AFMU excretion correlated with the classical SMZ plasma acetylation index (24) in healthy subjects, despite a lack of knowledge concerning the precise pathway of AFMU formation (25). The present study not only verifies the simplicity, safety, and convenience of the caffeine acetylator phenotyping test in clinical settings, but also provides the first direct validation of the AFMU/1X ratio as a remarkably precise indicator of human liver *N*-acetyltransferase activity. The reasons for this high degree of sensitivity are still unknown, but may relate to the liver-localized formation of the proximal *N*-acetyltransferase substrate (25) and the high polarity of AFMU and 1X, eliminating possible variations due to absorption and excretion characteristics of other commonly used test drugs.

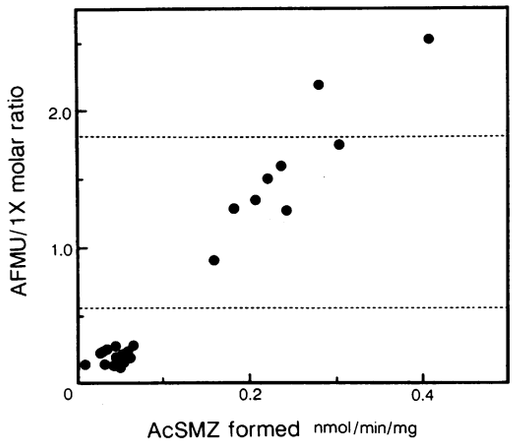

Figure 2. Correlation between in vivo and in vitro measures of acetylation capacity in 26 surgical patients. *Ordinate,* Urinary AFMU/1X molar ratio after caffeine ingestion; *abscissa,* formation rate of AcSMZ in cytosols from liver specimens. *Dotted lines,* Positions of published population antimodes separating apparent homozygous slow (< 0.55), heterozygous, and homozygous rapid (> 1.80) acetylators, taken from reference 14.

Relationship between N-acetyltransferase activity and immunodetectable protein. An immunopurified polyclonal serum recognizing human NAT-1 and NAT-2 (12) was reacted with Western blots of liver cytosols from the 26 phenotyped surgical patients and from 24 human organ donors. Immunoreactive protein with an apparent M_r of 31 kD was quantified by densitometric scanning of x-ray films and compared with in vitro SMZ-*N*-acetyltransferase activity (Fig. 3). The two measures correlated very highly (r = 0.92, P < 0.001), although with some degree of scatter due to the semi-quantitative nature of immunoblots and densitometric film scanning. Most importantly, in all cases (n = 26) where enzyme activity was consistent with the slow acetylator phenotype, including 17 in vivo phenotyped slow acetylators, the amount of immunoreactive 31-kD protein was below the detection limit (< 0.05 U) of the scanner. Conversely, *N*-acetyltransferase protein was detected in all cytosols with high enzyme activity. This indicates that activity differences between the two phenotypes relate to the quantity of enzyme present, as had previously been suggested on the basis of enzyme kinetic studies by Jenne (7). Lack of detectable immunoreaction in slow acetylator cytosols still possessing residual *N*-acetyltransferase activity (Fig. 3) may be due to the lower sensitivity of the immunodetection method. On the other hand, the presence of enzyme activity even in slow acetylators indicates either that the protein(s) are not completely absent, or that other acetylating enzymes not detected by our antiserum may contribute a low level of SMZ *N*-acetylation capacity.

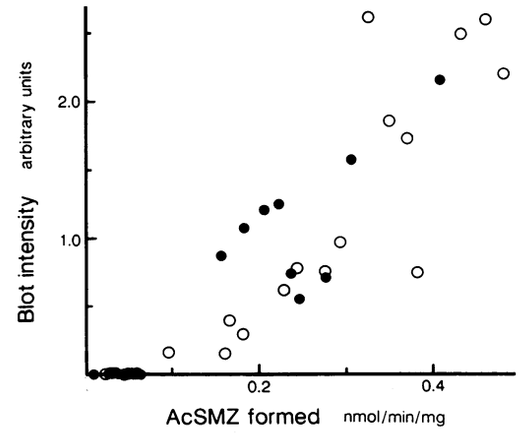

Figure 3. Correlation of cytosolic *N*-acetyltransferase activity and immunoreactive protein in liver cytosols from 26 surgical patients (●) and 24 kidney donors (○). *Ordinate,* Film scanner signal from immunoreactive 31-kD protein band after SDS-PAGE and Western transfer, expressed in arbitrary units of blot intensity; *abscissa,* as in Fig. 2.

The in vitro stability of *N*-acetyltransferase did not differ markedly in cytosols from in vivo phenotyped slow and rapid acetylators, displaying activity half-lives at 37°C of 3.4 and 5.6 h, respectively. This result is in agreement with that of Jenne (7), and makes it unlikely that all of the decrease in enzyme protein in slow acetylators is due to inherent instability of a mutant protein. Enzyme solubility characteristics were also indistinguishable between slow and rapid acetylator liver, eliminating the recently described possibility (26) that a protein structural alteration could cause a different apparent pattern of enzyme distribution between soluble and particulate subcellular fractions.

Content of NAT-1 and NAT-2 in different human livers. Our recent observation (12) that two kinetically distinct *N*-acetyltransferase activities, designated NAT-1 and NAT-2, could be resolved by DEAE-Sephacel anion exchange chromatography of cytosol from human livers prompted the speculation that these two closely related proteins could represent allelic variants expressed from a single polymorphic *N*-acetyltransferase gene locus. To test this hypothesis, cytosols from six kidney donor livers with low, medium, and high enzyme activity were subjected to analytical DEAE-Sephacel chromatography (shown for three livers in Fig. 4). Enzyme activity measurements in column fractions revealed that the activity present in the NAT-1 and NAT-2 peaks varied markedly between livers, but always in parallel. In one low-activity liver (KDL 18), peaks corresponding to NAT-1 and NAT-2 were completely undetectable, representing at least a 200-fold reduction compared with those observed in KDL 23 (Fig. 4) and KDL 21 (not shown). In another liver (KDL 17), NAT-1 and NAT-2 were drastically reduced but clearly detectable (15–20-fold above the assay detection limit). Measurement of apparent Michaelis constants in the pooled peak fractions showed that the affinities (K_m) of NAT-1 and NAT-2 for SMZ were relatively constant between livers, while limiting velocities (V_{max}) varied over at least a 20-fold range. Moreover, the variation in V_{max} values was accompanied by parallel differences in the amounts of immunoreactive NAT-1 and NAT-2 proteins present in the peak fractions (Fig. 4).

These observations are consistent with a model for slow acetylation involving a coordinated decrease in the quantity of each of two closely related *N*-acetyltransferase proteins in human liver. The results make it highly unlikely that NAT-1 and NAT-2 represent normal and mutant allele products of the polymorphic *N*-acetyltransferase gene locus. Assuming that the presence of NAT-1 and NAT-2 is not a purification artifact (12), the underlying mechanism leading to defective *N*-acetylation would then also need to account for this coordi-

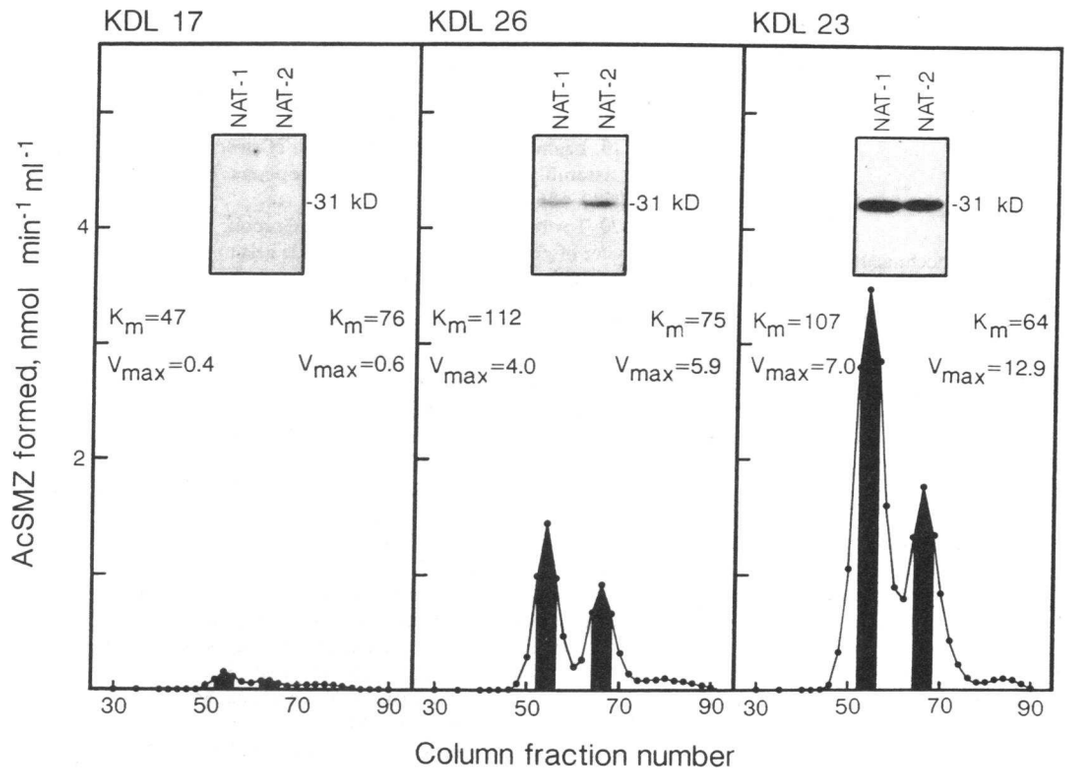

Figure 4. DEAE-Sephacel anion exchange chromatography of extracts from human kidney donor livers with low (KDL 17), intermediate (KDL 26), and high (KDL 23) cytosolic *N*-acetyltransferase activity (0.022, 0.293, and 0.433 nmol AcSMZ formed/min per mg cytosol protein, respectively). Shaded regions denote fractions from NAT-1 and NAT-2 peaks that were pooled and concentrated for determination of the Michaelis constants (K_m and V_{max}) indicated, and for SDS-PAGE (0.02 mg protein/lane) followed by Western blotting and immunoreaction (*insets*) as described in Methods.

nated pattern of expression. At present, the simplest explanation would involve polymorphism in the production of a single gene product, followed by proportional posttranslational modification giving rise to a second functional enzyme with distinct kinetic and charge properties. Also worthy of mention are the intermediate levels of NAT-1 and NAT-2 expression observed in KDL 26 (Fig. 4) and KDL 27 (not shown, result identical), consistent with heterozygosity for rapid acetylation in an additive gene dosage model.

Mechanisms for decreased enzyme content in slow acetylators. It remains to be seen which of many different mutational processes could underlie our observations of decreased *N*-acetyltransferase activity and immunoreactive protein in livers from slow acetylator individuals. At the protein level, it is still conceivable that a gross alteration in enzyme structure could alter both its immunoreactivity to a polyclonal antiserum and its capacity for arylamine substrates with little change in substrate affinity. However, we consider it highly unlikely that a mutant enzyme with such characteristics is actually present in slow acetylator livers but not detectable using the methods we report here. Although another possibility would be that a structurally altered protein with decreased stability is produced, the present results and those of Jenne (7) suggest that the enzyme activities in rapid and slow acetylator livers have similar in vitro stabilities. Our findings would therefore suggest that slow acetylation may result from decreased synthesis of enzyme protein.

At the DNA level, both point mutations and gross gene abnormalities (deletions, insertions, rearrangements) have been shown to cause a wide variety of human genetic disorders (27), many of which result in an absence of protein. For example, one of the most common haplotypes in hereditary phenylketonuria, caused by a single base substitution in the phenylalanine hydroxylase gene, results in an amino acid change that destabilizes the resulting enzyme protein (28). Point mutations may also have a crucial effect on gene transcription (29), transcript processing (28, 30), and the initiation, efficiency, or frame recognition of protein translation systems. In this context, it is obviously important to measure levels of *N*-acetyltransferase-specific mRNA in rapid and slow acetylator livers to distinguish between transcriptional and translational defects associated with the slow acetylator phenotype. Our recent isolation of a human gene encoding an arylamine *N*-acetyltransferase (31) has provided us with a specific tool to perform such studies. Although our preliminary results from RNA blot hybridization analyses (Grant, D. M., M. Blum, M. Heim, and U. A. Meyer, unpublished observations) have failed to find a clear relationship between levels of *N*-acetyltransferase transcripts and protein in rapid and slow acetylator human livers, these findings require further confirmation.

On the other hand, gene deletion or rearrangement, as has been detected by hybridization analysis at human loci for cytochrome P-450IID6 (P450db1) (32) and glutathione transferase (33) representing other drug-metabolizing enzyme systems, may be even more likely to result in protein deficiency. We have recently established that absence of the gene encoding arylamine *N*-acetyl-transferase in slow acetylator rabbits leads to a complete lack of specific mRNA and enzyme protein (34). Thus, at the protein level defective *N*-acetylation in man and rabbit appear very similar. However, our Southern analyses of genomic DNA from slow and rapid acetylator humans (35) suggest that in man a deletion mechanism is either unlikely or uncommon. Therefore, we expect that the molecular mechanism of the acetylation polymorphism in man is different from that observed in the rabbit model. Moreover, although our finding that all slow acetylators so far tested exhibit decreased

enzyme content might imply that the acetylation defect may be relatively homogeneous in nature, recent experience (30, 32, 36) has taught us that different molecular defects may produce the same phenotypic pattern in vivo or at the level of functional protein expression.

Acknowledgments

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