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EFFECT OF GROUP-SELECTIVE MODIFICATION REAGENTS ON ARYLAMINE *N*-ACETYLTRANSFERASE ACTIVITIES

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Abstract—Two forms of hamster hepatic arylamine *N*-acetyltransferase (NAT; EC 2.3.1.5), designated NAT I and NAT II, were purified 200- to 300-fold by sequential 35–50% ammonium sulfate fractionation, Sephadex G-100 gel filtration chromatography, AAB affinity chromatography, DEAE ion exchange chromatography, and P-200 gel filtration chromatography. Treatment of either NAT I or NAT II with *N*-ethylmaleimide (NEM), a cysteine selective reagent, caused a concentration-dependent loss of enzymatic activities. Acetyl coenzyme A (AcCoA) protected NAT I against inactivation by NEM, whereas both 2-acetylaminofluorene (2-AAF) and AcCoA protected NAT II against inactivation. Incubation of either NAT I or NAT II with phenylglyoxal (PG), an arginine selective reagent, caused a time-dependent and a concentration-dependent loss of both NAT I and NAT II activities; the inactivations followed pseudo first-order kinetics. The reaction order with respect to PG was approximately two for each enzyme, consistent with the expected stoichiometry for the reaction of PG with arginine. The presence of AcCoA provided full protection of NAT I against inactivation by PG. However, neither AcCoA nor 2-AAF provided protection of NAT II against inactivation by PG. Diethylpyrocarbonate (DEPC), a histidine selective reagent, caused time-dependent and concentration-dependent pseudo first-order inactivation of both NAT I and NAT II. Neither AcCoA nor products of NAT-catalyzed reactions protected NAT I and NAT II against inactivation by DEPC. These results suggest that cysteine, arginine and histidine residues are essential to the catalytic activity of both NAT I and NAT II; the cysteine(s) is located at or near the binding site of NAT I and NAT II, and the arginine residue appears to be located in the AcCoA binding site of NAT I. In contrast, the essential arginine residue(s) of NAT II and the essential histidine residue(s) of both NAT I and NAT II are not likely to reside in the binding site of the enzymes.

N-Acetylation is an important metabolic pathway for arylamine drugs and other xenobiotics, and is considered to be a determinant in drug toxicity and arylamine-induced carcinogenesis [1–3]. Acetyl coenzyme A (AcCoA§) dependent arylamine *N*-acetyltransferases (NAT; EC 2.3.1.5), cytosolic enzymes found in numerous mammalian tissues, catalyze the arylamine N-acetylation reaction [1].

NATs exhibit genetically determined polymorphisms and multiple forms of NATs have been reported in several species, including humans [1–7]. Studies employing mechanism-based inactivation and chromatographic techniques have indicated that at least two forms of NAT activities exist in hamster liver [8, 9], hamster intestine [9] and rat liver cytosol [10], and that the various forms have different substrate specificities [11–13]. In hamster and rat liver, one isozyme (NAT I) preferentially catalyzes AcCoA-dependent transacetylation of *p*-aminobenzoic acid (PABA), whereas the other isozyme (NAT II) catalyzes *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) dependent transacetylation of 4-aminoazobenzene (AAB) (N,NAT activity), AcCoA-dependent transacetylation of procainamide (PA), and the bioactivation of carcinogenic *N*-arylhydroxamic acids via *N*,*O*-acyltransfer (AHAT activity) [4, 7, 9, 10].‖ Following the isolation of a cDNA clone encoding the chicken liver NAT [14], complete nucleotide sequences of *NAT* genes from humans, rabbits and hamsters were reported [15–19]. These genes display high intra- and interspecies sequence homology [15–19].

Although the multiplicity of NATs and their biochemical and kinetic properties have been studied extensively [4, 8, 9, 20–23], the catalytic mechanism and the basis for the differential substrate specificities of NAT isozymes are not known. Andres *et al.* [24] presented evidence that the acetyl moiety which is transferred from AcCoA is bound as a thioester to a cysteine residue of the rabbit liver enzyme. However, information on the amino acid residues involved in substrate binding and in catalysis is limited.

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§ Abbreviations: AcCoA, acetyl coenzyme A; NAT, arylamine *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; N-OH-AAF, *N*-hydroxy-2-acetylaminfluorene; AAB, 4-aminoazobenzene; DTT, D,L-dithiothreitol; N,NAT, *N*,*N*-acetyltransferase; PA, procainamide; AHAT, *N*-arylhydroxamic acid *N*,*O*-actyltransferase; NEM, *N*-ethylmaleimide; PG, phenylglyoxal; DEPC, diethylpyrocarbonate; p-AABA, *p*-acetamidobenzoic acid; 2-AAF, 2-acetylaminofluorene; and 2-AF, 2-aminofluorene.

‖ The enzyme referred to as NAT I in the present paper corresponds to the polymorphic enzyme designated as AT-II in Ref. 7, and NAT II corresponds to the monomorphic enzyme designated as AT-I in Ref. 7.

Reports from this laboratory have described the design, synthesis and evaluation of both mechanism-based inhibitors and affinity-labeling agents for NAT isozymes [10, 12, 13, 25]. Particular efforts have been directed toward the design of reagents that exhibit specificity for those NATs that can use N-OH-AAF as an acetyl donor and which exhibit AHAT activity (NAT II) [12]. Because PABA is an efficient acetyl acceptor in NAT I-catalyzed reactions, but is not a good substrate for NAT II, the development of selective NAT I inhibitors also should be feasible if sufficient information were available with regard to the critical amino acid residues involved in binding and/or catalysis of PABA acetylation and with regard to the types of chemical functional groups that can inactivate the enzyme. In connection with the effort to design isozyme-selective reagents, chemical modification studies were undertaken to obtain information regarding the nature of catalytically essential amino acid residues that might reside in the active sites of the enzymes. Chemical modification of enzymes has been used widely to identify amino acids at the active sites of enzymes [26, 27]. In the present paper we describe studies on the inactivation of two NAT isozymes from hamster liver by *N*-ethylmaleimide (NEM), phenylglyoxal (PG) and diethylpyrocarbonate (DEPC), and evidence is presented that cysteine, arginine and histidine residues are required for the catalytic activity of both NATs. Preliminary reports of some of these results have been published [28, 29].

MATERIALS AND METHODS

Chemicals and reagents

PABA (sodium salt), D,L-dithiothreitol (DTT), *S*-acetyl coenzyme A (AcCoA, trilithium salt), bovine serum albumin, tetrasodium pyrophosphate, Sephadex G-100, NEM, phenylglyoxal hydrate (PG), ammonium sulfamate, DEPC and *N*-acetylprocainamide (N-Ac-PA) were purchased from the Sigma Chemical Co. (St. Louis, MO). 2-Aminofluorene (2-AF), histidine hydrochloride, *p*-acetamidobenzoic acid (p-AABA) and 2-acetylaminofluorene (2-AAF) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). AAB and *N*-1-(naphthyl)ethylenediamine dihydrochloride were purchased from the Eastman Kodak Co. (Rochester, NY). Epoxy-activated Sepharose 6B and PD-10 desalting columns (1.5 × 5 cm) prepacked with Sephadex G-25M were purchased from the Pharmacia Chemical Co. (Piscataway, NJ). Sodium nitrite, trichloroacetic acid (TCA) and EDTA were purchased from the Fisher Scientific Co. (Fairlawn, NJ). Potassium phosphate (monobasic) was obtained from Malinckrodt Inc. (St. Louis, MO). DEAE Biogel A, Bio-Rad dye Reagent Concentrate and Biol gel P-200 were obtained from Bio-Rad Laboratories (Richmond, CA). Ultrafiltration membranes (YM-10) were obtained from the Amicon Corp. (Danvers, MA). Ammonium sulfate was obtained from the Spectrum Chemical Co. (Redondo Beach, CA). N-OH-AAF was prepared as previously described [25]. The coupling of AAB to epoxy-activated Sepharose 6B was performed as described previously [9, 30]; dimethylformamide (15 mL) was used as a cosolvent for AAB. The efficiency of AAB coupling to epoxy groups was approximately 42–62% [31].

Tissue preparation

Male Golden Syrian hamsters (50–70 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Hamsters were lightly anesthetized with diethyl ether and decapitated. The livers were removed immediately and the gall bladders were dissected away from the livers. Livers were rinsed in cold 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT and were homogenized with 1 mL of this buffer per g of liver with a motor-driven Teflon/glass homogenizer. The homogenate was centrifuged at 105,000 *g* for 60 min at 4°.

Partial purification of N-*acetyltransferases*

Step 1. *Ammonium sulfate fractionation.* The 105,000 *g* supernatant fraction was brought to 35% saturation by adding a cold saturated solution of ammonium sulfate in 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT) with stirring in an ice bath. The precipitate obtained after centrifugation (9000 *g*, 10 min) was discarded and the supernatant was brought to 50% saturation by further addition of cold saturated ammonium sulfate solution. The 50% saturated solution was centrifuged at 9000 *g* for 10 min. The pellets were stored at −70°. All subsequent purification steps were carried out at 4°.

Step 2. *Sephadex G-100 gel filtration chromatography.* The pellets obtained from step 1 were suspended in 0.02 M potassium phosphate buffer (pH 7.4) containing 1 mM DTT and 1 mM EDTA. Protein concentration was approximately 50 mg/mL. Six millilitres of the diluted ammonium sulfate fraction was applied to a column (2.5 × 45 cm) of Sephadex G-100 which had been equilibrated with degassed 0.02 M potassium phosphate buffer (1 mM DTT, 1 mM EDTA, pH 7.4). The column was eluted with 250 mL of degassed buffer. Fractions (12 mL) were collected at a flow rate of 30 mL/hr. Aliquots of each fraction were assayed for transacetylase activities as described under *Enzyme Assays.* Fractions containing the majority of transacetylation activity were combined.

Step 3. *AAB coupled affinity chromatography.* The pooled fractions from step 2 (1.5 mg protein/mL, 30 mL) were applied to an AAB coupled epoxy-activated Sepharose 6B affinity column (1.5 × 15 cm) equilibrated with degassed 0.02 M potassium phosphate buffer containing 1 mM DTT and 1 mM EDTA (pH 7.4). Fractions (7 mL) were collected at a flow rate of approximately 40 mL/hr. At fraction number 20, 0.4 M potassium chloride in degassed 0.02 M potassium phosphate buffer (1 mM DTT, 1 mM EDTA, pH 7.4) was applied. Transacetylation activities of each fraction were determined as described under *Enzyme Assays.* This step resulted in the separation of NAT I and NAT II activities. The fractions containing the majority of the AcCoA/PABA transacetylation activity (NAT I) were pooled, concentrated to approximately 25% of the original volume under a nitrogen atmosphere by using an Amicon ultrafiltration cell with a YM 10 membrane. Glycerol (10% final concentration) was

added to stabilize the enzymatic activity before storage at −70°. The fractions containing the N-OH-AAF/AAB N,NAT activity (NAT II) were pooled, concentrated as described for NAT I, and stored at −70° in 30% glycerol.

Step 4. DEAE anion exchange chromatography. The NAT I preparation resulting from step 3 (1.2 mg protein/mL, 20 mL) was loaded onto a DEAE anion exchange column (1.5 × 27 cm) equilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.1, 1 mM DTT, 1 mM EDTA). Fractions (8 mL) were collected at a flow rate of 30 mL/hr. At fraction number 6, a potassium chloride gradient (0 to 0.35 M) in the degassed 0.02 M potassium phosphate buffer was begun. The AcCoA/PABA transacetylation activity of each fraction was determined as described under *Enzyme Assays*. The fractions which contained the majority of the AcCoA/PABA NAT activity were pooled and concentrated under a nitrogen atmosphere to approximately 25% of the original volume by using an Amicon ultrafiltration cell with a YM-10 membrane. Glycerol was added to a concentration of 10% to stabilize enzymatic activity. The NAT II preparation from step 3 (0.2 mg protein/mL, 15 mL) was desalted by passing it through PD-10 columns of Sephadex G-25 M that had been preequilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.4, 1 mM DTT, 1 mM EDTA); a volume of 2.5 mL was added to each PD-10 column, and the eluent was discarded. Then the sample was eluted with 3.5 mL of the 0.02 M potassium phosphate buffer, and the eluent from each PD-10 column was collected and pooled. The desalted NAT II preparation (21 mL) was applied to a DEAE anion exchange column (1.5 × 27 cm). The subsequent procedures were the same as described for NAT I. The fractions containing N-OH-AAF/AAB N,NAT activity (NAT II) were pooled, concentrated and stored at −70° in 20% glycerol.

Step 5. Polyacrylamide P-200 gel filtration chromatography. The NAT I preparation which was purified by step 4 was further subjected to polyacrylamide P-200 gel filtration chromatography. The preparation (3.6 mg protein/mL, 1 mL) was applied to a P-200 gel filtration column (1 × 50 cm) and eluted with degassed 0.02 M potassium phosphate buffer (pH 7.4, 1 mM DTT, 1 mM EDTA) at a flow rate of 24 mL/hr. Fractions (3 mL) were collected. The fractions which contained the majority of AcCoA/PABA NAT activity (NAT I) were pooled and stored at −70° in 10% glycerol.

Inactivation of NATs by NEM

Incubation mixtures contained partially purified NAT I (final protein concentration 0.088 mg/mL) or NAT II (final protein concentration 0.027 mg/mL) obtained by DEAE ion exchange chromatography, 0.05 M sodium pyrophosphate buffer (pH 7.0) and 0.25 mM DTT in a volume of 0.675 mL. Incubations were started by addition of NEM (0.05 to 0.4 mM final concentration) in a volume of 0.075 mL at 37°. At the end of a 1-min incubation, 0.7 mL of the reaction mixture was applied to a PD-10 column pre-packed with Sephadex G-25M to remove the unreacted reagent. The column was then eluted with two portions (2.3 and 1.3 mL) of 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT). The second portions of the buffer, which contained the majority of transacetylase activities, were collected and kept on ice. The protein concentrations of these fractions were determined and adjusted for measurement of the remaining AcCoA/PABA or N-OH-AAF/AAB transacetylase activities as described under *Enzyme Assays*.

Inactivation of NATs by PG

Incubation mixtures contained partially purified NAT I (final protein concentration 0.033 mg/mL) obtained by P-200 gel filtration chromatography and 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT) in a volume of 0.675 mL. Incubations were started by the addition of PG (4–10 mM final concentration) in a volume of 0.075 mL at 37°. At different time intervals, 0.7 mL of the reaction mixture was withdrawn and filtered through a PD-10 column of Sephadex G-25M exactly as described for the inactivation of NATs by NEM. The second portions of the buffer, which contained the majority of enzyme activity, were collected and the protein concentrations of these fractions were determined. The residual AcCoA/PABA NAT activity was assayed as described under *Enzyme Assays*. To determine the inactivation of NAT II activity by PG, partially purified NAT II obtained by DEAE ion exchange chromatography was diluted with 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT) to a final protein concentration of 0.03 mg/mL in a volume of 0.675 mL. Incubations at 37° were started by the addition of PG (5–15 mM final concentration) in a volume of 0.075 mL. The remaining N-OH-AAF/AAB N,NAT activity was determined with a 0.7-mL portion of the incubation mixture as described for the inactivation of NAT II by NEM.

Inactivation of NATs by DEPC

The concentration of DEPC used in the incubation mixtures was determined by its reaction with histidine. Aliquots (0.03 mL) of an ethanol solution of DEPC (approximately 25 mM) were mixed with 1.02 mL of 600 mM L-histidine at pH 7.0. The blank contained 0.03 mL of absolute ethanol instead of DEPC. The concentration was calculated from the increase in absorbance at 240 nm due to the formation of *N*-carbethoxyhistidine by using a molar extinction coefficient of 3200 M^{-1} [32].

Incubation of partially purified NAT I (final protein concentration 0.02 mg/mL) obtained by P-200 gel filtration chromatography or NAT II (final protein concentrations 0.018 mg/mL) obtained by DEAE ion exchange chromatography with DEPC (0.05 to 1.6 mM) was carried out at 37° in a volume of 0.95 mL of 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT). The incubation was started by the addition of an ethanol solution (0.03 mL) of DEPC. The final concentration of ethanol (3%) had no effect on control enzyme activity. At the end of the incubation period, excess L-histidine (40 mM final concentration) in a volume of 0.07 mL was added to the incubation tubes to terminate the reaction. A 1.0-mL portion of the incubation mixtures was applied to a PD-10 column pre-packed

with Sephadex G-25M. The column was then eluted with two portions (2.0 and 1.3 mL) of 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT). The second portion of the buffer, which contained the majority of enzymatic activity, was collected and the protein concentrations were determined, and adjusted for assay of AcCoA/PABA or N-OH-AAF/AAB tranacetylase activity as described under *Enzyme Assays*.

Effect of substrates and products on NAT inactivation

The ability of various substrates or products to protect NATs against inactivation by NEM, PG or DEPC was tested by preincubation of the partially purified NAT I or NAT II with the potential protecting agents at 37° for 1 min followed by incubation with the modifying agents. Incubation conditions were the same as described above for experiments with the inactivating agents except that incubation mixtures contained AcCoA (0.01 to 0.5 mM), an ethanol solution of either 2-AAF (0.02 to 0.5 mM) or N-Ac-PA (1.0 mM) or a DMSO solution of p-AABA (1.0 mM) in a volume of 0.075 mL (for NEM and PG) or 0.05 mL (for DEPC). In control experiments, the potential protecting agents did not affect transacetylase activities when they were incubated with the enzyme in the absence of NEM, PG or DEPC and were removed by filtration through PD-10 columns.

Effect of hydroxylamine on DEPC-treated NAT I and NAT II

The NAT I or NAT II was purified and separated from the cytosolic fractions by 35–50% ammonium sulfate fractionation and DEAE anion exchange chromatography. Partially purified NAT I or NAT II (final protein concentration 0.5 mg/mL) in 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 6.5) was incubated with DEPC at 4° in a final volume of 2.1 mL. The final concentration of DEPC was 0.3 mM in the experiments with NAT I and 2.0 mM in the experiments with NAT II. DEPC was added as an ethanol solution. The concentration of ethanol was 3%. At the end of 4 hr (NAT I) or 2 hr (NAT II), 0.7 mL of 600 mM L-histidine was added to the incubation mixtures, and a 2.5-mL aliquot of the mixture was applied to a PD-10 column of Sephadex G-25M. The column was then eluted with 3.5 mL of 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) and the eluent was collected. The remaining AcCoA/PABA or N-OH-AAF/AAB transacetylase activity and the protein concentrations were determined. To test the reversibility of DEPC-inactivated NAT I activity by hydroxylamine, aliquots of the eluents (3.0 mL) from PD-10 columns were incubated with hydroxylamine (0.2 mL, 0.2 M final concentration), pH 7.0, at room temperature. A portion (0.7 mL) was periodically withdrawn and filtered through a PD-10 column of Sephadex G-25M. The column was eluted as described for the inactivation of NATs by NEM, and assayed for AcCoA/PABA transacetylase activity as described under *Enzyme Assays*. To examine the effect of hydroxylamine on DEPC-inactivated NAT II activity, aliquots of the eluents (1.8 mL) from PD-10 columns containing DEPC-treated NAT II were incubated with hydroxylamine (0.06 mL, 0.1 M final concentration), pH 7.0, at 4°. At different time intervals, aliquots (0.7 mL) were filtered through PD-10 columns of Sephadex G-25M as described above, and assayed for N-OH-AAF/AAB N,NAT activity as described under *Enzyme Assays*. Hydroxylamine treatment at the concentrations used in these experiments did not affect control NAT activities. The three sets of controls for these experiments included enzyme preparations that were not treated with either DEPC or hydroxylamine, preparations that wete treated with DEPC only, and preparations that were treated with hydroxylamine only. All control preparations were incubated for the same time periods and under the same conditions as the preparations that were treated with both DEPC and hydroxylamine.

Enzyme assays

The procedures were carried out at protein concentrations and over time periods determined from the linear portions of the plots of enzyme activity versus time.

AcCoA/PABA NAT assay. AcCoA-dependent PABA NAT activity was determined spectrophotometrically by measuring the AcCoA-dependent decrease in the concentration of PABA. Reaction mixtures contained either 0.1 mL of the enzyme preparation (3–10 μg protein/mL) in 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) or aliquots (0.03 mL) of the fractions from column chromatography and a sufficient amount of the buffer to bring the incubation volume to 0.1 mL. AcCoA (0.0167 mL of a 6 mM solution) was added and the incubations were then initiated by adding 0.05 mL of 0.33 mM PABA. Incubations were carried out at 37° in air and were terminated by the addition of 0.333 mL of cold 5% TCA. After centrifugation to remove the precipitated proteins, the reaction rate was determined by diazotization of the unacetylated PABA according to the Bratton Marshall procedure [33], and was expressed as nanomoles per milligram of protein per minute. Incubations without AcCoA were included as controls.

N-OH-AAF/AAB transacetylation assay. The N-OH-AAF-dependent N,NAT activity was determined spectrophotometrically by the method of Booth [34]. Incubation tubes (1.5 mL) contained either 0.48 mL of the enzyme preparation (5–8 μg protein/mL) in 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT or aliquots (0.2 mL) of the fractions from column chromatography and a sufficient amount of 0.05 M sodium pyrophosphate buffer to bring the volume to 0.48 mL. The reactions were started by the addition of 0.02 mL of substrate solution (12.5 mM N-OH-AAF/3.75 mM AAB dissolved in 95% ethanol). Incubations were carried out for 2 min in air at 37°. The reactions were terminated by adding 0.5 mL of cold 20% TCA (ethanol:water, 1:1). After centrifugation of the mixture to remove the precipitated protein, acetylation of AAB was determined by the decrease in absorbance at 497 nm. The reaction mixture without enzyme preparation was used as a control.

Table 1. Partial purification of hamster hepatic NAT I and NAT II activities

Step	NAT I			NAT II		
	Activity* (nmol/mg/min)	Purification (fold)	Recovery (%)	Activity† (nmol/mg/min)	Purification (fold)	Recovery (%)
Cytosol	28.6	1.00	100	15.7	1.00	100
Ammonium sulfate	50.8	1.77	50	25.0	1.59	43
Sephadex G-100	157	5.46	37	76.5	4.87	29
Sepharose 6B AAB	393	13.7	30	1910	122	19
DEAE ion exchange	3090	108	19	2980	190	11
P-200 gel filtration	7820	273	10			

* Activities are expressed as AcCoA/PABA transacetylation rates.
† Activities are expressed as N-OH-AAF/AAB transacetylation rates.

Protein determination

Protein concentration was determined by the method of Bradford [35] with bovine serum albumin as the standard.

RESULTS

Partial purification of hamster hepatic NATs

Two NAT isozymes were partially purified from hamster liver cytosol by 35–50% ammonium sulfate fractionation, G-100 gel filtration chromatography, AAB Sepharose 6B affinity chromatography and DEAE ion exchange chromatography. AAB affinity chromatography resulted in separation of the NAT I and NAT II activities as previously described [9]. NAT I and NAT II were further purified by DEAE ion exchange chromatography and NAT I was then subjected to polyacrylamide P-200 gel filtration chromatography. Table 1 summarizes a representative purification scheme for the two isozymes. An overall 200- to 300-fold purification of both NAT I and NAT II was accomplished with this purification scheme. Total recovery was about 10% for each NAT.

Inactivation of NAT I and NAT II with NEM

Maintenance of NAT I and NAT II activities *in vitro* requires the presence of a thiol-containing

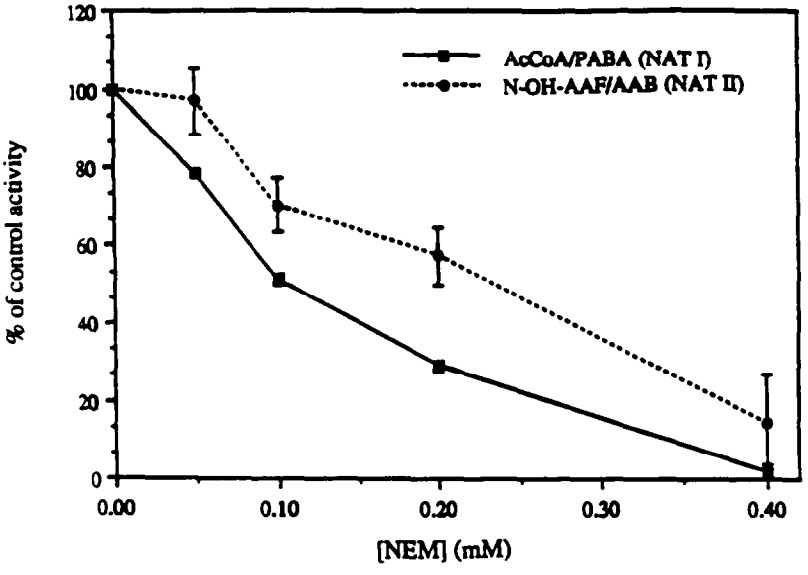

Fig. 1. Inactivation of hamster hepatic NAT I and NAT II by *N*-ethylmaleimide. Partially purified hamster hepatic NAT I and NAT II were incubated at 37° in the presence of 0.25 mM DTT and various concentrations of NEM for 1 min. The remaining AcCoA/PABA (NAT I) or *N*-OH-AAF/AAB (NAT II) transacetylase activity was measured as described under Materials and Methods after filtration of the incubation mixtures through Sephadex G-25M. Results are the means ± SD of three experiments, each conducted in triplicate. Control activities (expressed in nmol/mg protein/min) were 2651 ± 323 for AcCoA/PABA (NAT I) and 2778 ± 235 for N-OH-AAF/AAB (NAT II) transacetylase activities (mean ± SD, N = 3).

reagent such as DTT. Because of the possibility that the sulfhydryl groups of DTT would react with NEM, experiments were conducted to determine a concentration of DTT that would support enzyme activity but would not prevent NEM-mediated inactivation. Preliminary studies demonstrated that 0.25 mM DTT was sufficient to maintain maximal levels of enzyme activation in control experiments (data not presented).

As shown in Fig. 1, NEM caused a concentration-dependent loss of both NAT I and NAT II activities in the presence of 0.25 mM DTT. Inactivation was very rapid, with a complete loss of NAT I activity and an 80% loss of NAT II activity being achieved within 1 min in the presence of 0.4 mM NEM. Total inactivation of NAT II occurred when it was incubated with 0.5 mM NEM for 1–2 min (data not presented). The complete loss of the enzyme activities in the presence of NEM indicates that essential cysteine residues are modified by the reagent.

To determine whether the essential cysteine residues modified by NEM reside in or near the active sites of the enzymes, substrates and products of NAT-catalyzed reactions were tested for their abilities to prevent the NEM-mediated inactivation process. Incubation of partially purified preparations of both isozymes with AcCoA afforded complete protection from inactivation by NEM (Fig. 2, A and B). Additionally, the acetylation product of 2-AF, 2-AAF, prevented inactivation of NAT II by NEM (Fig. 2B). These results indicate that active site cysteine residues of both NAT I and NAT II are alkylated by NEM.

It was also found that a modest degree of protection (25–30%) of NAT I was afforded by both PABA and its acetylation product (p-AABA), 2-AF, and 3′,5′-adenosine diphosphate (ABP) when the potential protecting agents were present in 1.0 mM concentrations (data not presented). Neither PA (0.5 mM) nor N-Ac-PA (1.0 mM) reduced the extent of inactivation of NAT II by NEM, although 2-AF (0.5 mM) reduced the extent of inactivation by approximately 30% (data not presented).

Inactivation of NAT I and NAT II with PG

Figures 3 and 4 show that the inactivation of either NAT I or NAT II with various concentrations of PG followed pseudo first-order kinetics over the concentration range of the reagent used. First-order inactivation rate constants (k_{obs}) were calculated from the slopes of the lines obtained from the plots of the logarithm of percent control activity versus time, according to the method of Kitz and Wilson [36]. The plots of log k_{obs} against log [PG] had a slope of approximately 2, which indicates that the reaction is second order in phenylglyoxal and is consistent with the usual 2:1 stoichiometry of the reaction between PG and an arginine residue [37]. Also, consistent with this kinetic order, replots of the pseudo first-order rate constants (k_{obs}) as a function of the *square* of the PG concentration were linear for each enzyme (results not shown). Enzyme activity was not restored upon passage of the incubation mixtures through Sephadex G-25M, indicating that the inactivation of NAT I and NAT II by PG was irreversible.

The effects of substrates and products on the inactivation of NAT I and NAT II by PG were investigated. The results illustrated in Fig. 5 show that AcCoA protected NAT I against inactivation by PG. The presence of PABA, p-AABA, 2-AAF or CoA had little effect on the inactivation of NAT I by PG (results not shown). The protection of NAT I by AcCoA from inactivation suggests that at least one arginine residue is involved in AcCoA binding. In contrast, little protection of NAT II against PG-induced inactivation was seen when AcCoA, 2-AAF or N-Ac-PA was present in the incubation mixtures (Table 2).

Inactivation of NAT I and NAT II with DEPC

Incubation of either NAT I or NAT II with DEPC caused time-dependent and concentration-dependent inactivation of both enzymatic activities. Plots of the logarithm of percent remaining enzymatic activities against time were linear (Figs. 6 and 7), indicating that the inactivation process approximates first-order kinetics with respect to time at any fixed concentration of DEPC. Double logarithmic plots of k_{obs} versus [DEPC], presented in the insets of Figs. 6 and 7 show that the inactivation process was first-order with respect to DEPC, indicating that there may be one essential DEPC-sensitive residue in each active NAT molecule. From these plots, second-order rate constants of $47\ M^{-1}\ sec^{-1}$ and $3.9\ M^{-1}\ sec^{-1}$ for inactivation of NAT I and NAT II, respectively, were obtained [38].

Although histidyl residues are selectively modified by DEPC, the reagent also can react with amino acids such as tyrosine and lysine. Reaction of DEPC with histidine causes an increase in absorbance at 240 nm, whereas modification of tyrosyl residues results in a decrease in absorbance at 278 nm [32]. The difference spectra of DEPC-treated NAT I and NAT II showed an increase in the absorption maximum at 240 nm, but there was no significant decrease at 280 nm (results not shown). Thus, no evidence for modification of tyrosine by DEPC was obtained.

The ethoxyformyl (carbethoxy) group that is introduced into histidyl residues by DEPC can be removed from those residues by cleavage with hydroxylamine. Thus, incubation of DEPC-inactivated enzymes with hydroxylamine would be expected to result in recovery of enzyme activity if the inactivation is due to ethoxyformylation of histidine. When preparations of NAT I that had been inactivated by treatment with DEPC (pH 7.0, 37°) were incubated with hydroxylamine for 22 hr, no recovery of enzymatic activity occurred (data not shown). It was demonstrated in control experiments that the concentrations of hydroxylamine used in these experiments had no effect on NAT I activity when incubated with enzyme preparations that had not been treated with DEPC.

Because the selectivity of DEPC for reaction with histidyl residues is greater at pH values somewhat below 7, NAT I and NAT II preparations were treated with the reagent at pH 6.5 (4°). The results shown in Table 3 indicate that the inactivation of

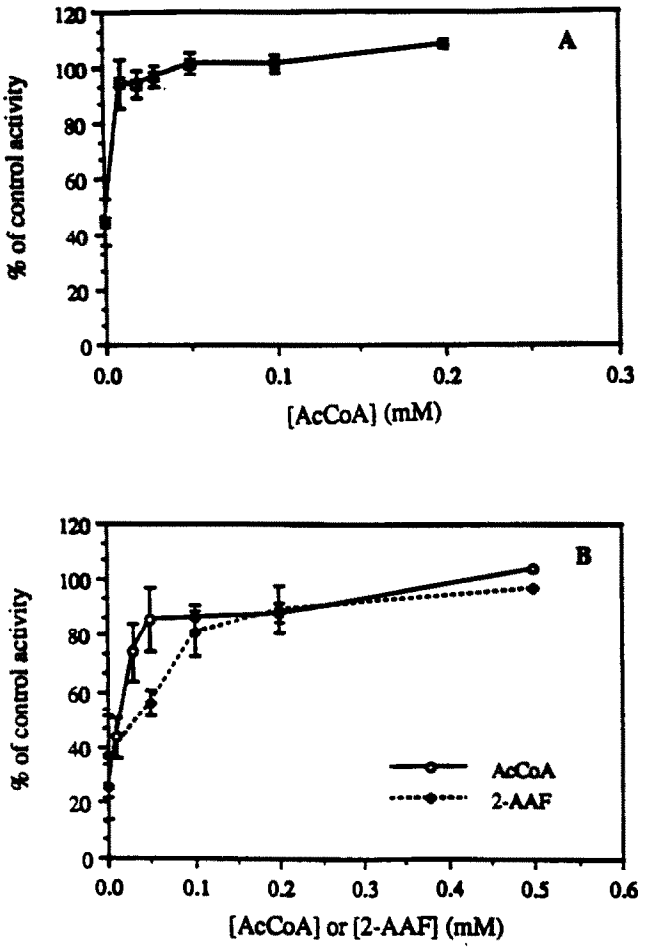

Fig. 2. Inactivation of hamster hepatic NAT I and NAT II by NEM: Effects of AcCoA and 2-AAF. (A) Hamster hepatic NAT I, purified by DEAE ion exchange chromatography, was incubated with AcCoA for 1 min at 37°. NEM (0.1 mM) was then added and the reaction mixture was incubated for an additional 1 min. The remaining AcCoA/PABA NAT activity was measured as described under Materials and Methods after filtration through Sephadex G-25M. Results are presented as means ± SD for three experiments, except where the AcCoA concentration was 0.2 mM (N = 1). Control activity was 2678 ± 189 nmol/mg protein/min (mean ± SD, N = 3). (B) Incubations of hamster hepatic NAT II, purified by DEAE ion exchange chromatography, were conducted at 37° in the presence of 0.3 mM NEM and the indicated concentrations of AcCoA or 2-AAF. Incubation mixtures were then filtered through Sephadex G-25M prior to measurement of N-OH-AAF/AAB N,NAT activity as described under Materials and Methods. Values are the means ± SD for three experiments, except where the concentration of AcCoA or 2-AAF was 0.5 mM (N = 1). Control activity was 2457 ± 148 nmol/mg protein/min (mean ± SD, N = 3).

NAT I with DEPC under these conditions was reversed by incubation with hydroxylamine, but when a preparation of NAT II that had been inactivated under similar conditions was incubated with hydroxylamine, a further loss of enzyme activity resulted (Table 3).

The effects of AcCoA and of N-acetylated products of NAT-catalyzed reactions on the DEPC-mediated inactivation process were investigated. None of the potential protecting agents had a substantial effect on the ability of DEPC to inactivate either NAT I or NAT II (Table 4).

DISCUSSION

N-Acetylation is a common biotransformation process which plays an important role in determining the pharmacological and toxicological effects of both arylamines and *N*-arylhydroxylamines [1–3]. The use of group-selective modification reagents as inhibitors of NAT activity can provide not only information about the types of chemical functional groups that may influence NAT activity *in vitro* or *in vivo*, but also insight into the probable identity of amino acid residues that are critical to NAT activity.

In the present study, the sulfhydryl reagent *N*-ethylmaleimide was employed to investigate the importance of cysteine residues in the catalytic activity of hamster hepatic NATs. Previously, the requirement of a reducing agent (e.g. dithiothreitol) for the stabilization of AHAT activity was reported,

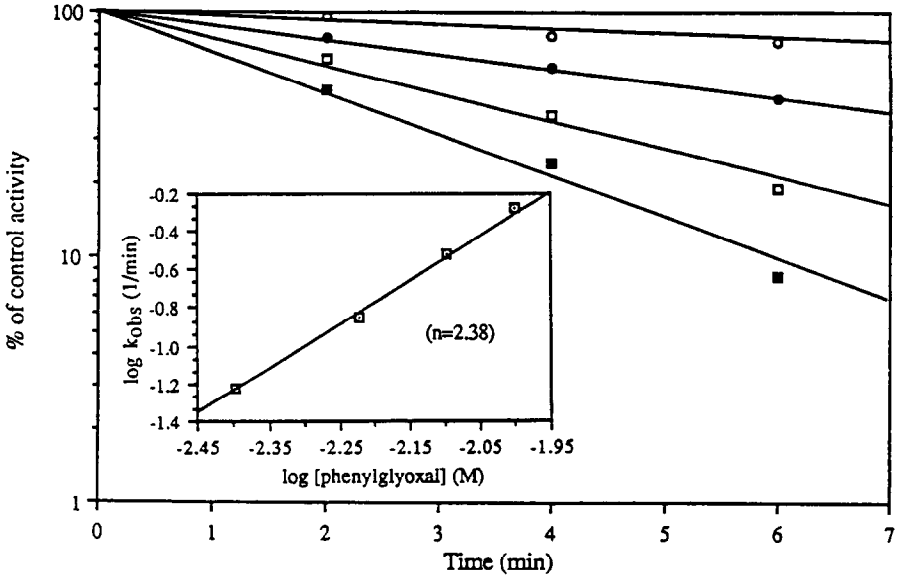

Fig. 3. Inactivation of hamster hepatic NAT I by phenylglyoxal. The reaction mixture contained hamster hepatic NAT I fractions from P-200 gel filtration chromatography and the indicated concentrations of phenylglyoxal: (○) 4 mM; (●) 6 mM; (□) 8 mM; and (■) 10 mM. At the indicated intervals, aliquots were removed and filtered through Sephadex G-25M. The remaining AcCoA/PABA NAT activity was determined as described under Materials and Methods. Data are the means of three experiments. Control activities, expressed in nmol/mg protein/min, were: 7427 ± 535 (4 mM), 7473 ± 646 (6 mM), 7890 ± 835 (8 mM), and 8069 ± 677 (10 mM) (N = 9; mean ± SD). *Inset*: The log k_{obs} was plotted against log phenylglyoxal concentration to yield a reaction order of 2.38 with respect to phenylglyoxal.

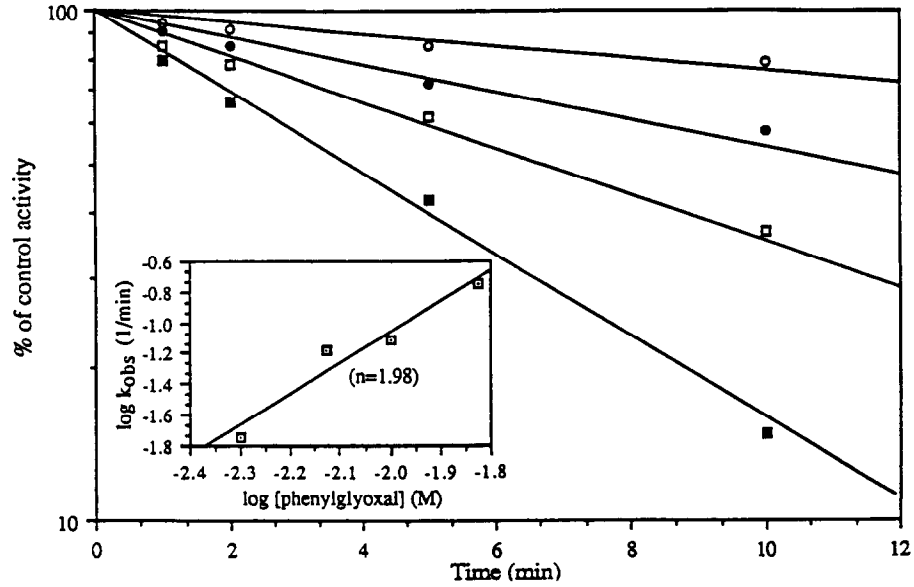

Fig. 4. Inactivation of hamster hepatic NAT II by phenyglyoxal. Hamster hepatic NAT II fractions from DEAE ion exchange chromatography were incubated for various time periods with the indicated concentrations of phenylglyoxal: (○) 5 mM; (●) 7.5 mM; (□) 10 mM; and (■) 15 mM. The remaining N-OH-AAF/AAB N,NAT activity was measured after filtration of the incubation mixture through Sephadex G-25M, as described under Materials and Methods. Results are expressed as the means of three experiments. Control activities, expressed in nmol/mg protein/min, were: 1931 ± 183 (5 mM), 1679 ± 221 (7.5 mM), 1894 ± 127 (10 mM) and 1550 ± 289 (15 mM) (N = 12; mean ± SD). *Inset*: A double logarithmic plot of k_{obs} vs phenylglyoxal concentration yielded a reaction order of 1.98 with respect to phenylglyoxal.

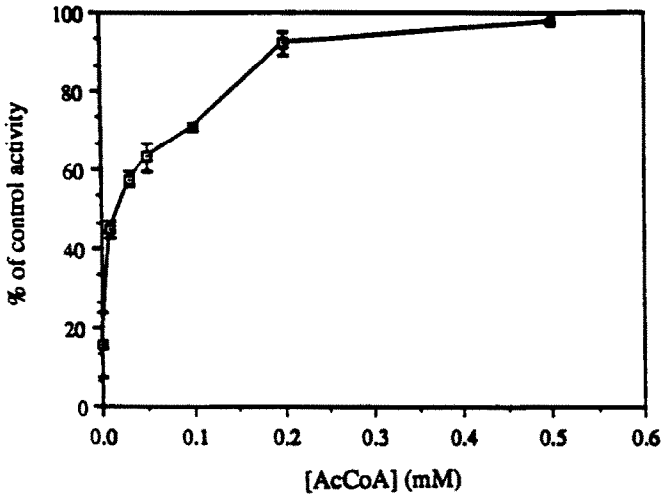

Fig. 5. Inactivation of hamster hepatic NAT I by phenylglyoxal: Effect of AcCoA. Hamster hepatic NAT I, purified by P-200 gcl filtration chromatography, was incubated at 37° in the presence of 10 mM phenylglyoxal and the indicated concentrations of AcCoA. Incubation mixtures were filtered through Sephadex G-25M prior to measurement of AcCoA/PABA NAT activity as described in Materials and Methods. Each value is the mean ± range of two experiments, each carried out in triplicate. Control activity (determined in the absence of phenylglyoxal and expressed in nmol/mg protein/min, mean ± SD, N = 3) was 8674 ± 1474.

Table 2. Inactivation of hamster hepatic NAT II activity by phenylglyoxal: Effects of AcCoA and N-acetylated products

Protection agent	Percent of control activity*
None	26.6 ± 3.5†
AcCoA (0.5 mM)	33.9 ± 4.1
2-AAF (0.5 mM)	10.4 ± 4.9
N-Ac-PA (1.0 mM)	32.5 ± 2.3

* Hamster hepatic NAT II, purified by DEAE ion exchange chromatography, was incubated with 15 mM phenylglyoxal and the indicated protecting agents. At the end of a 10-min incubation, incubation mixtures were filtered through Sephadex G-25M and the remaining N-OH-AAF/AAB N,NAT activity was measured as described in Materials and Methods. Each value is the mean ± range of two experiments, each conducted in triplicate, except where indicated. Control activity (determined in the absence of phenylglyoxal and expressed in nmol/mg protein/min, mean ± SD, N = 5) was 1580 ± 207.

† Mean ± SD, N = 6.

suggesting that the enzymes contain sulfhydryl groups essential for catalysis [39]. In addition, Mangold and Hanna [40] have shown that sulfhydryl reagents such as iodoacetamide, NEM and *p*-chloromercuribenzoate inhibit hamster hepatic, N,NAT activity. The results reported herein showed that both NAT I and NAT II are inactivated rapidly by NEM in a concentration-dependent manner. The NEM-mediated inactivation of NAT I and NAT II indicates that essential cysteine residues have been modified [27]. An alternative, but less likely, possibility is that the inactivation is the result of reaction between NEM and the sulfhydryl groups of DTT, causing a depletion of the DTT which is required for stabilization of NAT activity. However, the protection of both NAT I and NAT II by low concentrations of AcCoA and the 2-AAF-mediated protection of NAT II strongly suggest that essential residues are modified by NEM and that the residues reside within the active site domains of both NAT I and NAT II.

It is known that the reactions catalyzed by NATs proceed according to a ping-pong Bi-Bi mechanism [41, 42]. Thus, the reaction of the enzymes with AcCoA leads the formation of a covalently bound acetyl enzyme intermediate. Then, after dissociation of CoA from the enzyme, the acetyl group is transferred to an amine with release of the free enzyme. The acetyl enzyme intermediate has been isolated after denaturation of the protein from pigeon liver and rabbit liver and has been characterized as a cysteinyl thioester [23, 43]. In addition, recent site directed mutagenesis studies suggested that Cys_{68} is a part of the active site domain of polymorphic human NAT and is involved in catalysis [44]. Thus, it is likely that the essential cysteine residues modified by NEM in the present study function as nucleophilic targets for the binding of the acetyl group by NAT I and NAT II.

The functional role of arginyl residues in the binding of negatively charged substrates and cofactors has been documented for numerous enzymes [45]. Because the NAT cofactor, AcCoA, contains anionic phosphate groups, it is reasonable to expect that cationic amino acids such as arginine may constitute

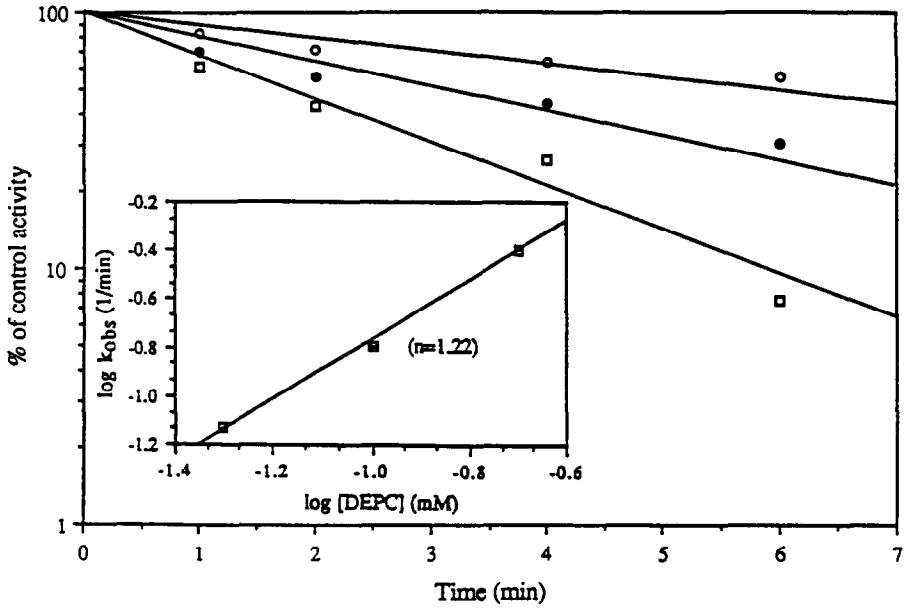

Fig. 6. Inactivation of hamster hepatic NAT I by DEPC. The enzyme preparation was partially purified by P-200 gel filtration chromatography, and incubated with the indicated concentrations of DEPC in 50 mM sodium pyrophosphate buffer (pH 7.0) at 37°. At various time intervals, the reaction was terminated by addition of excess L-histidine. The residual AcCoA/PABA NAT activity was measured after filtration of the incubation mixture through Sephadex G-25M. DEPC concentrations were: (○) 0.05 mM, (●) 0.1 mM, and (□) 0.2 mM. Results are means of three experiments. Control activities were (in nmol/mg protein/min): 7720 ± 912 (0.05 mM), 7636 ± 356 (0.1 mM), and 8078 ± 477 (0.2 mM) (N = 12; mean ± SD). *Inset*: A double logarithmic plot of log k_{obs} vs DEPC concentration yielded a reaction order of 1.22 with respect to DEPC.

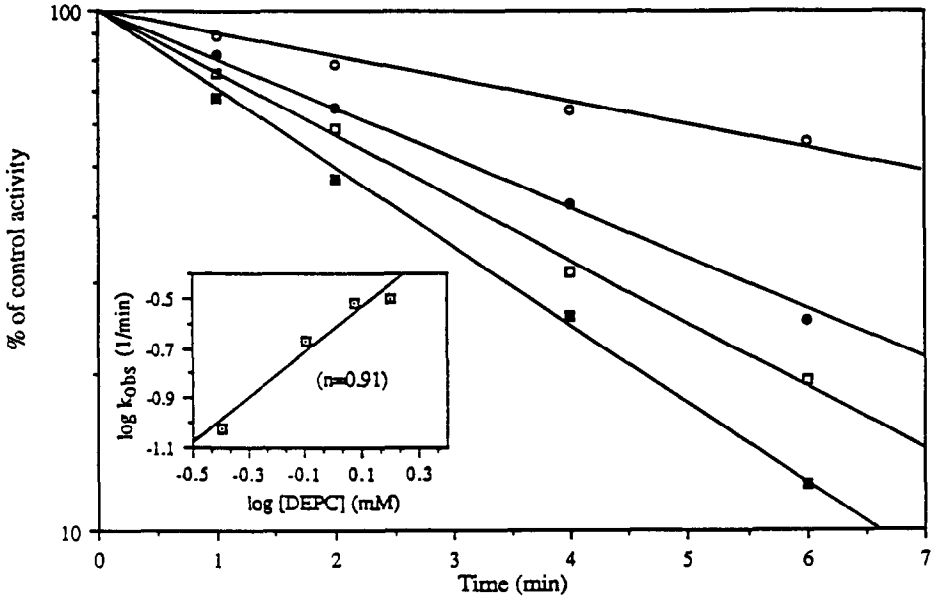

Fig. 7. Inactivation of hamster hepatic NAT II by DEPC. The enzyme preparation, partially purified by DEAE ion exchange chromatography was incubated with DEPC in 50 mM sodium pyrophosphate buffer (pH 7.0) at 37°. The reaction was terminated at designated time intervals by adding excess L-histidine. The remaining N-OH-AAF/AAB N,NAT activity was measured after filtration of the incubation mixture through Sephadex G-25M. DEPC concentrations were: (○) 0.04 mM, (●) 0.8 mM, (□) 1.2 mM, and (■) 1.6 mM. Results are means of two experiments, each conducted in triplicate. Control activities were (in nmol/mg protein/min): 2865 ± 126 (0.4 mM), 2771 ± 189 (0.8 mM), 2583 ± 112 (1.2 mM), and 2864 ± 46.6 (1.6 mM) (N = 8; mean ± SD). *Inset*: the log k_{obs} was plotted against log DEPC concentration to yield a reaction oder of 0.91 with respect to DEPC.

Table 3. Effect of hydroxylamine on DEPC-inactivated NAT I and NAT II

Period of incubation with NH_2OH (hr)	Percent of control activity	
	NAT I*	NAT II†
0	47.7 ± 5.2	48.9 ± 1.4
22	68.2 ± 0.2	7.73 ± 0.2
45	84.6 ± 1.2	7.80 ± 2.2
65	85.5 ± 0.4	ND‡

* Partially purified NAT I, obtained by 35–50% ammonium sulfate fractionation and DEAE anion exchange chromatography, was incubated with 0.3 mM DEPC at 4°, pH 6.5. After a 4-hr incubation, the reaction was terminated by the addition of excess L-histidine, and the reaction mixture was filtered through a PD-10 column of Sephadex G-25M. The eluent was treated with 0.2 M hydroxylamine, pH 7.0, at room temperature. At the indicated times, the mixture was filtered through a PD-10 column and assayed for the AcCoA/PABA NAT activity as described in Materials and Methods. Control activity (determined in the absence of DEPC and expressed in nmol/mg protein/min, mean ± SD, N = 3) was 781.4 ± 9.4. Each value is the mean ± range of two experiments, each conducted in triplicate.

† Incubation of partially purified NAT II, obtained by 35–50% ammonium sulfate fractionation and DEAE anion exchange chromatography, was carried out at 4°, pH 6.5, in the presence of 2.0 mM DEPC. At the end of a 2-hr incubation, excess L-histidine was added to the reaction mixture. After filtration of the mixture through a PD-10 column of Sephadex G-25M, the eluent was incubated with 0.1 M hydroxylamine, pH 7.0, at 4°. At the indicated times, the remaining N-OH-AAF/AAB N,NAT activity was determined after filtration of the incubation mixture through a PD-10 column. Control activity (determined in the absence of DEPC and expressed in nmol/mg protein/min, mean ± SD, N = 3) was 307.5 ± 24.8. Each value is the mean ± range of two experiments, each conducted in triplicate.

‡ Not determined.

Table 4. Inactivation of hamster hepatic NAT I and NAT II by DEPC: Effects of AcCoA and N-acetylated products

Protecting agent	Percent of control activity	
	NAT I*	NAT II‡
None	21.8 ± 2.3‡	24.4 ± 4.9§
AcCoA (0.5 mM)	33.9 ± 8.8	30.9 ± 2.9
p-AABA (1.0 mM)	32.6 ± 4.0	ND‖
2-AAF (0.5 mM)	ND	33.1 ± 1.1
N-Ac-PA (1.0 mM)	ND	26.6 ± 3.9

* The enzyme solution, obtained by P-200 gel filtration chromatography, was preincubated with the indicated protecting agents for 1 min at 37° prior to the addition of DEPC (0.2 mM). After 4 min of incubation with DEPC, the reaction was terminated by addition of excess L-histidine. The incubation mixtures were filtered through Sephadex G-25M and the remaining AcCoA/PABA NAT activity was assayed as described in Materials and Methods. Values are the means ± range of two experiments, each conducted in triplicate, except where indicated. Control activity (determined in the absence of DEPC) was 8037 ± 1128 nmol/mg protein/min (mean ± SD, N = 4).

† The enzyme solution, obtained by DEAE ion exchange chromatography, was preincubated with the indicated protecting agents for 1 min at 37° prior to the addition of DEPC (1.6 mM). After 6 min of incubation with DEPC, the reaction was terminated by the addition of excess L-histidine. The incubation mixtures were filtered through Sephadex G-25M and the remaining N-OH-AAF/AAB N,NAT activity was assayed as described in Materials and Methods. Values are the means ± range of two experiments, each carried out in triplicate, except where indicated. Control activity (determined in the absence of DEPC) was 2644 ± 445 nmol/mg protein/min (mean ± SD, N = 6).

‡ Mean ± SD, N = 4.

§ Mean ± SD, N = 6.

‖ Not determined.

a portion of the cofactor binding site. Both NAT I and NAT II are inactivated in a concentration- and time-dependent manner by the arginine-selective reagent, phenylglyoxal. The reaction order was approximately two with respect to phenylglyoxal for both NATs. A second-order reaction with respect to phenylglyoxal is consistent with the 2:1 stoichiometry that is common observed for reactions between PG and arginyl residues [37].

AcCoA afforded complete protection of NAT I against inactivation by PG, whereas potential protecting agents such as AcCoA, 2-AAF and N-Ac-PA did not protect NAT II against inactivation by PG. These results suggest that critical arginine residues may be located in the AcCoA binding site of NAT I, but that the essential arginine of NAT II may not be located in the active site. It is possible that the essential arginine residue(s) of NAT I is involved in the binding and orientation of AcCoA by ionic interactions. Interestingly, the presence of CoA did not provide protection of NAT I against inactivation by PG, suggesting that the acetyl moiety is required for the binding of the molecule at the active site.

Both NAT I and NAT II were inactivated by the histidine-selective reagent, DEPC. The inactivation process followed pseudo first-order kinetics, and the reaction order with respect to DEPC was one, indicating the likelihood that a single essential residue was modified in each enzyme. The absorption maximum that was observed at 240 nm after treatment of either NAT with DEPC provides support for the conclusion that histidine residues had been modified. Because partially purified enzyme preparations were used for the inactivation experiments, the spectroscopic data were not used to estimate the total number of modified histidines in the enzymes.

Riddle and Jencks [41] observed that the saponification velocity of the acetyl-enzyme intermediate of pigeon liver NAT varied with the basicity of the acceptor amines, suggesting that a general base, such as histidine, is involved in the catalysis. Andres *et al.* [43] also proposed the involvement of a general base in the catalysis of arylamine N-acetylation by the pigeon liver enzyme. However, the presence of AcCoA did not alter significantly the inactivation of either NAT I or NAT II by DEPC, and no protection was afforded by reaction

products such as p-AABA, 2-AAF and N-Ac-PA (Table 4). These results may indicate that the essential histidine residues that are modified by DEPC do not reside within the binding sites of the enzymes and are not directly involved in catalysis of the transacetylation process. Alternatively, the modified histidine residues may participate in catalysis, but are brought into the immediate proximity of the active sites as a result of conformational changes induced by the binding of cofactor and/or arylamine substrates.

Although DEPC reacts preferentially with histidine residues, other amino acids such as tyrosine, lysine and cysteine may also be modified. The increase in the absorption maximum at 240 nm and the absence of a change in absorption at 280 nm are consistent with the modification of histidine, but not tyrosine, in both the NAT I and NAT II preparations. As a further test for the modification of histidine, the DEPC-treated enzymes were incubated with hydroxylamine which is known to remove the ethoxyformyl group from DEPC-modified histidine and tyrosine residues, but not from modified lysine residues [32]. It was found that NAT I that had been inactivated by DEPC at pH 7 (37°) could not be reactivated by hydroxylamine, but that hydroxylamine treatment did reactivate NAT I that had been incubated with DEPC at pH 6.5 (4°). Since both DEPC-inactivated NAT I preparations showed an increased absorption maximum at 240 nm, it is possible that the reaction with DEPC at pH 7 (37°) caused dicarbethoxylation of histidine and the reaction at pH 6.5 (4°) formed the mono-*N*-carbethoxy derivatives. Both the mono- and the di-*N*-carbethoxyhistidyl residues exhibit an absorption maximum at 240 nm, but only the mono-*N*-carbethoxy modification can be reversed by hydroxylamine [32]. Also, the ethoxyformyl group would not have been cleaved from any essential lysine groups that had been derivatized by DEPC at pH 7 (37°).

In contrast to the results obtained with NAT I, DEPC-treated NAT II was further inactivated by incubation with 0.1 M hydroxylamine, a concentration of hydroxylamine that had no effect on NAT II activity when it was incubated with enzyme preparations that had not been treated with DEPC. The reason for the deleterious effect of hydroxylamine on the DEPC-treated NAT II preparations is unknown.

Although the molecular basis of the hereditary polymorphism [1, 3, 8, 46] and the biochemical and kinetic characterization of *N*-acetyltransferases [4, 20, 23, 43] have received considerable attention, relatively little information is available regarding either the types of chemical agents that inactivate NATs or the types of amino acid residues that are required for the catalytic activity of the enzymes. Wick *et al.* [12] found that a conjugated ketone, 1-(fluoren-2-yl)-2-propen-1-one, is a selective and irreversible inactivator of rat liver NAT II, and Andres *et al.* [24] used bromoacetanilide as an active site directed inhibitor of rabbit liver NAT. Another α-bromoacetamide, 2-(bromoacetyl)aminofluorene, has been found to alkylate an active site cysteine residue in hamster hepatic NAT II.* The chemical modification studies reported herein provide further support for the presence of catalytically essential active site cysteine residues in NATs. The ability of an α-dicarbonyl reagent such as PG to inactivate both NAT I and NAT II indicates that arginine residues have an important function in maintaining the activity of the enzymes, and the essential nature of histidine is indicated by the experiments with DEPC. From the standpoint of designing selective inhibitors, it is of interest that the NAT I-catalyzed AcCoA/PABA transacetylation requires an arginine residue that appears to reside in, or near, the active site, whereas the NAT II-catalyzed N-OH-AAF/AAB transacetylation reaction apparently does not require a similarly located arginine.

The information derived from these chemical modification studies is expected to be useful for the design of more potent and selective inhibitors of the NAT isozymes. Important advances in the purification of hamster liver NATs have been reported recently [7, 47], and the isolation of a cDNA clone for monomorphic hamster hepatic NAT (NAT II) has been described [19]. It is anticipated that selective NAT inhibitors will be used in conjunction with purification methodologies and the tools of molecular biology to provide insight into the active site topologies and catalytic mechanisms of this important class of enzymes.

* Cheon HG and Hanna PE, unpublished results.

REFERENCES

1. Weber WW and Hein DW, N-Acetylation pharmacogenetics. *Pharmacol Rev* **37**: 25–79, 1985.
2. Evans DAP, *N*-Acetyltransferase. *Pharmacol Ther* **42**: 157–234, 1989.
3. Hein DW, Acetylator genotype and arylamine-induced carcinogenesis. *Biochim Biophys Acta* **948**: 37–66, 1988.
4. Trinidad A, Kirlin WG, Ogolla F, Andrews AF, Yerokun T, Ferguson RJ, Brady PK and Hein DW, Kinetic characterization of acetylator genotype-dependent and -independent *N*-acetyltransferase isozymes in homozygous rapid and slow acetylator inbred hamster liver cytosol. *Drug Metab Dispos* **17**: 238–247, 1989.
5. Grant DM, Blum M, Beer M and Meyer UA, Monomorphic and polymorphic human arylamine *N*-acetyltransferases: A comparison of liver isozymes and expressed products of two cloned genes. *Mol Pharmacol* **39**: 184–191, 1991.
6. Kirlin WG, Trinidad A, Yerokun T, Ogolla F, Ferguson RJ, Andrews AF, Brady PK and Hein DW, Polymorphic expression of acetylcoenzyme A-dependent arylamine *N*-acetyltransferase and acetylcoenzyme A-dependent *O*-acetyltransferase mediated activation of *N*-hydroxyarylamines by human bladder cytosol. *Cancer Res* **49**: 2448–2454, 1989.
7. Ozawa S, Abu-Zeid M, Kawakubo Y, Toyama S, Yamazoe Y and Kato R, Monomorphic and polymorphic isozymes of arylamine *N*-acetyltransferases in hamster liver: Purification of the isozymes and genetic basis of *N*-acetylation polymorphism. *Carcinogenesis* **11**: 2137–2144, 1990.
8. Hein DW, Kirlin WG, Ferguson RJ and Weber WW, Biochemical investigation of the basis for the genetic N-acetylation polymorphism in the inbred hamster. *J Pharmacol Exp Ther* **234**: 358–364, 1985.
9. Smith TJ and Hanna PE, *N*-acetyltransferase multiplicity and the bioactivation of *N*-arylhydroxamic

acids by hamster hepatic and intestinal enzymes. *Carcinogenesis* **7**: 697–702, 1986.
10. Wick MJ, and Hanna PE, Bioactivation of *N*-arylhydroxamic acids by rat hepatic *N*-acetyltransferase. Detection of multiple enzyme forms by mechanism-based inactivation. *Biochem Pharmacol* **39**: 991–1003, 1990.
11. Weber WW, Radtke HE and Tannen RH, Extrahepatic *N*-acetyltransferases and *N*-deacetylases. In: *Extrahepatic Metabolism of Drugs and Other Foreign Compounds* (Ed. Gram TE), pp. 493–531. SP Medical and Scientific Books, New York, 1980.
12. Wick MJ, Yeh H-M and Hanna PE, An isozyme-selective affinity label for rat hepatic acetyltransferases. *Biochem Pharmacol* **40**: 1389–1398, 1990.
13. Hanna PE, Banks RB and Marhevka VC, Suicide inactivation of hamster hepatic arylhydroxamic acid *N,O*-acyltransferase. A selective probe of *N*-acetyltransferase multiplicity. *Mol Pharmacol* **21**: 159–165, 1982.
14. Ohsako S, Ohtomi M, Sakamoto Y, Uyemura K and Deguchi T, Arylamine *N*-acetyltransferase from chicken liver. II. Cloning of cDNA and expression in Chinese hamster ovary cells. *J Biol Chem* **263**: 7534–7538, 1988.
15. Blum M, Grant DM, McBride W, Heim M and Meyer UA, Human arylamine *N*-acetyltransferase genes: Isolation, chromosomal localization, and functional expression. *DNA Cell Biol* **9**: 193–203, 1990.
16. Ohsako S and Deguchi T, Cloning and expression of cDNAs for polymorphic and monomorphic arylamine *N*-acetyltransferases from human liver. *J Biol Chem* **265**: 4630–4634, 1990.
17. Blum M, Heim M and Meyer UA, Nucleotide sequence of rabbit NAT 1 encoding monomorphic arylamine *N*-acetyltransferase. *Nucleic Acids Res* **18**: 5287, 1990.
18. Blum M, Heim M and Meyer UA, Nucleotide sequence of rabbit NAT 2 encoding polymorphic arylamine *N*-acetyltransferase. *Nucleic Acids Res* **18**: 5295, 1990.
19. Abu-Zeid M, Nagata K, Miyata M, Ozawa S, Fukuhara M and Yamazoe Y, An arylamine acetyltransferase (AT-1) from Syrian Golden hamster liver: Cloning, complete nucleotide sequence, and expression in mammalian cells. *Mol Carcinogenesis* **4**: 81–88, 1991.
20. Yerokun T, Kirlin WG, Trinidad A, Ferguson RJ, Ogolla F, Andrews AF, Brady PK and Hein DW, Identification and kinetic characterization of acetylator genotype-dependent and -independent arylamine carcinogen *N*-acetyltransferases in hamster bladder cytosol. *Drug Metab Dispos* **17**: 231–237, 1989.
21. Allaben WT and King CM, The purification of rat liver arylhydroxamic acid *N,O*-acyltransferase. *J Biol Chem* **259**: 12128–12134, 1984.
22. Weber WW and Cohen SN, N-Acetylation of drugs: Isolation and properties of an *N*-acetyltransferase from rabbit liver. *Mol Pharmacol* **3**: 266–273, 1967.
23. Andres HH, Vogel RS, Tarr GE, Johnson L and Weber WW, Purification, physicochemical and kinetic properties of liver AcCoA:arylamine *N*-acetyltransferases from rapid acetylator rabbits. *Mol Pharmacol* **31**: 446–456, 1987.
24. Andres HH, Klem AJ, Schopfer LM, Harrison JK and Weber WW, On the active site of liver AcCoA arylamine *N*-acetyltransferase from rapid acetylator rabbits (III/J). *J Biol Chem* **263**: 7521–7527, 1988.
25. Marhevka VC, Ebner NA, Sehon RD and Hanna PE, Mechanism-based inactivation of *N*-arylhydroxamic acid *N,O*-acyltransferase by 7-substituted-*N*-hydroxy-2-acetamidofluorenes. *J Med Chem* **28**: 18–24, 1985.
26. Kaiser ET, Lawrence DS and Rokita SE, The chemical modification of enzymatic specificity. *Annu Rev Biochem* **54**: 565–595, 1985.
27. Glazer AN, The chemical modification of proteins by group-specific and site-specific reagents. In: *The Proteins* (Eds. Neurath H and Hill RL), 3rd Edn, Vol. 2, pp. 1–103. Academic Press, New York, 1975.
28. Cheon HG and Hanna PE, Evidence for essential arginine residues in hamster hepatic arylamine *N*-acetyltransferases. *FASEB J* **4**: A610, 1990.
29. Cheon HG and Hanna PE, Involvement of cysteine and histidine residues in the catalytic activity of hamster hepatic arylamine *N*-acetyltransferases. *Pharmacologist* **32**: 156, 1990.
30. Gelsema WJ, De Ligny CL, Roozen AMP and Wilms GP, Optimal conditions for the coupling of aromatic amines to epoxy-activated Sepharose 6B. *J Chromatogr* **209**: 363–368, 1981.
31. Lowe CR, The chemical technology of affinity chromatography. In: *Laboratory Techniques in Biochemistry and Molecular Biology* (Eds. Work TS and Work E), Vol. 7, Part II, pp. 344–400. North Holland Publishing, Amsterdam, 1979.
32. Miles EW, Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol* **47**: 431–442, 1977.
33. Weber WW, *N*-Acetyltransferase (mammalian liver). *Methods Enzymol* **17B**: 805–811, 1971.
34. Booth J, Acetyltransfer in arylamine metabolism. *Biochem J* **100**: 745–753, 1966.
35. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
36. Kitz R and Wilson IB, Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J Biol Chem* **237**: 3245–3249, 1962.
37. Takahashi K, The reaction of phenylglyoxal with arginine residues in proteins. *J Biol Chem* **243**: 6171–6179, 1968.
38. Levy HM, Leber PD and Ryan EM, Inactivation of myosin by 2,4-dinitrophenol and protection by adenosine triphosphate and other phosphate compounds. *J Biol Chem* **238**: 3654–3659, 1963.
39. King CM, Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res* **34**: 1503–1515, 1974.
40. Mangold BLK and Hanna PE, Arylhydroxamic acid *N,O*-acyltransferase substrates. Acetyl transfer and electrophile generating activity of *N*-hydroxy-*N*-(4-alkyl, 4-alkenyl, and 4-cyclohexylphenyl)acetamides. *J Med Chem* **25**: 630–638, 1982.
41. Riddle B and Jencks WP, Acetyl-coenzyme A:arylamine *N*-acetyltransferase. Role of the acetyl-enzyme intermediate and the effects of substituents on the rate. *J Biol Chem* **246**: 3250–3258, 1971.
42. Weber WW, Cohen SN and Steinberg MS, Purification and properties of *N*-acetyltransferase from mammalian liver. *Ann NY Acad Sci* **151**: 734–741, 1968.
43. Andres HH, Kolb HJ, Schreiber RJ and Weiss L, Characterization of the active site, substrate specificity and kinetic properties of acetylcoenzyme A: arylamine *N*-acetyltransferase from pigeon liver. *Biochim Biophys Acta* **746**: 193–201, 1983.
44. Dupret J-M and Grant DM, Involvement of Cys_{68} in the catalytic mechanism of polymorphic human arylamine *N*-acetyltransferase NAT 2. *FASEB J* **5**: A842, 1991.
45. Riordan JF, Arginyl residues and anionic binding sites in proteins. *Mol Cell Biochem* **26**: 71–92, 1979.
46. Grant DM, Morike K, Eichelbaum M and Meyer UA, Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine *N*-acetyltransferase in human liver. *J Clin Invest* **85**: 968–972, 1990.
47. Trinidad A, Hein DW, Rustan TD, Ferguson RJ,

Miller LS, Bucher KD, Kirlin WG, Ogolla F and Andrews AF, Purification of hepatic polymorphic arylamine *N*-acetyltransferase from homozygous rapid acetylator inbred hamster: Identity with polymorphic *N*-hydroxyarylamine-*O*-acetyltransferase. *Cancer Res* **50**: 7942–7949, 1990.