Biochemical Pharmacology, Vol. 37, No. 7, pp. 1225–1231, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

IRREVERSIBLE INHIBITION OF RAT HEPATIC TRANSACETYLASE ACTIVITY BY *N*-ARYLHYDROXAMIC ACIDS

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

(*Received* 23 *March* 1987; *accepted* 10 *August* 1987)

Abstract—Both *N*-hydroxy-2-acetamidofluorene (*N*-OH-AAF) and the heterocyclic analogue, 2-(*N*-hydroxyacetamido)carbazole (*N*-OH-AAC), were shown to be mechanism-based irreversible inhibitors (suicide inhibitors) of partially purified rat hepatic *N*-acetyltransferase (NAT) activity. Although *N*-OH-AAC exhibited an apparent first-order inactivation rate constant (k_i) that was 7-fold lower than that of *N*-OH-AAF, their relative k_i/K_D values indicate that *N*-OH-AAC was the more potent and efficient inactivator of transacetylase activity. Inactivation of NAT activity by these *N*-arylhydroxamic acids appeared to involve contributions by electrophiles that react with the enzyme subsequent to release from the active site and by electrophiles that remain complexed with the active site. The irreversible nature of the enzyme inactivation was demonstrated by the failure to recover transacetylase activity upon either extensive dialysis or gel filtration of preparations that had been subjected to incubation with *N*-OH-AAF or *N*-OH-AAC. The use of the nucleophile *N*-acetylmethionine to trap the electrophilic reactants formed in the transacetylase-catalyzed bioactivation process resulted in a lower rate and extent of formation of methylthio adducts with *N*-OH-AAC than with *N*-OH-AAF. The results of this study indicate that *N*-OH-AAF and *N*-OH-AAC have potential for use as tools in the investigation of rat hepatic transacetylases.

N-Arylhydroxamic acid *N,O*-acyltransferase (AHAT)§ is a cytosolic mammalian enzyme that catalyzes the conversion of a variety of arylhydroxamic acids, including several carcinogens, to reactive electrophiles [1–3]. The ability of AHAT to transform carcinogenic arylhydroxamic acids into reactants that become covalently bound to biological macromolecules and its identity with certain acetyl coenzyme A (CoASAc) dependent arylamine *N*-acetyltransferases (NAT, EC 2.3.1.5) have stimulated investigations of its distribution, biochemical properties, and substrate specificity [4–8].

The sequence of events involved in the bioactivation of arylhydroxamic acids by AHAT *in vitro* is illustrated in Fig. 1, pathway b. Studies conducted in this laboratory with partially purified hamster hepatic AHAT demonstrated that the generation of electrophiles during the bioactivation of arylhydroxamic acids is accompanied by irreversible inactivation of *N*-hydroxy-2-acetamidofluorene:4-aminoazobenzene (*N*-OH-AAF:AAB) transacetylase activity (Fig. 1, pathway a) [9, 10]. A rat liver preparation that had been partially purified by ammonium sulfate precipitation in a similar fashion, however, underwent little inactivation of *N*-OH-AAF:AAB transacetylase activity upon incubation with arylhydroxamic acids [9].

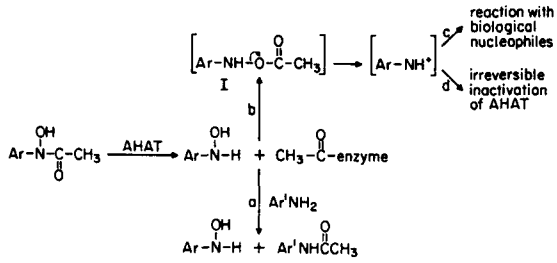

Fig. 1. Transacetylation (a) and bioactivation reactions (b) catalyzed by *N*-arylhydroxamic acid *N,O*-acyltransferase (AHAT).

Kinetic analysis of the inactivation of hamster hepatic transacetylase activity showed that *N*-OH-AAF (Fig. 2) is a mechanism-based inhibitor (suicide inhibitor) [9, 10]. Subsequently, a quantitative structure–activity investigation revealed that those arylhydroxamic acids whose physicochemical properties favor the formation of a positively charged inter-

‡ To whom correspondence should be addressed.

§ Abbreviations: AHAT, *N*-arylhydroxamic acid *N,O*-acyltransferase; CoASAc, acetyl coenzyme A; NAT, *N*-acetyltransferase; *N*-OH-AAF, *N*-hydroxy-2-acetamidofluorene; AAB, 4-aminoazobenzene; *N*-OH-AAC, 2-(*N*-hydroxyacetamido)carbazole; DTT, dithiothreitol; and DMSO, dimethyl sulfoxide.

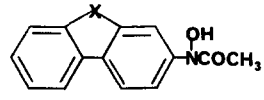

Fig. 2. Structures of *N*-OH-AAF (X = CH_2) and *N*-OH-AAC (X = NH).

mediate (resonance-stabilized nitrenium ion) upon bioactivation exhibit the highest rates of enzyme inactivation [11]. In contrast, the formation of methylthio adducts as a result of the reaction of the bioactivated arylhydroxamic acids with *N*-acetylmethionine is not facilitated by the same physicochemical properties that enhance the formation of positively charged intermediates [12].

Because of the demonstrated usefulness of mechanism-based inhibitors (suicide inhibitors) as molecular probes for the study of transacetylases [10, 13], and because of the importance of rat tissues as sources of enzyme activities for studies of bioactivation processes [1, 2, 4, 5], a further examination of the possibility that *N*-OH-AAF would function as an inactivator of a partially purified rat hepatic preparation of AHAT was undertaken. Additionally, experiments were conducted with a new carbazole analog (*N*-OH-AAC, Fig. 2) of *N*-OH-AAF in order to gain information regarding structural influences on the inactivation process and on the reaction of the bioactivated forms of arylhydroxamic acids with *N*-acetylmethionine. The results presented herein provide conclusive evidence that *N*-OH-AAF is a mechanism-based inhibitor of rat hepatic transacetylase activity and demonstrate significant differences in the behavior of *N*-OH-AAF and *N*-OH-AAC upon bioactivation by AHAT.

EXPERIMENTAL PROCEDURES

Materials and methods. *N*-OH-AAF was prepared as described previously [11]. All chemical reagents were of the highest grade available. 2-Nitrobiphenyl, 2-nitrofluorene and acetyl chloride were purchased from the Aldrich Chemical Co., Milwaukee, WI. AAB was purchased from the Eastman Kodak Co., Rochester, NY. The following reagents were purchased from the Sigma Chemical Co., St. Louis, MO: dithiothreitol (DTT), cysteine, bovine serum albumin, grade III NAD, Sephadex G-100, cellulose dialysis tubing (10 in. × 0.62 in.) and L-methionine. PD-10 Columns packed with Sephadex G-25M were purchased from Pharmacia, Piscataway, NJ; the columns were washed with 50 ml of elution buffer prior to use. The dialysis tubing was rinsed in distilled water and in 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT) prior to use. *N*-Acetyl-L-[^{14}C-CH_3]methionine (0.2 to 0.3 mCi/mmol) was prepared from L-[^{14}C-CH_3]methionine and acetic anhydride according to the procedure of Wheeler and Ingersoll [14]. Protein concentrations were measured by the method of Lowry *et al.* [15] with bovine serum albumin as the standard. NMR spectra were obtained with a JEOL Fx-90Q spectrometer, and mass spectral (MS) data were obtained with an Associated Electronic Industries MS-30 (electron impact) in the University of Minnesota Mass Spectrometry Laboratory, Department of Chemistry; samples were introduced by direct inlet. Analytical TLC was carried out with Eastman plastic backed plates (13181 silica gel with fluorescent indicator, No. 6060); hydroxamic acids were visualized with UV light and with a 2.5% $FeCl_3$ in 0.5 N HCl spray reagent. Elemental analyses were performed by Midwest Microlab, Indianapolis, IN. All enzymatic reactions were performed at 37° in air with a Dubnoff metabolic shaking incubator; enzymatic activities were measured with a Beckman model 34 spectrophotometer.

Synthesis of 2-(N-hydroxyacetamido)carbazole [N-9H-*carbazol-2-yl*-N-*hydroxyacetamide*]. The method of Westra [16] was used. A solution of 2-nitrocarbazole [17] (2.0 g, 0.009 mol) in tetrahydrofuran (460 ml) was cooled to −10° under a nitrogen atmosphere. Palladium (5% on carbon, 2.4 g) was added to the solution. Hydrazine hydrate (85%, 5 ml) was added slowly to ensure that the temperature did not rise above −5°. After 2.5 to 3 hr, TLC (ethanol) of the reaction mixture indicated that the starting material was no longer present. Triethylamine (2.8 ml) was added. Then, a solution of freshly distilled acetyl chloride (13.8 ml, 0.19 mol) in tetrahydrofuran (50 ml) was added at such a rate that the temperature did not rise above 0°. The reaction mixture was allowed to warm to room temperature, and the reaction was monitored by TLC (ethyl acetate). After the reaction was complete (2 hr), the mixture was filtered and the solids were washed with diethyl ether. A saturated sodium bicarbonate solution (180 ml) was added to the filtrate, and the mixture was stirred for 30 min. The organic layer was separated and was extracted three times with 50-ml portions of 1% NaOH. The basic solution was acidified (pH <3) with concentrated HCl and was extracted with 50-ml portions of ether which were combined and dried ($MgSO_4$). Evaporation of the solvent afforded the crude hydroxamic acid, which, after six recrystallizations from ethyl acetate, yielded 0.48 g (22%) of the desired product, m.p. 188–190°. MS analysis (70 eV): *m/e*, 240 $(M)^+$, 224 $(M—O)^+$. ^{1}H-NMR (Me_2SO-d_6): δ 11.23 (S, 1H), 10.64 (S, 1H), 7.64 (m, 7H), 2.23 (S, 3H). Elemental analysis: calc. ($C_{14}H_{12}N_2O_2$): C, 69.99; H, 5.03; N, 11.66. Found: C, 70.07; H, 4.93; N, 11.47.

Enzyme preparation. Male Sprague–Dawley rats (175–200 g) were obtained from Bio-Lab (White Bear Lake, MN). The *N*-acetyltransferase activities were purified 5-fold from hepatic cytosol by the following procedures. The rats were lightly anesthetized with ether prior to decapitation. Livers were removed immediately, were rinsed with cold 0.05 M sodium pyrophosphate buffer (pH 7, 1 mM DTT), and were minced and homogenized in 1 ml of buffer per gram of liver with a motor-driven glass and Teflon homogenizer. The homogenate was centrifuged at 105,000 *g* (7°) for 60 min. A 5- to 6-fold purification of the arylhydroxamic acid:AAB transacetylation activity in the 105,000 *g* supernatant fraction was achieved by gel filtration of an ammonium sulfate precipitate that was prepared as described by King [2]. The following procedures were performed at 4°. The ammonium sulfate pellets from the 45–65% fraction were dissolved in a sufficient amount of 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the protein concentration to approximately 50 mg/ml. Five to six millilitres of this protein solution was applied to a 2.5 × 45 cm Sephadex G-100 column that had been equilibrated with degassed 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DDT. Following sample application, the column was eluted with 200 ml of the buffer at a flow rate of approxi-

mately 30 ml/hr; 12-ml fractions were collected. A 1.0-ml aliquot from each fraction was analyzed for N-OH-AAF:AAB transacetylation activity. The fractions containing the peak transacetylation activity were combined and concentrated to approximately 30% of the original volume in an Amicon model 52 ultrafiltration cell with a PM10 membrane under a nitrogen atmosphere. To the concentrated NAT activity was added sufficient glycerol to achieve a 30% concentration. This preparation could be stored for several weeks at −70° without loss of N-acetyltransferase activities.

Aminoazobenzene transacetylation assays. Incubation flasks (25 ml) contained either 0.3 to 0.5 ml of the partially purified enzyme preparation (2.5 mg protein) or 1.0 ml of column chromatography eluate, 0.05 ml of substrate solution and enough 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the final volume to 2.5 ml. The substrate solutions contained both AAB and the N-arylhydroxamic acid dissolved in either 95% ETOH (N-OH-AAF) or DMSO/methoxyethanol (1:1) (N-OH-AAC). The final concentrations of N-OH-AAF and N-OH-AAC were 1.0 mM. The final concentration of AAB was 0.15 mM. Reactions were initiated by the addition of substrate and were carried out over a 4-min (N-OH-AAF) or 6-min (N-OH-AAC) period. The reaction times were determined from the linear portions of graphs of the AAB transacetylation rate vs time. Hydroxamic acids were omitted from incubation mixtures that were used as reference standards. Reactions were terminated by the addition of 2.5 ml of cold 20% trichloroacetic acid (in ethanol/water, 1:1) and, after centrifugation to remove the precipitated protein, transacetylation rates were measured spectrophotometrically as described by Booth [18].

Inactivation of rat hepatic transacetylation activity. The incubation mixtures contained 0.3 to 0.5 ml of partially purified enzyme preparation (2.5 mg protein), arylhydroxamic acid dissolved in either 0.05 ml of 95% EtOH (N-OH-AAF) or 0.05 ml of DMSO/methoxyethanol, 1:1 (N-OH-AAC), and sufficient 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7) to give a final volume of 2.45 ml. The final concentrations of the arylhydroxamic acids were 2–5 μM (N-OH-AAF) and 0.5–5 μM (N-OH-AAC). Control flasks contained 0.05 ml of the appropriate solvent instead of the arylhydroxamic acid solution. The incubations were started by the addition of the arylhydroxamic acid solution and were continued for 1–8 min. At the end of the incubation period, a substrate solution containing AAB and an arylhydroxamic acid was added in order to measure the amount of remaining transacetylation activity. The substrate solution contained AAB and the same arylhydroxamic acid that had been used in the incubation. The volume, solvents, and concentrations of the substrate solutions, and the method of measurement of the transacetylation rates are described under "Aminoazobenzene transacetylation assays."

Kinetic constants. Semilog plots of percent remaining transacetylation activity versus incubation time were generated for at least four concentrations of each arylhydroxamic acid, and k_{obs} values were calculated from the slopes of the apparent first-order lines. The kinetic parameters, k_i and K_D, were determined from the vertical and horizontal intercepts, respectively, of the double-reciprocal plots of k_{obs} vs arylhydroxamic acid concentration according to the method of Kitz and Wilson [19]. All plots were determined by least squares linear regression analysis. The correlation coefficients for the fit of the points to the lines generated in the double-reciprocal plots were 0.97 (N-OH-AAF) and 0.98 (N-OH-AAC).

Inactivation and dialysis of enzyme. Incubation mixtures contained 1.5 to 2.5 ml of the partially purified and concentrated enzyme preparation to which glycerol had been added (see "Enzyme preparation"). The arylhydroxamic acids were dissolved in either 0.20 ml of 95% ethanol (N-OH-AAF) or 0.32 ml of DMSO/methoxyethanol (1:1) (N-OH-AAC). A sufficient volume of sodium pyrophosphate buffer (0.05 M, pH 7, 1 mM DTT) was added to give a final volume of 10 ml for experiments with N-OH-AAF and 16 ml for experiments with N-OH-AAC. The final concentration of N-OH-AAF was 0.0175 mM and the final concentration of N-OH-AAC was 0.25 mM. Protein concentrations were 1.75 mg/ml in the experiments with N-OH-AAF and 1.0 mg/ml in the experiments with N-OH-AAC; incubation time was 15 min for both compounds. At the end of the incubation period, 1.429- and 2.45-ml samples (2.5 mg protein) were removed for analysis of the N-OH-AAF:AAB and the N-OH-AAC:AAB transacetylation activities respectively. The remaining incubation mixture was dialyzed for 4 hr with two 300–500 ml portions of cold sodium pyrophosphate buffer (0.05 M, pH 7, 1 mM DDT) containing 3–7% glycerol and either 2% ethanol (N-OH-AAF) or 2% DMSO/methoxyethanol, 1:1 (N-OH-AAC). Nitrogen was passed through the buffer during the dialysis period. At the end of the dialysis period, samples of the dialysate were removed and assayed for transacetylation activity as described under "Aminoazobenzene transacetylation assays."

Inactivation and gel filtration of enzyme. Incubation mixtures contained sodium pyrophosphate buffer (0.05 M, pH 7, 1 mM DTT), 0.058 ml of arylhydroxamic acid dissolved in ethanol or DMSO/methoxyethanol (1:1), and sufficient partially purified enzyme preparation to give a protein concentration of 1.75 mg/ml in a final volume of 2.9 ml. The final concentrations of N-OH-AAF and N-OH-AAC were the same as in the dialysis experiments (0.0175 and 0.25 mM respectively). Incubation time was 15 min. At the end of the incubation period, 0.286-ml samples (0.5 mg protein) were removed for analysis of the N-OH-AAF:AAB and N-OH-AAC:AAB transacetylation activities. A 1.75-ml portion of the remaining incubation mixture was applied to a PD-10 column prepacked with Sephadex G-25M. The column was then eluted with two portions (1.2 and 1.8 ml) of buffer [0.05 M sodium pyrophosphate, 1 mM DTT, pH 7, 7.5% glycerol, and 2% ethanol or DMSO/methoxyethanol (1:1)]. The majority of the arylhydroxamic acid:AAB transacetylation activity was collected during the second elution (1.8 ml). Protein concentrations were determined and adjusted to 1.0 mg/ml for assay of N-

Table 1. 4-Aminoazobenzene transacetylation rates, kinetic constants for arylhydroxamic acid:AAB transacetylase inactivation, and rates of methylthio adduct formation

	AAB transacetylation rate* [nmol·(mg protein)$^{-1}$·min^{-1}]	k_i† (min^{-1})	K_D‡ (μM)	k_i/K_D (sec^{-1} M^{-1})	Methylthio adduct formation rate§ [nmol·(mg protein)$^{-1}$·(30 min)$^{-1}$]
N-OH-AAF	10.7 ± 0.2	2.33	115.6	335	9.1
N-OH-AAC	4.6 ± 0.4	0.32	0.7	7571	0.5

* Activities are expressed as AAB transacetylation rates (mean ± SD, N = 4).
† Apparent limiting first-order rate constant for inactivation of AAB transacetylation activity.
‡ Apparent dissociation constant for inactivation of AAB transacetylation activity.
§ Activity is expressed as the mean of the rate of methylthio adduct formation catalyzed by the partially purified rat hepatic enzyme (N = 2).

OH-AAF:AAB and *N*-OH-AAC:AAB activities as described under "Aminoazobenzene transacetylation assays."

Electrophile generation assay. The production of electrophiles was measured by the method of Bartsch *et al.* [1]. Incubation flasks contained sodium phosphate buffer (41 μmol, pH 6.8), 0.8 μmol NAD^+, 10 μmol *N*-acetyl-L-[^{14}C-CH_3]methionine (0.2 to 0.3 mCi/mmol), 1.0 mg of partially purified enzyme preparation, hydroxamic acid dissolved in either 0.05 ml of 95% ethanol (*N*-OH-AAF) or 0.05 ml of DMSO/methoxyethanol, 1:1 (*N*-OH-AAC), and sufficient distilled water to give a final volume of 1 ml. The final concentrations of hydroxamic acids were 0.075 mM (*N*-OH-AAF) and 0.25 mM (*N*-OH-AAC). A 2-min temperature equilibration period was initiated by addition of the enzyme solution to 25-ml Erlenmeyer flasks, containing the buffer, NAD^+, *N*-acetylmethionine and distilled water in a 37° shaker bath. Reactions were started by addition of substrates and were carried out in air at 37° for 30 min. At the end of the incubation period, the flasks were placed on ice and 2.5 ml of Et_2O was added. Within 2–5 min of terminating the reaction, the contents of the flasks were transferred to test tubes and mixed thoroughly with a vortex mixer. The test tubes were then immediately immersed in a dry ice–acetone bath until the aqueous layer was frozen. The ether layer was decanted and the aqueous layer was allowed to melt and then was placed in a 80–90° water bath for 45 min. The aqueous layer was extracted with benzene–heptane (5 ml, 3:7). The organic extract was washed two times with 2 ml of distilled water and was dried for 1 hr ($MgSO_4$). One milliliter of the organic extract was placed in a scintillation counting vial containing 10 ml of Econofluor scintillation fluid. The disintegrations per minute of ^{14}C present in each sample were counted in a Beckman LS 250 scintillation counter for 20 min (pre set error = 0.02%). Control experiments were carried out with heat-denatured enzyme, and all results were adjusted for any nonenzymatic adduct formation.

RESULTS

Previous investigations have shown that gel filtration chromatography of rat hepatic cytosol [1], the 45–65% ammonium sulfate fraction of rat hepatic cytosol [2], or the 35–50% ammonium sulfate precipitate obtained from hamster hepatic cytosol [13] results in the coelution of AHAT and *N*-OH-AAF:AAB transacetylase activities. Also, in the present study, the two activities coeluted when an ammonium sulfate precipitate obtained from rat hepatic cytosol was subjected to gel filtration on a Sephadex G-100 column. The addition of glycerol to the product obtained by gel filtration enhanced the stability of the preparation.

The *N*-OH-AAF:AAB and *N*-OH-AAC:AAB transacetylation rates were proportional to protein concentration up to at least 1 mg protein/ml of incubation mixture and were linear with time for at least 4 min with *N*-OH-AAF and for at least 6 min with *N*-OH-AAC (data not presented). The AAB transacetylation rate for *N*-OH-AAF was approximately twice that of *N*-OH-AAC (Table 1). The relatively small difference in transacetylation rates indicates that the structural differences between *N*-OH-AAF and *N*-OH-AAC have little influence on their abilities to function as acetyl donors and is consistent with previous results obtained with analogues of *N*-OH-AAF and hamster hepatic transacetylases [11].

Inactivation of rat hepatic AAB transacetylation activity by hydroxamic acids. Earlier attempts to demonstrate the inactivation of rat hepatic transacetylase activity by arylhydroxamic acids were conducted with an enzyme preparation that was obtained by ammonium sulfate precipitation [9]. Because it was believed that a more purified enzyme preparation might be more susceptible to this type of inactivation, the ammonium sulfate fraction was purified further by gel filtration. Incubation of either *N*-OH-AAF or *N*-OH-AAC with the partially purified rat liver preparation resulted in a time-dependent loss of AAB transacetylation activity. The inactivation process with each hydroxamic acid exhibited apparent first-order kinetics, as indicated by the linear semilog plots that were obtained when the percent remaining activity was plotted versus incubation time; the process was saturable at higher concentrations of the hydroxamic acids. First-order inactivation rate constants (k_{obs}) were determined for several concentrations of each hydroxamic acid, and limiting rates of inactivation (k_i) and apparent dissociation constants (K_D) were obtained from double-reciprocal plots of k_{obs} versus inhibitor concentration. The k_i and K_D values for *N*-OH-AAF and *N*-OH-AAC are presented in Table 1. These data indicate that *N*-OH-AAF and *N*-OH-AAC are

Table 2. Effect of cysteine on the rate of inactivation of transacetylation by hydroxamic acids

Inhibitor	Cysteine (10 mM)	k_{obs}* $(min)^{-1}$	r†
N-OH-AAF	–	0.13	0.99
(0.075 mM)	+	0.07	0.99
N-OH-AAC	–	0.10	0.99
(0.25 mM)	+	0.06	0.99

* Pseudo first-order rate constants for the inactivation of AAB transacetylation activity at the indicated concentrations of inhibitors in the presence and absence of the nucleophile cysteine.

† Correlation coefficients for the fit of the points to the lines generated in the semilog plots of percent remaining AAB transacetylation activity versus time.

mechanism-based inhibitors of rat hepatic AAB transacetylase activity.

In previous studies with hamster hepatic enzyme preparations, it was shown that the inclusion of nucleophiles in the incubation mixture reduced the rate of transacetylase inactivation by N-OH-AAF and related hydroxamic acids [5, 10, 11] The reduction in the rate of inactivation is attributable to the ability of the nucleophiles to scavenge those electrophilic reactants that are released from the enzyme active site subsequent to bioactivation, thereby preventing the released electrophiles from participating in the enzyme inactivation process. In the present study, the presence of cysteine in the incubation mixture with N-OH-AAF and the partially purified transacetylase preparation caused a reduction of approximately 50% in the apparent first-order inactivation rate constant (Table 2). Similarly, the k_{obs} for N-OH-AAC was lowered by cysteine. The effect of cysteine on the inactivation rates suggests that the inactivation process includes contributions both from reactive intermediates that remain complexed with the enzyme active site and from reactive intermediates that are released from the active site prior to reaction with the enzyme.

The irreversible nature of the inactivation of the AAB transacetylation activities was established both by dialysis and by gel filtration of samples of the enzyme preparations that had been incubated with either N-OH-AAF or N-OH-AAC. Neither extensive dialysis nor gel filtration of the inactivated enzyme preparations resulted in the recovery of AAB transacetylase activity (Tables 3 and 4).

Reaction of bioactivated hydroxamic acids with N-*acetylmethionine.* The relative abilities of N-OH-AAF and N-OH-AAC to undergo AHAT-catalyzed conversion to electrophilic species that react with N-acetylmethionine were determined according to the method of Bartsch *et al.* [1]. The arylhydroxamic acids were incubated with the partially purified rat hepatic preparation in the presence of radiolabeled N-acetylmethionine. The methylthio adducts were extracted and quantified by liquid scintillation counting, as described under Experimental Procedures. The rate of formation of methylthio adducts subsequent to the bioactivation of N-OH-AAF was 18-fold greater than that which occurred with N-OH-AAC (Table 1). Thus, the replacement of the CH_2 in the 9-position of the fluorene ring of N-OH-AAF with NH not only results in a reduction of the apparent inactivation rate constant, k_i, but also decreases the extent of methylthio adduct formation.

DISCUSSION

One objective of this study was to determine whether or not N-OH-AAF is a mechanism-based inactivator (suicide inhibitor) of rat hepatic transacetylase activity. Previously it was shown that N-OH-AAF was an efficient irreversible inactivator of hamster hepatic transacetylase activities that had been purified 2- to 3-fold by ammonium sulfate precipitation, but that a similarly purified rat liver preparation was resistant to inactivation by N-OH-AAF [9, 10]. In the present study, passage of the rat liver preparation that was obtained by ammonium sulfate fractionation through a gel filtration column and stabilization of the latter product with glycerol resulted in a transacetylase preparation that was readily susceptible to inactivation by N-OH-AAF.

Since N-OH-AAF lacks inherent electrophilic properties, it must undergo activation prior to causing irreversible inhibition of N-OH-AAF:AAB transacetylation activity. Such activation may be catalyzed either by the transacetylase that undergoes inactivation or by another enzyme that is present in the preparation. The inclusion of a nucleophile, such

Table 3. Effect of dialysis on rat hepatic transacetylation activity following inactivation with hydroxamic acids

	Transacetylation rate* [nmol·(mg protein)$^{-1}$·min^{-1}]			
	Before dialysis		After dialysis	
Inhibitor	Control	Inhibited	Control	Inhibited
N-OH-AAF	10.5, 10.9	0.68, 0.88	10.4, 11.6	0.31, 0.51
N-OH-AAC	4.5, 4.5	0.09, 0.29	4.2, 4.8	0.22, 0.82

* Activities are expressed as the AAB transacetylation rates (N = 2) following incubation of the partially purified rat hepatic preparation in the absence (control) or presence (inhibited) of either N-OH-AAF or N-OH-AAC, as described under Experimental Procedures.

Table 4. Effect of gel filtration on rat hepatic transacetylation activity following inactivation with hydroxamic acids

	Transacetylation rate* [nmol·(mg protein)$^{-1}$·min^{-1}]			
	Before gel filtration		After gel filtration	
Inhibitor	Control	Inhibited	Control	Inhibited
N-OH-AAF	12.58, 12.62	2.61, 3.41	13.3, 13.5	3.2, 4.0
N-OH-AAC	5.68, 5.72	0.06, 0.46	6.0, 6.8	0.6, 0.8

* Activities are expressed as the AAB transacetylation rates (N = 2) following incubation of the partially purified rat hepatic preparation in the absence (control) or presence (inhibited) of either *N*-OH-AAF or *N*-OH-AAC, as described under Experimental Procedures.

as cysteine, in the incubation mixture is useful for determining whether or not electrophiles that are released from the active site of the bioactivating enzyme contribute to the inactivation process. If cysteine either prevents the inactivation or retards the inactivation rate, it can be concluded that at least a portion of the electrophiles that participate in the inactivation are being trapped by cysteine and are thereby prevented from participating in the inactivation process. The presence of cysteine caused a significant reduction in the pseudo first-order rate of inactivation of rat hepatic transacetylase activity by *N*-OH-AAF, but the inactivation was not prevented completely (Table 2). That the effect of cysteine was due to its nucleophilic properties, rather than to its ability to prevent oxidation of critical sulfhydryl groups on the enzyme, is indicated by the extensive inactivation caused by the hydroxamic acids in the presence of the reducing agent, DTT, which was included in all incubation mixtures. Also, methionine, which is a nucleophile that does not have reducing properties, provides substantial protection of the enzyme from inactivation by *N*-OH-AAF [20]. These results indicate that there is a contribution to the inactivation process both by electrophiles that are released from the active site of the bioactivating enzyme (AHAT) and by electrophiles that remain complexed with the enzyme active site. Thus, both the pseudo first-order kinetic properties of the *N*-OH-AAF-mediated inactivation of *N*-OH-AAF:AAB transacetylation activity and the results of the experiments with cysteine are consistent with the involvement of a mechanism-based inhibition process, and the results are analogous to those obtained with the partially purified hamster hepatic preparation [10]. Additionally, the results support the previously suggested association of *N*-OH-AAF:AAB transacetylation activity with AHAT activity in rat liver [1, 2]. The results, however, do not strictly preclude the possibility that the portion of electrophiles that are released into the medium prior to their reaction with the transacetylase enzyme may be produced, at least in part, by an *N,O*-acyltransferase other than that which catalyzes the *N*-OH-AAF:AAB transacetylation reaction.

Previous experiments with a series of 7-substituted-2-(*N*-hydroxyacetamido)fluorenes provided evidence that electronic effects of the substituents play a significant role in determining the rate of inactivation of hamster hepatic transacetylases and in the extent of reaction of the bioactivated compounds with *N*-acetylmethionine [11, 12]. In the present work, *N*-OH-AAC was selected for study because its steric properties are virtually identical to those of *N*-OH-AAF and because the presence of the electron-donating 9-NH group in the position meta to the hydroxamic acid substituent would be expected to facilitate the formation of the positively charged intermediates believed to be generated in AHAT-catalyzed bioactivation reactions (Fig. 1, pathway b). In addition, the interaction of *N*-OH-AAC with mammalian bioactivation enzymes is of interest because the compound is a potential active metabolite of 2-aminocarbazole, a mutagenic component of tobacco [21].

Both *N*-OH-AAC and *N*-OH-AAF served as acetyl donors in the AAB transacetylation reaction (Table 1). In the previous study with the hamster liver enzyme preparation, it was shown that the steric bulk of substituents in the 7-position of *N*-OH-AAF influenced the ability of the compounds to function as acetyl donors, but that the electronic or lipophilic properties of the molecules had little influence on the rate of the transacetylation reactions [11]. Similarly, the differences in the electronic and lipophilic characteristics of *N*-OH-AAF and *N*-OH-AAC caused little difference in their abilities to serve as acetyl donors in the transacetylase reaction catalyzed by the partially purified rat liver preparation (Table 1). In contrast, there were more substantial differences in the effectiveness of the two compounds as inactivators of rat hepatic AAB transacetylase activity and in the extent of the formation of methylthio adducts subsequent to bioactivation in the presence of radiolabeled *N*-acetylmethionine. Whereas the presence of electron donating substituents in the 7-position of *N*-OH-AAF enhanced the apparent first-order rate of inactivation of hamster hepatic transacetylase activity [11], the replacement of the 9-CH_2 of *N*-OH-AAF with the stronger electron releasing 9-NH group caused a 7-fold reduction in the k_i value obtained with the rat liver preparation (Table 1). There was a much greater difference in the apparent dissociation constants (K_D) of the two hydroxamic acids, with that of *N*-OH-AAC being 165 times smaller than that of *N*-OH-AAF. Because

the experiments were conducted with a partially purified enzyme preparation, it cannot be stated with certainty that the differences in the K_D values are due exclusively to differences in the interaction of the two hydroxamic acids with the active site of the enzyme. However, the bimolecular rate constants for the inactivation process, as reflected by the k_i/K_D ratios (Table 1), clearly illustrate that, under the conditions of these experiments, *N*-OH-AAC functions as a more potent and efficient inactivator of rat hepatic transacetylase activity than does *N*-OH-AAF.

The extent of formation of methylthio adducts upon the reaction of the bioactivated arylhydroxamic acids with *N*-acetylmethionine was 18-fold greater with *N*-OH-AAF than with *N*-OH-AAC (Table 1). The very low rate of methylthio adduct formation exhibited by *N*-OH-AAC is consistent with the results of the previous study in which it was observed that arylhydroxamic acids in which the physicochemical properties favor the formation of positively charged intermediates react to a very limited extent with *N*-acetylmethionine after activation by AHAT [12]. The latter result may be due to a low level of electrophile generation, to the ineffectiveness of *N*-acetylmethionine as a scavenging agent for electrophiles produced from such compounds, or to a combination of these factors. It was observed previously that *N*-acetylmethionine was a much less effective trapping agent than the thiol, 2-mercaptoethanol, for the electrophilic reactants produced during the bioactivation of *N*-hydroxy-*N*-(4-cyclohexylphenyl)acetamide [3]. The significant decrease in the rate of transacetylase inactivation by *N*-OH-AAC in the presence of cysteine (Table 2) indicates that thiol compounds may be better scavenging agents than *N*-acetylmethionine for the bioactivated forms of this *N*-arylhydroxamic acid. Coles [22] has discussed the importance of the chemical and physicochemical properties of both bioactivated electrophiles and biological nucleophiles as determinants of reaction rate and selectivity.

The findings that *N*-OH-AAF and *N*-OH-AAC are mechanism-based inactivators of partially purified rat hepatic *N*-arylhydroxamic acid:AAB *N*-acetyltransferase activity, and that differences in the physiochemical properties of the two compounds influence their abilities to serve as substrates and inactivators of the enzyme, indicate their potential utility as molecular probes of rat hepatic transacetylase multiplicity [23]. Such studies are currently in progress.

REFERENCES

1. H. Bartsch, M. Dworkin, J. A. Miller and E. C. Miller, *Biochim. biophys. Acta* **286**, 272 (1972).
2. C. M. King, *Cancer Res.* **34**, 1503 (1974).
3. B. L. K. Mangold and P. E. Hanna, *J. med. Chem.* **25**, 630 (1982).
4. C. M. King and I. B. Glowinski, *Environ. Hlth Perspect.* **49**, 43 (1983).
5. P. E. Hanna and R. B. Banks, in *Bioactivation of Foreign Compounds* (Ed. M. W. Anders), p. 375. Academic Press, New York (1985).
6. I. B. Glowinski, W. W. Weber, J. M. Fysh, J. B. Vaught and C. M. King, *J. biol. Chem.* **255**, 7883 (1980).
7. W. T. Allaben and C. M. King, *J. biol. Chem.* **259**, 12128 (1984).
8. K. Saito, A. Shinohara, T. Kamataki and R. Kato, *J. Biochem., Tokyo* **99**, 1689 (1986).
9. R. B. Banks and P. E. Hanna, *Biochem. biophys. Res. Commun.* **91**, 1423 (1979).
10. P. E. Hanna, R. B. Banks and V. C. Marhevka, *Molec. Pharmac.* **21**, 159 (1982).
11. V. C. Marhevka, N. A. Ebner, R. D. Sehon and P. E. Hanna, *J. med. Chem.* **28**, 18 (1985).
12. A. A. Elfarra and P. E. Hanna, *J. med. Chem.* **28**, 1453 (1985).
13. T. J. Smith and P. E. Hanna, *Carcinogenesis* **7**, 697 (1986).
14. G. Wheeler and A. Ingersoll, *J. Am. chem. Soc.* **73**, 4604 (1951).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. J. G. Westra, *Carcinogenesis* **2**, 355 (1981).
17. G. D. Mendenhall and P. A. S. Smith, *Org. Synth. Coll.* **5**, 829 (1973).
18. J. Booth, *Biochem. J.* **100**, 745 (1966).
19. R. J. Kitz and I. B. Wilson, *J. biol. Chem.* **237**, 3245 (1962).
20. M. J. Wick and P. E. Hanna, *Fedn Proc.* **46**, 1138 (1987).
21. E. J. LaVoie, A. Govil, G. Briggs and D. Hoffman, *Mutation Res.* **90**, 337 (1981).
22. B. Coles, *Drug Metab. Rev.* **15**, 1307 (1984–85).
23. M. J. Wick and P. E. Hanna, *Fedn Proc.* **44**, 1115 (1985).