Biochemical Pharmacology, Vol. 39, No. 6, pp. 991–1003, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

BIOACTIVATION OF *N*-ARYLHYDROXAMIC ACIDS BY RAT HEPATIC *N*-ACETYLTRANSFERASE

DETECTION OF MULTIPLE ENZYME FORMS BY MECHANISM-BASED INACTIVATION

MARILEE J. WICK* and PATRICK E. HANNA*†‡
Departments of * Pharmacology and † Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, U.S.A.

(*Received* 23 *May* 1988; *accepted* 4 *August* 1989)

Abstract—Enzymatic N,O-acyltransfer of carcinogenic *N*-arylhydroxamic acids such as *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) results in the production of reactive electrophiles that can bond covalently with nucleophiles and also can cause inactivation of acyltransferase activity in a mechanism-based manner. Incubation of partially purified rat hepatic *N*-acetyltransferases (NAT) with N-OH-AAF resulted in extensive inactivation of N-OH-AAF/4-aminoazobenzene (AAB) *N,N*-acetyltransferase and acetyl coenzyme A (AcCoA)/procainamide (PA) *N*-acetyltransferase activities, whereas AcCoA/*p*-aminobenzoic acid (PABA) *N*-acetyltransferase activity was inhibited only slightly. Affinity chromatography with Sepharose 6B 2-aminofluorene (2-AF) resulted in the separation of two NAT activities. NAT I primarily catalyzed the AcCoA-dependent acetylation of PABA; NAT II catalyzed, *N,N*-acetyltransfer (*N*-OH-AAF/AAB), AcCoA/PA *N*-acetyltransfer and N-OH-AAF N,O-acyltransfer (AHAT) activities. Most of the AcCoA/2-AF *N*-acetyltransferase activity eluted in the NAT II fraction. Results of inactivation experiments with N-OH-AAF and the NAT II fractions suggested that one NAT isozyme was responsible for catalyzing the N-OH-AAF/AAB, AcCoA/PA and N,O-acyltransfer reactions and that inactivation of NAT II correlated with the extent of covalent binding to protein. Further purification of the NAT II fractions by chromatofocusing resulted in a 1300-fold purification of the N-OH-AAF/AAB activity and the coelution of N-OH-AAF/AAB, AcCoA/PA and *N,O*-acyltransferase activities. These studies indicate that N,O-acyltransfer, arylhydroxamic acid-dependent N-acetylation of arylamines (N,N-acetyltransfer), and AcCoA-dependent N-acetylation of PA may be catalyzed by a common enzyme in rat liver, whereas a second enzyme is responsible for the AcCoA-dependent N-acetylation of PABA.

Both the toxicity and the carcinogenicity of many *N*-arylhydroxamic acids depend on their further metabolic transformation [1]. *N*-Arylhydroxamic acid *N,O*-acyltransferase (AHAT),§ a cytosolic enzyme found in a number of mammalian tissues, catalyzes intramolecular *N,O*-acyl group transfer of *N*-arylhydroxamic acids, resulting in the production of electrophiles that are capable of reacting with various nucleophiles, including those on proteins and nucleic acids (Fig. 1, pathway A) [1–4]. Thus, AHAT has been implicated as one of several enzymes that may be ultimately responsible for the carcinogenicity of *N*-arylhydroxamic acids such as *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) [5, 6].

Arylhydroxamic acid : arylamine transacetylase activity (N,N-acetyltransfer) (Fig. 1, pathway B), originally described by Booth [7], is believed to be closely associated with *N,O*-acyltransferase activity. The two activities coelute during partial purification of both rat hepatic cytosol [2, 3] and hamster hepatic and intestinal cytosol [8]. Recent studies also have indicated an association between the *N*-arylhydroxamic acid-dependent *N,N*-acetyltransferase activities and the genetically polymorphic acetyl coenzyme A (AcCoA)-dependent *N*-acetyltransferase activity (NAT; EC 2.3.1.5). A single *N*-acetyltransferase enzyme that is capable of catalyzing both *N*-arylhydroxamic acid-dependent and AcCoA-dependent acetyltransferase reactions has been detected in rabbit liver [9]. Mattano *et al.* [10] reported that NAT and AHAT activities, as well as *N*-hydroxyarylamine *O*-acetyltransferase activity, are catalyzed by a common enzyme in mouse liver.

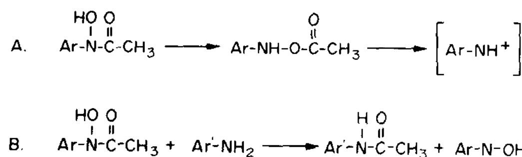

Fig. 1. (A) Bioactivation of *N*-arylhydroxamic acids by N,O-acyltransfer. (B) Arylhydroxamic acid-dependent transacetylation (N,N-acetyltransfer) of arylamines.

‡ Address correspondence to: Dr Patrick E. Hanna, Department of Pharmacology, University of Minnesota Medical School, 3-260 Millard Hall, 435 Delaware Street, SE, Minneapolis, MN 55455.

§ Abbreviations: AHAT, *N*-arylhydroxamic acid *N,O*-acyltransferase; NAT, *N*-acetyltransferase; AcCoA, acetyl coenzyme A; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; 2-AF, 2-aminofluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene; AAB, 4-aminoazobenzene; PA, procainamide; PABA, *p*-aminobenzoic acid; DTT, dithiothreitol; BNPP, bis(*p*-nitrophenyl) phosphate; SMZ, sulfamethazine; and DMSO, dimethyl sulfoxide.

The *O*-acetyltransferase reaction is believed to be involved in the activation of toxic and carcinogenic *N*-hydroxyarylamines and has been shown to be associated with partially purified NAT activities obtained from hamster liver [11].

An investigation that utilized the ability of *N*-arylhydroxamic acids to function as mechanism-based inactivators (suicide inhibitors) of hamster hepatic arylhydroxamic acid:arylamine *N,N*-acetyltransferase activity indicated the presence of multiple acetyltransferase enzymes in hamster hepatic cytosol [12]. Whereas the N-OH-AAF/4-aminoazobenzene (AAB) *N,N*-acetyltransferase and the AcCoA/sulfamethazine (SMZ) *N*-acetyltransferase activities were inactivated extensively by preincubation with N-OH-AAF, the AcCoA/*p*-aminobenzoic acid (PABA) transacetylase activity was inhibited only partially. These results suggested that hamster hepatic N-OH-AAF/AAB and AcCoA/SMZ transacetylation activities are associated with the same enzyme; however, these two activities appeared to be distinct from the AcCoA/PABA transacetylation activity [12]. Further evidence in support of the proposal that hamster hepatic *N*-arylhydroxamic acid-dependent acetyltransferase activities are catalyzed by an enzyme that is distinct from the PABA-acetylating enzyme was obtained by chromatographic separation of the activities [8].

Because the rat is a widely used animal model in studies of the metabolism of N-substituted aromatic drugs and carcinogens, the present work was undertaken to study the rat hepatic *N*-acetyltransferase multiplicity and substrate specificities by employing a combination of mechanism-based inactivation and protein purification techniques. We report the chromatographic separation and characterization with regard to substrate specificities of two transacetylase activities from rat liver. A preliminary report of some of these results has been published [13].

MATERIALS AND METHODS

Materials

All chemical reagents were of the highest grade available. 4-Aminoazobenzene (AAB) and *N*-1-(naphthyl)ethylenediamine dihydrochloride were purchased from the Eastman Kodak Co., (Rochester, NY). D,L-Dithiothreitol, *S*-acetyl coenzyme A (AcCoA, trilithium salt), grade III NAD, *N*-acetyl D,L-methionine, L-cysteine, L-methionine, guanosine 2′- and 3′-monophosphoric acid, bovine serum albumin, procainamide hydrochloride, *p*-aminobenzoic acid (sodium salt), tetrasodium pyrophosphate, Sephadex G-100, Polybuffer exchanger 94, Polybuffer 74 and cellulose dialysis tubing were purchased from the Sigma Chemical Co., (St Louis, MO). The dialysis tubing was rinsed in distilled water and in 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT) prior to use. 2-Nitro[9-^{14}C]fluorene (8.37 Ci/mol) was purchased from Sigma Radiochemicals (Pathfinder Laboratories) (St Louis, MO). Acetyl chloride, 2-aminofluorene, 2-nitrofluorene, histidine HCl, paraoxon (diethyl 4-nitrophenyl phosphate) and *p*-dimethylaminobenzaldhyde were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Bis(*p*-nitrophenyl) phosphate was purchased from Calbiochem (San Diego, CA). Ultrafiltration membranes (PM10) were from the Amicon Corp., (Danvers, MA). Epoxy-activated Sepharose 6B and PD-10 desalting columns (1.5 × 5 cm) packed with Sephadex G-25M were purchased from the Pharmacia Chemical Co., (Piscataway, NJ). The PD-10 columns were washed with 50 mL of elution buffer prior to use.

N-Hydroxy-2-acetylaminofluorene (N-OH-AAF) was synthesized from 2-nitrofluorene as previously described [14]. *N*-Acetyl-L-[^{14}C-CH_3]methione (0.28 to 0.35 mCi/mmol) was prepared from L-[^{14}C-CH_3]methionine (ICN Pharmaceuticals, Irvine, CA) and acetic anhydride according to the procedure of Wheeler and Ingersoll [15]. The procedure of Gelsema *et al.* [16], as previously described [8], was followed for the coupling of 2-aminofluorene to epoxy-activated Sepharose 6B except that 2-aminofluorene (2-AF) was substituted for aniline. The concentration of 2-aminofluorene immobilized on the Sepharose 6B matrix was determined to be approximately 27 μmol/mL of hydrated gel by direct spectroscopy of a suspension of the derivatized gel in ethylene glycol as described by Lowe [17].

Enzyme preparation and partial purification

Protein concentrations were measured by the method of Lowry *et al.* [18] with bovine serum albumin as the standard. Male Sprague–Dawley rats (170–220 g) were obtained from Bio-Lab (White Bear Lake, MN). Immediately prior to excision of livers, rats were lightly anesthetized with diethyl ether and decapitated. Livers were rinsed in cold 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT and were homogenized with 1 mL buffer/g of liver in a motor-driven teflon/glass homogenizer. The homogenate was centrifuged at 105,000 *g* for 60 min at 4°. The resulting supernatant fraction was diluted with an equal volume of 0.05 M sodium pyrophosphate buffer and subjected to ammonium sulfate fractionation by the method of King [3].

All subsequent purification steps were carried out at 4°. For gel filtration chromatography, the precipitated 45–65% ammonium sulfate fraction was diluted to a protein concentration of approximately 50 mg/mL with buffer. Six milliliters of the diluted ammonium sulfate fraction was applied to a 2.5 × 45 cm column of Sephadex G-100 which had been equilibrated with degassed 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7). The column was then eluted with 200 mL of degassed buffer. Fractions (12 mL) were collected at a flow rate of 30 mL/hr. Aliquots of each fraction were analyzed for N-OH-AAF/AAB, AcCoA/PA and AcCoA/PABA transacetylation activities, as described under "Enzyme assays." The fractions that contained the highest levels of transacetylation activity were combined and concentrated to approximately 25% of the original volume under a nitrogen atmosphere in an Amicon model 52 ultrafiltration cell equipped with a PM10 membrane. Glycerol was added to a concentration of 30% to stabilize the enzymic activity. This preparation was used for the experiments to determine the effect of N-OH-AAF on the unresolved transacetylase activities.

Rat hepatic NAT activities were prepared for purification by affinity chromatography according to the procedures described above (ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography) except that gel filtration was performed with degassed potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The fractions that contained the majority of the NAT activity were combined and applied directly to a 1.5 × 14 cm column of Sepharose 6B 2-AF. The column was then eluted with 200 mL of 0.02 M potassium phosphate (1 mM EDTA, 1 mM DTT), pH 7.4 buffer. Fractions (6 mL) were collected at a flow rate of approximately 48 mL/hr. At fraction 39, the elution buffer was changed to 0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0. The N-OH-AAF/AAB, AcCoA/PA and AcCoA/PABA transacetylation activities of each fraction were determined as described under "Enzyme assays." The fractions containing the majority of the AcCoA/PABA transacetylation activity (NAT I) were pooled, concentrated to approximately 25% of the original volume by ultrafiltration under nitrogen, and stored at −70° in 10% glycerol. The fractions containing the N-OH-AAF/AAB and AcCoA/PA activities (NAT II) were similarly pooled, concentrated, and stored at −70° in 30% glycerol. Enzyme activities stored under these conditions were stable for several months.

In preparation for chromatofocusing, the glycerol-stabilized NAT II fractions were passed through a PD-10 column containing Sephadex G-25M that had been pre-equilibrated with degassed 0.025 M histidine HCl buffer (pH 6.2, containing 10% glycerol and 1 mM DTT). The sample (4 mL, 3 mg protein) was applied to a column packed with Polybuffer exchanger 94 (1 × 17 cm) which had been equilibrated with histidine HCl buffer. The column was eluted with 150 mL of Polybuffer 74 (diluted 9-fold, pH 4.0, containing 10% glycerol and 1 mM DTT). Fractions (3 mL) were collected at a flow rate of 70 mL/hr. The pH of each fraction was measured immediately, and 0.4 mL of 0.4 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) was added to each fraction to stabilize acetyltransferase activity. The N-OH-AAF/AAB transacetylation, AcCoA/PA transacetylation and methylthio adduct-forming activities were determined by sampling aliquots of the fractions as described under "Enzyme assays."

Enzyme assays

N-OH-AAF/AAB N,N-*transacetylation assay*. Transacetylation of AAB by N-OH-AAF was determined by a modification of the previously described method [7, 8]. Incubations were carried out in 1.5-mL centrifuge tubes that contained 0.02 to 0.48 mL of the enzyme preparation (or 0.2-mL aliquots of the fractions from column chromatography), 0.02 mL of substrate solution, and sufficient 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the volume to 0.5 mL. The substrate solution contained both AAB and N-OH-AAF dissolved in 95% ethanol. Final concentrations in the reaction mixture were 1.0 mM N-OH-AAF and 0.15 mM AAB. Reactions were started by addition of substrate and were carried out at 37° in air for 4–5 min. Reactions were terminated by addition of 0.5 mL of cold 20% trichloroacetic acid (in ethanol: water, 1:1) and the precipated protein was removed by centrifugation. The absorbance of the supernatant fraction was measured at 497 nm against an incubation blank containing ethanol instead of N-OH-AAF/AAB.

AcCoA-dependent acetylation of PABA. Reaction mixtures contained 0.01 to 0.15 mL of enzyme preparation (or 0.1-mL aliquots of the fractions from column chromatography), 0.025 mL of 6 mM AcCoA (aqueous), 0.075 mL of 0.33 mM PABA (aqueous) and sufficient 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the incubation volume to 0.25 mL. Incubations were carried out at 37° in air for 10–20 min, and the supernatant fraction was analyzed by Weber's modification [19] of the Bratton–Marshall procedure as previously described [8].

AcCoA-dependent acetylation of PA and 2-AF. Acetylation of PA and 2-AF by AcCoA was determined by the method of Hein *et al.* [20]. Reaction mixtures contained 0.035 to 0.05 mL of enzyme preparation (or 0.05-ml aliquots of the fractions from column chromatography), either 0.02 mL of 1 mM procainamide hydrochloride solution (aqueous) or 0.02 mL of 1 mM 2-AF (dissolved in DMSO), 0.02 mL of 10 mM AcCoA (aqueous) and enough 0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0 buffer to give a final incubation volume of 0.09 mL. The reactions were carried out for 10–30 min (as determined from the linear portion of the graphs of PA or 2-AF acetylation vs time).

Electrophile generation assay (methylthio adduct formation). The AHAT-mediated production of electrophiles from N-OH-AAF was measured by the *N*-acetylmethionine electrophile trapping assay of Bartsch *et al.* [2] as described previously [21]. The enzyme source was 0.2 to 0.4 mL of glycerol-stabilized NAT I or NAT II transacetylase preparations or 0.2 to 0.4 mL of the fractions from chromatofocusing. The concentration of N-OH-AAF was 0.05 mM and the concentration of *N*-acetylmethionine was 10 mM.

Inactivation experiments

Inactivation of unresolved NAT activities by N-OH-AAF. Incubation mixtures contained the glycerol-stabilized Sephadex G-100 NAT fractions (final protein concentration 5 mg/mL), 0.03 mM N-OH-AAF, 2% ethanol and 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT) in a final incubation volume of 0.9 mL. Incubations were conducted in the presence and absence of 10 mM cysteine. When present, cysteine was preincubated with enzyme at 37° for 2 min before the addition of N-OH-AAF. The incubation was continued in the presence of N-OH-AAF at 37° for 15 min. At the end of the incubation period, 0.105 mL of sodium pyrophosphate buffer (0.05 M, pH 7, 1 mM DTT) was added to each incubation mixture, and 1.0 mL of the resulting mixture was applied to a PD-10 column prepacked with Sephadex G-25M. The column was then eluted with two portions (2.0 and 1.3 mL) of buffer (0.05 M sodium pyrophosphate, pH 7, containing 1 mM DTT, 2% ethanol and 7% glycerol). The majority (approximately 95%) of the NAT activity

and protein was eluted with the second portion of buffer, whereas the elution of small organic molecules did not begin until at least 1–2 mL of additional buffer had passed through the column. Protein concentrations were determined and adjusted to 2.5 mg/mL for assay of N-OH-AAF/AAB, AcCoA/PABA and AcCoA/PA activities as described under "Enzyme assays."

Inactivation of NAT I and NAT II preparations by N-OH-AAF. Incubation mixtures contained the glycerol-stabilized transacetylase preparations from Sepharose 6B 2-AF affinity chromatography (NAT I or NAT II, final protein concentration 0.06 mg/mL), 0.01 mM N-OH-AAF, 2% ethanol and enough 0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0 buffer to give a final incubation volume of 15.0 mL (for the NAT II transacetylase preparation) or 5.0 mL (for the NAT I transacetylase preparation). Incubations were started with the addition of N-OH-AAF and were continued for 15 min at 37° in air. At the end of the incubation period, the flasks were placed on ice. Aliquots of the incubation mixtures were then analyzed for their respective transacetylase activities (N-OH-AAF/AAB activity in incubation mixtures containing NAT II or AcCoA/PABA activity in incubation mixtures containing NAT I). To test for reversibility of inhibition of enzyme activity, portions of the incubation mixture containing NAT II were subjected to 4 hr of dialysis at 4° against two portions of 0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0 buffer (containing 2% ethanol and 4–5% glycerol). The buffer was purged with nitrogen throughout the dialysis period. At the end of the dialysis period, aliquots of the dialysate were reassayed for N-OH-AAF/AAB transacetylation activity. Performance of the dialysis step was omitted for incubation mixtures containing NAT I transacetylase preparation, as no appreciable inactivation was observed.

Experiments which examined the effects of nucleophiles and AcCoA on the N-OH-AAF-mediated inactivation of the N-OH-AAF/AAB and AcCoA/PA activities of NAT II were conducted in a fashion similar to those described above for the Sephadex G-100 NAT preparation. Incubation mixtures contained 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT), N-OH-AAF (0.03 mM) and glycerol-stabilized NAT II preparation (final protein concentration 0.15 mg/mL). Incubations were conducted in the presence or absence of 10 mM cysteine, 10 mM guanosine phosphate or 1 mM AcCoA, which were preincubated with enzyme for 2 min prior to the addition of N-OH-AAF. Incubation in the presence of N-OH-AAF was continued for 15 min at 37°. Incubation volume was 1.05 mL. At the end of the incubation period, 1.0 mL of the incubation mixture was applied to a PD-10 column (Sephadex G-25M) and eluted as described above. After determination of protein concentration and adjustment to 0.06 mg/mL, N-OH-AAF/AAB and AcCoA/PA assays were performed on the filtrate as described under "Enzyme assays."

In separate experiments, the electrophile-generating (AHAT) activity of the NAT II preparation was measured after incubation with N-OH-AAF. The incubations and filtration through PD-10 columns were conducted exactly as described above for the N-OH-AAF-mediated inactivation of the N-OH-AAF/AAB and AcCoA/PA activities of NAT II. The electrophile-generating ability (methylthio adduct formation) of the filtered enzyme preparation was measured as described under "Enzyme assays."

*Effects of paraoxon and bis(*p*-nitrophenyl) phosphate (BNPP) on rat hepatic transacetylase activities.* The effects of paraoxon and BNPP on rat hepatic NAT II and NAT I transacetylase activities were determined in experiments which were similar in design to those described above for the N-OH-AAF-mediated inactivation of the NAT II transacetylase activities. Incubation mixtures contained the glycerol-stabilized NAT I or NAT II preparations (final protein concentration 0.15 mg/mL) and 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT). Incubations were conducted for 5 min at 37° both in the presence and absence of either 0.1 mM paraoxon or 0.1 mM BNPP, which were added to the incubation mixtures from 2 mM aqueous (pH 6.0) solutions. The incubation volume was 1.05 mL. At the end of the incubation period the incubation mixtures were filtered through Sephadex G-25M as described above. N-OH-AAF/AAB and AcCoA-PA transacetylation activity (for NAT II incubations) or AcCoA/PABA transacetylation activity (for NAT I incubations) was determined as described under "Enzyme assays."

To determine the effects of paraoxon and BNPP on NAT II methylthio adduct formation, glycerol-stabilized NAT II fractions (0.05 mg/mL) were preincubated for 4 min at 37° both in the presence and absence of either 0.1 mM paraoxon or 0.1 mM BNPP. Incubations were continued in the presence of *N*-acetylmethionine, N-OH-AAF and paraoxon or BNPP for 20 min. Methylthio adduct formation was measured as described under "Enzyme assays."

Inactivation of rat hepatic NAT II N-OH-AAF/AAB transacetylase activity by N-OH-AAF and quantitation of N-OH-AAF protein binding. Inactivation of rat hepatic NAT II N-OH-AAF/AAB transacetylase activity was accomplished in incubations containing the glycerol-stabilized NAT II preparation (0.2 mg/mL), 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0), N-OH-AAF (0.05 mM) and 2% DMSO. The incubation volume was 0.1125 mL. Incubations were carried out at 37° for 2, 3, 5 and 7 min. At the end of the incubation period, buffer (0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0) and substrates (N-OH-AAF and AAB) were added to bring the volume to 0.5 mL for assay of remaining N-OH-AAF/AAB *N,N*-transacetylation activity as described under "Enzyme assay."

Separate experiments were conducted to determine the binding of [9-^{14}C]N-OH-AAF to NAT II protein. Incubations were conducted under conditions identical to those described above for the N-OH-AAF-mediated inactivation of the NAT II N-OH-AAF/AAB *N,N*-transacetylation activity, except that incubations contained 0.05 mM [9-^{14}C]N-OH-AAF (1.56 Ci/mol) and the final incubation volume was 1.0 mL. Incubations were carried out for 2, 3, 5 or 7 min at 37° and were terminated by extraction with 2 mL of ice-cold water-saturated 1-butanol.

The aqueous layer was further extracted with ten 2-mL portions of water-saturated 1-butanol. Two milliliters of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the mixture was shaken for 30 min. After centrifugation, the aqueous layer was discarded and protein was precipitated by the addition of 3 mL of cold (−20°) acetone. The precipitated protein was further extracted with six 2-ml portions of acetone, dried *in vacuo*, and solubilized in 1 mL of 1 M NaOH for quantitation of incorporated radioactivity by liquid scintillation counting. Control incubations were performed with denatured NAT II protein; results were corrected for binding to denatured protein, which was typically 0.2 nmol adduct/mg protein/min.

RESULTS

Partial purification of unresolved rat hepatic NAT activities

It was reported previously that ammonium sulfate fractionation of rat hepatic cytosol and gel filtration of the resulting 45–65% fraction result in coelution of AHAT and N-OH-AAF/AAB activities [3]. In the present study, ammonium sulfate fractionation of rat hepatic cytosol resulted in similar extents of purification and recovery of N-OH-AAF/AAB, AcCoA/PA, AcCoA/PABA, and AcCoA/2-AF transacetylation activities (Table 1). Although gel filtration chromatography of the 45–65% ammonium sulfate fraction resulted in coelution of the four activities, the AcCoA/PABA transacetylase activity was purified to a greater extent and with much higher recovery of activity than the N-OH-AAF/AAB, AcCoA/PA or AcCoA/2-AF transactylase activities (Table 1).

Inactivation of unresolved NAT activities by N-OH-AAF

Previous investigations have shown that incubation of a partially purified rat hepatic transacetylase preparation with N-OH-AAF results in an irreversible, mechanism-based inactivation of the N-OH-AAF/AAB *N,N*-acetyltransferase activity [22, 23]. Therefore, it was of interest to determine whether N-OH-AAF could also function as an irreversible inhibitor of the ACCoA/PABA and AcCoA/PA NAT activities that coeluted with N-OH-AAF/AAB *N,N*-acetyltransferase activity. As shown in Fig. 2, incubation of the rat hepatic Sephadex G-100 preparation with N-OH-AAF resulted in only 16% inhibition of the AcCoA/PABA transacetylation activity, whereas the N-OH-AAF/AAB and AcCoA/PA transacetylation activities were inhibited 73 and 76% respectively. These results suggest that AcCoA/PABA transacetylation activity is catalyzed by an enzyme that is distinct from the enzyme(s) catalyzing the N-OH-AAF/AAB and AcCoA/PA transacetylations.

Previous studies have shown that the inclusion of low molecular weight nucleophiles in the incubation mixture can reduce the rate of acetyltransferase inactivation by N-OH-AAF [12, 14, 23] and that the changes in rate are useful for detecting NAT multiplicity [8, 12]. Therefore, incubations which included the nucleophile cysteine were performed. Figure 2 shows that cysteine, in the absence of N-OH-AAF, had little effect on any of the transacetylase activities. However, cysteine afforded a very large degree of protection against inactivation by N-OH-AAF of both the N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA NAT activities. Differential N-OH-AAF-mediated inactivation of the N-OH-AAF/AAB (*N,N*-acetyltransferase) and AcCoA/PA activities in the presence of cysteine was not observed, as activities were maintained at similar levels (76 and 73% of control activity respectively).

Separation of rat hepatic NAT activities by affinity chromatography

Application of the unresolved transacetylase activities obtained by gel filtration to a Sepharose 6B 2-AF affinity chromatography column resulted in separation of the AcCoA/PABA NAT activity from the N-OH-AAF/AAB and AcCoA/PA transacetylation activities (Fig. 3). The AcCoA/PABA transacetylase activity, designated NAT I, was eluted from the column in the same potassium phosphate buffer in which it was applied. However, this elution took place after a major protein peak had been eluted, resulting in significant purification of the AcCoA/PABA transacetylase activity. The N-OH-AAF/AAB and AcCoA/PA transacetylase activities, designated NAT II, coeluted in sodium pyrophosphate buffer. Most of the AcCoA/2-AF NAT activity eluted with the NAT II fractions. A very small amount of AcCoA/PABA activity consistently eluted with the N-OH-AAF/AAB and AcCoA/PA activities. It is not known whether this activity is due to incomplete chromatographic separation of NAT I and NAT II, or whether NAT II is capable of catalyzing a low level of AcCoA/PABA activity. Although a small amount of AcCoA/PA activity was detected in two fractions preceding the major peaks of AcCoA/PA activity, this did not occur consistently upon repetition of the procedure.

Shown in Table 1 are data for a typical purification experiment. N-OH-AAF/AAB and AcCoA/PA activities were purified to the same extent with nearly identical recovery of transacetylase activity at each step of the purification procedure. Purification and recovery of AcCoA/PABA activity was higher than for the NAT II activities.

Inactivation of separated rat hepatic NAT I and NAT II transacetylase activities by incubation with N-OH-AAF

To determine whether the NAT I and NAT II transacetylase preparations separated by Sepharose 6B 2-AF affinity chromatography are capable of bioactivating N-OH-AAF to electrophiles which can inactivate the enzymic activity. the preparations were incubated with N-OH-AAF and subsequently assayed for their respective transacetylase activities. As shown in Fig. 4, incubation of N-OH-AAF with the NAT II transacetylase preparation resulted in the loss of 63% of the N-OH-AAF/AAB *N,N*-acetyltransferase activity. This loss of activity was not reversible upon dialysis of the inactivated enzyme preparation. When the NAT I transacetylase preparation was incubated with N-OH-AAF, no appreciable loss of AcCoA/PABA transacetylation activity

Table 1. Partial purification of rat hepatic NAT activities

Purification step	Transacetylation activity											
	N-OH-AAF/AAB			AcCoA/PA			AcCoA/PABA			AcCoA/2-AF		
	Activity*	Cumulative purification	Cumulative recovery	Activity*	Cumulative purification	Cumulative recovery	Activity*	Cumulative purification	Cumulative recovery	Activity*	Cumulative purification	Cumulative recovery
Cytosol	2.0	1.0	100.0	0.6	1.0	100.0	0.5	1.0	100.0	1.2	1.0	100.0
Ammonium sulfate	4.1	2.0	57.4	1.2	2.2	60.7	1.3	2.6	74.3	2.4	2.1	59.0
Sephadex G-100	11.0	5.5	36.2	3.2	5.5	36.5	5.5	11.0	73.3	6.6	5.7	37.5
Sepharose 6B 2-AF (NAT I)	0	0	0	0	0	0	42.1	84.1	30.4	10.0	8.6	3.1
Sepharose 6B 2-AF (NAT II)	122.4	60.6	11.9	35.1	60.6	11.9	3.7	7.4	1.4	57.6	49.7	9.7

* Activities are expressed as transacetylation rates (nmol/mg/min).

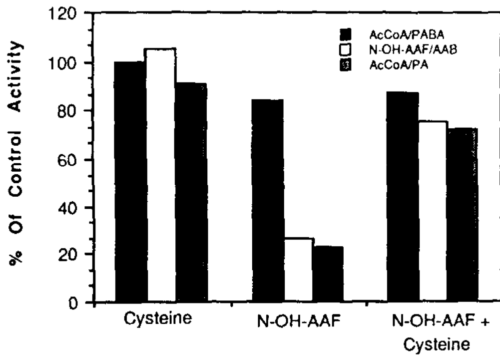

Fig. 2. Effects of N-OH-AAF and cysteine on the unresolved rat hepatic AcCoA/PABA, N-OH-AAF/AAB (*N,N*-acetyltransferase) and AcCoA/PA transacetylase activities contained in glycerol-stabilized fractions from Sephadex G-100 gel filtration. After incubation with N-OH-AAF and/or cysteine, the enzyme preparation was filtered through Sephadex G-25M and assayed for transacetylation activities as described in Materials and Methods. Results are the means of two experiments, each conducted in triplicate. Control activities determined in the absence of cysteine and without preincubation with N-OH-AAF were (in nmol/mg/min) 6.5 for AcCoA/PABA, 12.1 for N-OH-AAF/AAB and 3.8 for AcCoA/PA.

was demonstrated. These results indicate that the rat hepatic NAT II transacetylase preparation, but not the NAT I transacetylase preparation, is capable of converting N-OH-AAF, via N,O-acyltransfer, to electrophilic intermediates that cause irreversible loss of enzymic activity.

Inactivation of NAT II transacetylase activities by N-OH-AAF: Effect of nucleophiles and AcCoA

Because differential N-OH-AAF-mediated inactivation of the N-OH-AAF/AAB and AcCoA/PA transacetylation activities contained in the gel filtration fractions was not observed (Fig. 2), similar inactivation experiments were conducted on the more highly purified NAT II preparation obtained from affinity chromatography. The effects of cysteine, guanosine phosphate and AcCoA on the N-OH-AAF-mediated inactivation also were studied.

The results of these experiments are summarized in Table 2. Rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities were reduced to approximately 20 and 23% of control values, respectively, after incubation with N-OH-AAF. When cysteine was included in the incubation medium with N-OH-AAF, a very large portion of the inactivation of both activities was prevented. The percent of control activity remaining in the presence of N-OH-AAF and cysteine was 89% for N-OH-AAF/AAB *N,N*-acetyltransferase activity and 83% for AcCoA/PA *N*-acetyltransferase activity. Thus, differential inactivation of the two activities was not observed, either in the presence of N-OH-AAF alone or in the presence of N-OH-AAF and cysteine.

Guanosine phosphate was used as an electrophile

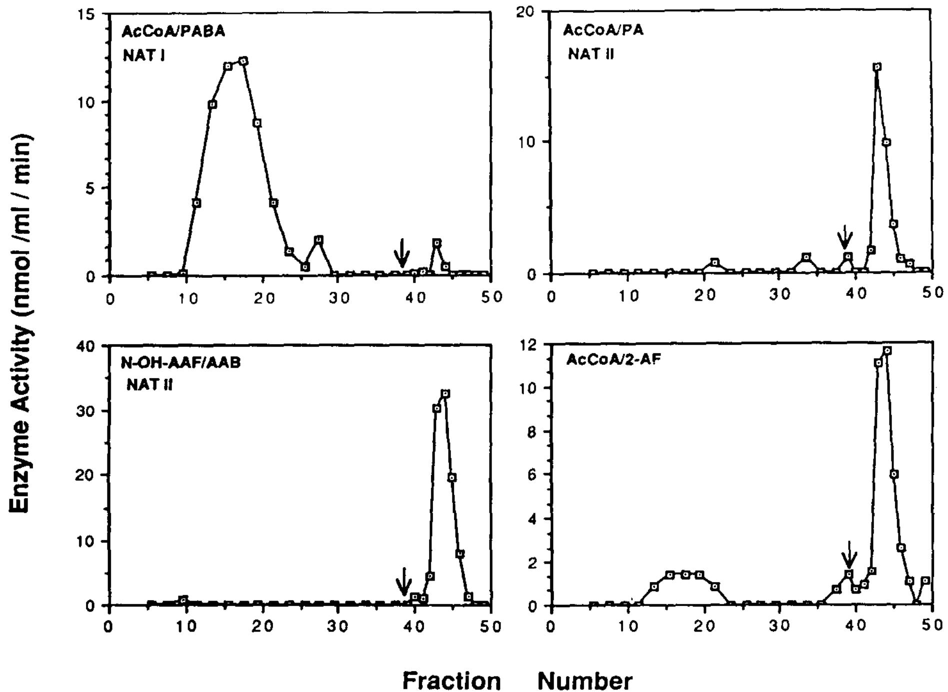

Fig. 3. Sepharose 6B 2-AF affinity chromatography of rat hepatic AcCoA/PABA, N-OH-AAF/AAB (*N,N*-acetyltransferase), AcCoA/PA and AcCoA/2-AF transacetylase activities. At fraction 39, the elution buffer was changed to 0.05 M sodium pyrophosphate (arrow). The chromatographic procedure and assay methods are described under Materials and Methods.

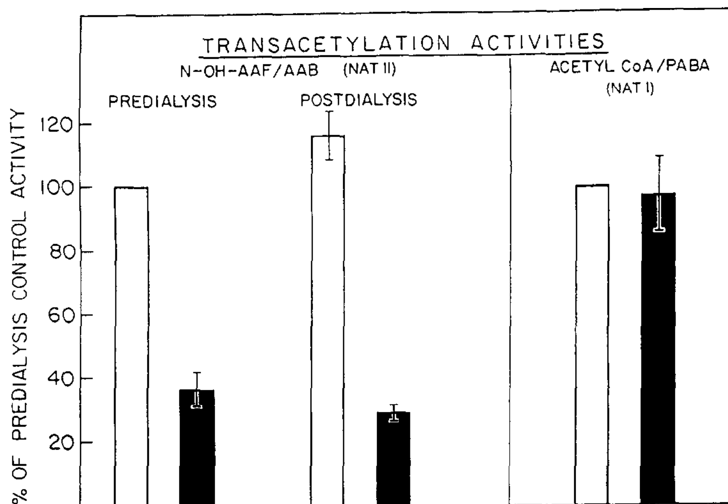

Fig. 4. Effect of N-OH-AAF on rat hepatic NAT II (N-OH-AAF/AAB*N,N*-acetyltransferase) and NAT I (AcCoA/PABA) transacetylase activities after separation by Sepharose 6B 2-AF affinity chromatography. The enzyme preparations were incubated with N-OH-AAF (■) and compared with control activities (□). The activity for the NAT II fraction was measured after dialysis to test for irreversibility of enzyme inhibition. Results are the means (±SD) of three experiments. Control incubations contained no N-OH-AAF. Control activities were (in nmol/mg/min) 140.4 ± 17.8 for N-OH-AAF/AAB and 55.9 ± 4.4 for AcCoA/PABA activity.

Table 2. Inactivation of rat hepatic NAT II transacetylase activities by N-OH-AAF: Effects of cysteine, guanosine phosphate and AcCoA

	Percent of control activity*	
Incubation conditions	N-OH-AAF/AAB†	AcCoA/PA‡
0.03 mM N-OH-AAF	19.8 ± 3.3§	22.6 ± 7.7§
0.03 mM N-OH-AAF + 10 mM cysteine	89.1	83.5
0.03 mM N-OH-AAF + 10 mM guanosine phosphate	77.7	75.6
0.03 mM N-OH-AAF + 1 mM AcCoA	105.3	102.8

* Values are presented as percent of control activity after incubation with the indicated agents. The incubation mixtures were filtered through Sephadex G-25M prior to measurement of transacetylase activity as described in Materials and Methods.

† The N-OH-AAF/AAB *N,N*-acetyltransferase activity in the control incubations was 252.8 ± 6.3 nmol/mg/min.

‡ The AcCoA/PA activity in the control incubations was 67.7 ± 2.7 nmol/mg/min.

§ Mean ± SD, N = 4; all other values are the means of two experiments.

trapping agent because its structure and nucleophilic properties are different from those of cysteine and because it has been shown previously to reduce the rate of N-OH-AAF-mediated inactivation of hamster hepatic N-OH-AAF/AAB *N,N*-acetyltransferase activity [12]. Furthermore, guanosine phosphate does not contain a thiol group and would not likely affect the level of NAT activity through an effect on the state of oxidation of essential thiol groups on the enzyme. As shown in Table 2, when guanosine phosphate was included in the incubation medium, the protection of both the N-OH-AAF/AAB and AcCoA/PA activities was only approximately 10% less than that afforded by cysteine. Again, differential inactivation of the two activities was not observed.

In a previous study it was shown that AcCoA, the endogenous cofactor for transacetylase-catalyzed N-acetylation of arylamines, provided, in a concentration-dependent manner, protection of hamster hepatic N-OH-AAF/AAB *N,N*-acetyltransferase activity from inactivation by N-OH-AAF [24]. Therefore, it was of interest to determine whether AcCoA could prevent the N-OH-AAF-mediated inactivation of rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylation activities present in the NAT II fractions. As reported in Table 2, 1 mM AcCoA provided complete protection from inac-

Table 3. Bioactivation of N-OH-AAF by separated rat hepatic NAT I and NAT II transacetylase preparations: Methylthio adduct formation*

Transacetylase preparation	Methylthio adduct formation†
NAT I	1.1, 0.0
NAT II	96.9, 95.0

* Shown are the results of two experiments, carried out in triplicate.
† Activity is expressed as the rate of methylthio adduct formation (nmol/mg/20 min).

tivation by N-OH-AAF of both the N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA *N*-acetyltransferase activities. The protection afforded to both activities by AcCoA was dependent on the concentration of AcCoA, as less protection was observed when concentrations of AcCoA lower than 1 mM were used (data not shown). Control experiments were conducted in which the transacetylation reactions were carried out in the presence of cysteine, guanosine phosphate and AcCoA. None of these agents affected the transacetylation activities.

Methylthio adduct formation catalyzed by separated rat hepatic NAT I and NAT II transacetylase preparations

The *N*-acetylmethionine electrophile trapping assay of Bartsch *et al.* [2] was used to test further the relative abilities of the NAT I and NAT II transacetylase preparations to catalyze the generation of electrophiles from N-OH-AAF. The results are summarized in Table 3. Incubation of the rat hepatic NAT II transacetylase preparation with N-OH-AAF and *N*-acetyl-L-[^{14}C-CH_3]methionine led to the formation of a substantial amount of methylthio adduct. In contrast, the NAT I transacetylase preparation catalyzed the formation of little or no methylthio adduct. These findings, in agreement with the inactivation experiments described above (Fig. 4), indicate that only one of the two rat hepatic transacetylase fractions that were separated by affinity chromatography was capable of catalyzing the generation of substantial amounts of reactive electrophilic intermediates.

Effects of paraoxon and BNPP on NAT I and NAT II transacetylase activities

Because of the possibility that the transacetylase or methylthio-adduct forming activities of the NAT I and NAT II fractions might be catalyzed to some extent by solubilized microsomal deacylase enzymes present in these fractions [25], the effects of the deacylase inhibitors paraoxon and bis(*p*-nitrophenyl)phosphate (BNPP) on the NAT I and NAT II acetyltransferase activities were investigated. As shown in Table 4, neither the NAT I AcCoA-PABA *N*-acetyltransferase activity nor the NAT II transacetylase and methylthio-adduct forming activities (AHAT) were inhibited by treatment with either 0.1 mM paraoxon or 0.1 mM BNPP.

Prevention of methylthio adduct formation by incubation with N-OH-AAF

In previous experiments with partially purified hamster NAT, it was found that incubation with N-OH-AAF inhibited both N-OH-AAF/AAB N,N-acetyltransfer and arylhydroxamic acid-methylthio adduct formation in a time-dependent fashion [22]. The finding herein that rat hepatic N,O-acyltransfer of N-OH-AAF was catalyzed by the NAT II fractions (Table 3) prompted the investigation of whether incubation of N-OH-AAF with NAT II, in addition to resulting in the inactivation of the N-OH-AAF/AAB and AcCoA/PA activities (Table 2), could also result in the inactivation of the transacetylase activity responsible for N-OH-AAF/methylthio adduct formation. Methylthio adduct formation occurred at a rate of 218.2 ± 17.8 nmol/mg/20 min (mean ± SD, N = 3) in control incubation mixtures, whereas the rate was 13.0 ± 2.7 nmol/mg/20 min with NAT II preparations that had been subjected to prior treatment with N-OH-AAF. Thus, incubation of NAT II with N-OH-AAF caused a 94% reduction in methylthio adduct formation.

Correlation of N-OH-AAF-mediated inactivation of NAT II transacetylase activity with protein binding

To provide further evidence that N-OH-AAF-mediated inactivation of the NAT II transacetylase activities is a result of binding of "activated" N-OH-AAF to the acetyltransferase enzyme, an attempt was made to correlate the N-OH-AAF-mediated inactivation of the NAT II N-OH-AAF/AAB *N,N*-acetyltransferase activity with covalent binding of radiolabeled N-OH-AAF to total NAT II protein as a function of time. As shown in Fig. 5, incubation of rat hepatic NAT II fractions with 0.05 mM N-OH-AAF or [9-^{14}C]N-OH-AAF for 2, 3, 5 and 7 min resulted in the time-dependent inactivation of the N-OH-AAF/AAB transacetylation activity and a corresponding time-dependent formation of ^{14}C-protein adducts. The correlation coefficient for the fit of the line to the points in Fig. 5 was 0.99.

Chromatofocusing: coelution of NAT II acetyltransferase activities

To gain additional evidence for the association of the N-OH-AAF/AAB N,N-acetyltransfer, AcCoA/PA N-acetyltransfer, and N,O-acyltransfer activities with a common enzyme, the NAT II preparation was further purified by chromatofocusing. Shown in Table 5 is the purification data resulting from chromatofocusing of the NAT II acetyltransferase activities. N-OH-AAF/AAB, AcCoA/PA and *N,O*-acetyltransferase (methylthio adduct formation) activities were coeluted in the same fractions, and were purified to similar extents by chromatofocusing,

Table 4. Effects of paraoxon and BNPP on rat hepatic NAT I and NAT II transacetylase activities

	Transacetylase activities*			
	NAT I	NAT II		
	AcCoA/PABA†	N-OH-AAF/AAB†	AcCoA/PA†	Methylthio adduct formation‡
Control	49.06	221.60	61.75	257.29
0.1 mM Paraoxon	51.37	233.35	63.95	269.88
0.1 mM BNPP	49.87	233.54	64.26	273.04

* Results are the means of two experiments.

† Activity, expressed as the transacetylation rate (nmol/mg/min), was determined after incubation in the presence or absence of 0.1 mM paraoxon or BNPP and filtration through Sephadex G-25M, as described in Materials and Methods.

‡ Activity, expressed as the rate of methylthio adduct formation (nmol/mg/20 min), was determined in the presence or absence of 0.1 mM paraoxon or BNPP, as described in Materials and Methods.

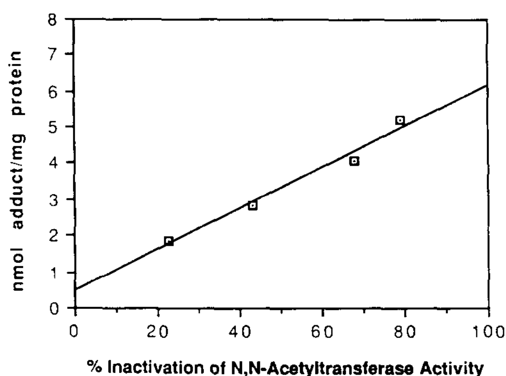

Fig. 5. Correlation of N-OH-AAF-mediated inactivation of NAT II N-OH-AAF/AAB transacetylase (*N,N*-acetyltransferase) activity with binding of [9-^{14}C]N-OH-AAF to NAT II protein. Incubations in the presence of 0.05 mM N-OH-AAF were carried out for 2, 3, 5 and 7 min. The extent of inactivation of *N,N*-acetyltransferase activity and the formation of ^{14}C-protein adducts were quantified as described under Materials and Methods. Results are the means of two experiments.

supporting the proposal that the three activities are associated with the same enzyme. The pH of the fractions containing the peak acetyltransferase activities was 4.3–4.4. Because proteins are eluted from a chromatofocusing system at pH values near their isoelectric point, these results are in close agreement with those of Allaben and King [26], who reported a value of 4.5 as the isoelectric point of rat hepatic *N,O*-acyltransferase. Recovery of the NAT II acetyltransferase activities upon chromatofocusing was approximately 50%. Comparison of the acetyltransferase activities obtained by chromatofocusing (Table 5) with the cytosolic acetyltransferase activities (Table 1) indicates that the cumulative purification for N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA *N*-acetyltransferase activities was 1300-fold and 1100-fold respectively.

DISCUSSION

Multiple forms of *N*-acetyltransferase have been demonstrated in various tissues and species [27]. In the rat small intestine, two forms of cytosolic AHAT activity, with different substrate specificities for the activation of *N*-arylhydroxamic acids and differences in their immunoprecipitation properties, have been separated by gel filtration [28]. Although two soluble forms of AHAT activity were also separated from rat liver preparations, one of them appears to have been a microsomal component that had been solubilized during the homogenization procedure [25].

The existence of multiple forms of AcCoA-dependent NATs in rat liver has been postulated by Tannen

Table 5. Effect of chromatofocusing on NAT II activities

	Acetyltransferase activity					
	N-OH-AAF/AAB		AcCoA/PA		Adduct formation	
	Activity*	Purification factor	Activity*	Purification factor	Activity†	Purification factor
NAT II fractions (Sepharose 6B 2-AF)	122.4		35.1		95.9	
Chromatofocusing	2632.8	21.5	632.8	18.0	1625.9	17.0

* Activity is expressed as the transacetylation rate (nmol/mg/min).

† Activity is expressed as the rate of methylthio adduct formation (nmol/mg/20 min).

and Weber [29]. The present study demonstrates the detection, via mechanism-based inactivation, of distinct rat hepatic *N*-acetyltransferase activities, and their separation by affinity chromatography. One fraction, designated NAT II, was capable of catalyzing the AcCoA-dependent N-acetylation of PA and the N-OH-AAF-dependent N-acetylation of AAB (N,N-acetyltransfer), as well as N,O-acyltransfer. In contrast, the NAT I activity, which catalyzed the majority of the AcCoA/PABA transacetylation, did not exhibit appreciable N,O-acetyltransfer, N-OH-AAF/AAB, or AcCoA/PA transacetylation activity. These findings are similar to those recently reported for hamster liver and intestine [8], from which two transacetylase activities were separated by means of affinity chromatography.

Although we recently reported [30] preliminary results from mechanism-based inactivation experiments that indicated differences in the relative amounts of inactivation of N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA *N*-acetyltransferase activities, the present, more extensive investigation of the mechanism-based inactivation of the NAT II activities did not reveal differences in the relative levels of inactivation of N-OH-AAF/AAB *N,N*-acetyltransferase activity as compared to AcCoA/PA *N*-acetyltransferase activity (Table 2). The present studies also demonstrate that *N,O*-acyltransferase activity was similarly inactivated by prior incubation with N-OH-AAF. Furthermore, rat hepatic AcCoA/PA and N-OH-AAF/AAB activities were coeluted and copurified at every step of the purification procedure, with 1100 to 1300-fold purification of AcCoA/PA and N-OH-AAF/AAB transacetylation activities ultimately being obtained.

The coelution and copurification of rat hepatic *N,O*-acyltransferase (AHAT) and N-OH-AAF/AAB *N,N*-acetyltransferase activities through ammonium sulfate precipitation and gel filtration have been reported previously [3, 23]. In the present study, *N,O*-acyltransferase activity was coeluted with N-OH-AAF/AAB and AcCoA/PA transacetylation activities through the affinity chromatography step and was coeluted and copurified with these two activities following chromatofocusing. Based on the results of both the mechanism-based inactivation experiments and the purification experiments, it is reasonable to conclude that *N,O*-acetyltransferase, N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA *N*-acetyltransferase activities from rat liver are catalyzed by the same protein.

Organophosphate compounds, such as paraoxon and BNPP, are commonly used to distinguish cytosolic *N*-acetyltransferase, *N,O*-acetyltransferase (AHAT), and *N,N*-acetyltransferase activities from microsomal esterases that can both deacetylate and bioactivate N-OH-AAF [24, 25]. The lack of inhibition of transacetylase activities in either the NAT I or NAT II fraction by paraoxon and BNPP (Table 4) indicates that none of the activities can be attributed to solubilized microsomal deacylases (esterases).

The inclusion of cysteine (Fig. 2, Table 2) or guanosine phosphate (Table 2) in the incubation medium in the presence of N-OH-AAF prevented a large portion of the N-OH-AAF-mediated inactivation of rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities, but the inactivation was not prevented completely. These results are similar to those recently reported for rat hepatic N-OH-AAF/AAB *N,N*-acetyltransferase activity that had been partially purified by gel filtration [23], and indicate that electrophiles that are released from the active site of the *N,O*-acetyltransferase enzyme, in addition to electrophiles that remain complexed with the enzyme active site, contribute to the inactivation of the enzyme. When the endogenous cofactor, AcCoA, was included in incubations with N-OH-AAF, complete protection of the enzyme activities from N-OH-AAF-mediated inactivation was afforded (Table 2). This protection was dependent on the concentration of AcCoA, indicating that the active site of the enzyme was acetylated by AcCoA, thus preventing the bioactivation of N-OH-AAF. This AcCoA-dependent protection from arylhydroxamic acid inactivation is similar to that observed with hamster hepatic N-OH-AAF/AAB *N,N*-acetyltransferase activity [24] and provides further support for the identity of *N,O*-acyltransferase activity (AHAT) with the AcCoA-dependent *N*-acetyltransferase activities in the NAT II fractions.

Previous reports from this laboratory described the inactivation of *N*-acetyltransferase, *N,N*-acetyltransferase and AHAT activities by *N*-arylhydroxamic acids both *in vivo* and *in vitro* [8, 12, 22–24]. The results of *in vitro* experiments demonstrated that the inactivation exhibited pseudo first-order kinetics and that a mechanism-based (suicide) process was involved. Based on the known propensity for bioactivated *N*-arylhydroxamic acids to form covalent adducts with biological macromolecules [6], the mechanism-based inactivation was proposed to involve covalent binding of the bioactivated *N*-arylhydroxamic acids to the enzyme. In the present study, the inactivation of rat hepatic N-OH-AAF/AAB *N,N*-acetyltransferase activity was found to correlate quantitatively with covalent binding of radiolabel to protein when the enzyme was incubated with [9-^{14}C]N-OH-AAF (Fig. 5). This result supports the contention that enzyme inactivation is due to irreversible formation of protein bound adducts rather than being the result of other processes such as oxidation of essential sulfhydryl groups on the enzyme.

Rat hepatic *N,O*-acetyltransferase activity has been purified to homogeneity [26]. Recently, Land *et al.* [31] reported the copurification to homogeneity of three rat hepatic acyltransferase activities: N,O-acyltransfer of N-OH-AAF (AHAT), AcCoA-dependent O-acetylation of *N*-hydroxy-3,2′-dimethyl-4-aminobiophenyl, and AcCoA-dependent N-acetylation of 2-aminofluorene. Others have provided evidence that a single protein catalyzes N,O-acyltransfer, N-acetyltransfer, and arylhydroxylamine O-acetylation reactions in hamster [32] and mouse [10] livers. In rabbit liver, a single enzyme catalyzes *N,O*-acyltransferase and NAT activity [9]. The results of the present study are consistent with the proposal that rat hepatic *N,O*-acyltransferase, N-OH-AAF/AAB *N,N*-acetyltransferase and AcCoA/PA *N*-acetyltransferase

activities may be catalyzed by a common enzyme. In agreement with the results of Land *et al.* [31], we found that rat hepatic AcCoA/2-AF activity coeluted from the Sepharose 6B 2-AF affinity chromatography column with the *N,O*-acyltransferase activity (NAT II) (Table 1). However, a second, smaller peak of AcCoA/2-AF transacetylase activity coeluted with the AcCoA/PABA activity. Thus, both fractions, NAT I and NAT II, are capable of catalyzing the AcCoA-dependent acetylation of 2-aminofluorene.

The findings of the present study point to similarities in the biochemical relationship between various hepatic acyltransferase activities in the rat and hamster models. As we report herein for rat liver, hamster liver *N,O*-acyltransferase activity is catalyzed primarily by an acyltransferase that is distinct and separable from the acetyltransferase that catalyzes AcCoA/PABA transacetylation [8, 11, 33], whereas AcCoA/2-AF transacetylase activity is catalyzed by both of these acyltransferase enzymes [11]. In addition to these biochemical similarities, previous studies have indicated that inbred rats may exhibit N-acetylation pharmacogenetics very similar to that found in inbred hamsters [27, 34]. Hein *et al.* have characterized the acetylation polymorphism for inbred hamsters [11, 20] and found that it differs from that described for rabbit in that those substrates that are monomorphically acetylated in the hamster are polymorphically acetylated in the rabbit, and vice versa [20].

The findings discussed herein, that rat hepatic tissue contains at least two forms of acyltransferase activity with different substrate specificities and that only one of these activities expresses appreciable *N,O*-acyltransferase activity, contribute to an understanding of the biochemical processes involved in the metabolism and bioactivation of various arylamines, arylhydroxylamines and arylhydroxamic acids. Further studies are necessary to define the exact processes that determine acyltransferase substrate specificity and mechanism.

Acknowledgement—The authors wish to thank Mr L. W. Boteju for synthesis of the radiolabeled N-OH-AAF and *N*-acetylmethionine.

REFERENCES

1. Miller JA and Miller EC, The metabolic activation of carcinogenic aromatic amines and amides. *Prog Exp Tumor Res* **11**: 273–301, 1969.
2. Bartsch H, Dworkin M, Miller JA and Miller EC, Electrophilic *N*-acetoxyaminoarenes derived from carcinogenic *N*-hydroxy-*N*-acetylaminoarenes by enzymatic deacetylation and transacetylation in liver. *Biochim Biophys Acta* **286**: 272–298, 1972.
3. King CM, Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res* **34**: 1503–1515, 1974.
4. King CM and Phillips M, *N*-Hydroxy-2-fluorenylacetamide. Reaction of the carcinogen with guanosine, ribonucleic acid, deoxyribonucleic acid and protein following enzymatic deacetylation or esterification. *J Biol Chem* **244**: 6209–6216, 1969.
5. King CM and Allaben WT, The role of arylhydroxamic acid *N-O* acyltransferase in the carcinogenicity of aromatic amines. In: *Conjugation Reactions in Drug Biotransformation* (Ed. Aitio A), pp. 431–441. Elsevier/North Holland, New York, 1978.
6. Hanna PE and Banks RB, Arylhydroxylamines and arylhydroxamic acids: conjugation reactions. In: *Bioactivation of Foreign Compounds* (Ed. Anders MW), pp. 375–402. Academic Press, New York, 1985.
7. Booth J, Acetyl transfer in arylamine metabolism. *Biochem J* **100**: 745–753, 1966.
8. 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.
9. Glowinski IB, Weber WW, Fysh JM, Vaught JB and King CM, Evidence that arylhydroxamic acid *N,O*-acyltransferase and the genetically polymorphic *N*-acetyltransferase are properties of the same enzyme in rabbit liver. *J Biol Chem* **255**: 7883–7890, 1980.
10. Mattano S, Land S, King CM and Weber WW, Purification of hepatic arylamine *N*-acetyltransferase from rapid and slow acetylator mice: Identity with arylhydroxamic acid *N,O*-acyltransferase. In: *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes, Proceedings of the Third International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, 25–28 April, 1987* (Eds. King CM, Romano LJ and Schuetzle D), pp. 155–159. Elsevier, New York, 1988.
11. Hein DW, Flammang TJ, Kirlin WG, Trinidad A and Ogalla F, Acetylator genotype-dependent metabolic activation of carcinogenic *N*-hydroxyarylamines by *S*-acetyl coenzyme A-dependent enzymes of inbred hamster tissue cytosols: Relationship to arylamine *N*-acetyltransferase. *Carcinogenesis* **8**: 1767–1774, 1987.
12. 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.
13. Wick MJ and Hanna PE, Separation and characterization of rat hepatic *N*-acetyltransferases. *Fed Proc* **44**: 1115, 1985.
14. Marhevka VC, Ebner NA, Sehon RD and Hanna PE, Mechanism-based inactivation of *N*-arylhydroxamic acid *N,O*-acetyltransferase by 7-substituted-*N*-hydroxy-2 acetamidofluorenes. *J Med Chem* **28**: 18–24, 1985.
15. Wheeler GP and Ingersoll AW, The resolution of amino acids. III. Methionine. *J Am Chem Soc* **73**: 4604–4606, 1951.
16. Gelsema WJ, Deligny 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.
17. Lowe CR, An introduction to affinity chromatography. In: *Laboratory Techniques in Biochemistry and Molecular Biology* (Eds. Work TS and Work E), Vol. 7, Part II, pp. 395–399. North Holland Publishing, New York, 1979.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
19. Weber WW, *N*-Acetyltransferases (mammalian liver). *Methods Enzymol* **17B**: 805–811, 1971.
20. Hein DW, Omichinski JG, Brewer JA and Weber WW, A unique pharmacogenetic expression of the N-acetylation polymorphism in the inbred hamster. *J Pharmacol Exp Ther* **220**: 8–15, 1982.
21. Elfarra AA and Hanna PE, Substituent effects on the bioactivation of 2-(*N*-hydroxyacetamido)fluorenes by *N*-arylhyroxamic acid *N,O*-acyltransferase. *J Med Chem* **28**: 1453–1460, 1985.

22. Banks RB and Hanna PE, Arylhydroxamic acid *N,O*-acyltransferase: Apparent suicide inactivation by carcinogenic *N*-arylhydroxamic acids. *Biochem Biophys Res Commun* **91**: 1423–1429, 1979.
23. Wick MJ, Jantan IB and Hanna PE, Irreversible inhibition of rat hepatic transacetylase activity by *N*-arylhydroxamic acids. *Biochem Pharmacol* **37**: 1225–1231, 1988.
24. Smith TJ and Hanna PE, Hepatic *N*-acetyltransferases: Selective inactivation *in vivo* by a carcinogenic *N*-arylhydroxamic acid. *Biochem Pharmacol* **37**: 427–434, 1988.
25. King CM and Glowinski IB, Acetylation, deacetylation and acyltransfer. *Environ Health Perspect* **49**: 43–50, 1983.
26. Allaben WT and King CM, The purification of rat liver arylhydroxamic acid *N,O*-acyltransferase. *J Biol Chem* **259**: 12128–12134, 1984.
27. Weber WW and Hein DW, N-Acetylation pharmacogenetics. *Pharmacol Rev* **37**: 25–79, 1985.
28. Olive CW and King CM, Evidence for a second arylhydroxamic acid acyltransferase species in the small intestine of the rat. *Chem Biol Interact* **11**: 599–604, 1975.
29. Tannen RH and Weber WW, Rodent models of the human isoniazid-acetylator polymorphism. *Drug Metab Dispos* **7**: 274–279, 1979.
30. Wick MJ and Hanna PE, Differential inactivation of rat hepatic *N*-acetyltransferase activities by N-OH-AAF. *Fed Proc* **46**: 1138, 1987.
31. Land S, Yamada H, Wang CY, Zukowski K and King CM, Metabolism of aromatic amines: Relationship of O-acetylation, N,O-acetyltransfer and N-acetyltransfer in rat liver. *Proc Am Assoc Cancer Res* **28**: 113, 1987.
32. Saito K, Shinohara A, Kamataki T and Kato R, *N*-Hydroxyarylamine *O*-acetyltransferase in hamster liver: Identity with arylhydroxamic acid *N,O*-acetyltransferase. *J Biochem (Tokyo)* **99**: 1689–1697, 1986.
33. 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.
34. Bond JT, Contino PJ and Weber WW, The rat acetylation polymorphism: *In vivo* differences in drug acetylation. *Fed Proc* **46**: 1292, 1987.