Arylhydroxamic Acid *N*,*O*-Acyltransferase Substrates. Acetyl Transfer and Electrophile Generating Activity of *N*-Hydroxy-*N*-(4-alkyl-, 4-alkenyl-, and 4-cyclohexylphenyl)acetamides

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Arylhydroxamic acid *N*,*O*-acyltransferase (AHAT) is an enzyme system that is capable of converting many *N*-arylhydroxamic acids into reactive electrophilic species. As part of an investigation into the influence of the structure of the aryl group upon the ability of *N*-arylhydroxamic acids to serve as substrates for AHAT, a series of *N*-hydroxy-*N*-(4-alkyl-, 4-alkenyl-, and 4-cyclohexylphenyl)acetamides was prepared and evaluated in vitro with partially purified rat and hamster hepatic AHAT. The nature of the 4-substituent markedly influenced the ability of the hydroxamic acids to serve as acetyl donors in the AHAT-catalyzed transacetylation of 4-aminoazobenzene (AAB). As the length of the 4-substituent was increased from methyl to pentyl, the compounds became increasingly more effective substrates. The compounds containing vinyl, propenyl, and 2-methylpropenyl 4-substituents were more effective acetyl donors than the corresponding compounds containing saturated 4-substituents. The three most effective AHAT substrates in the AAB transacetylation assay were *N*-hydroxy-*N*-(4-pentylphenyl)- (**7**), *N*-hydroxy-*N*-(4-propenylphenyl)- (**10**), and *N*-hydroxy-*N*-[4-(2-methylpropenyl)phenyl]acetamide (**11**), each of which was approximately as active as the standard compound, *N*-hydroxy-4-acetamidobiphenyl (**1**), with rat hepatic AHAT and approximately 60% as active as **1** with hamster hepatic AHAT. Both **1** and *N*-hydroxy-*N*-(4-cyclohexylphenyl)acetamide (**8**) were activated by hamster hepatic AHAT to yield electrophilic intermediates that formed adducts with 2-mercaptoethanol. The 2-mercaptoethanol adducts were characterized by mass spectrometry and were identified as 4-phenyl-2-[(2-hydroxyethyl)thio]aniline (**22**) and 4-cyclohexyl-2-[(2-hydroxyethyl)thio]aniline (**21**). The structure of compounds **21** and **22** were confirmed by an unambiguous chemical synthesis. Both compounds **1** and **8** irreversibly inactivated hamster hepatic AHAT by a time-dependent process. The results of the inactivation experiments confirmed that **1** inactivates AHAT primarily via a suicide substrate mechanism and revealed that **8** inactivates the enzyme by a process consisting primarily of a pathway in which electrophiles are released into the medium and subsequently react with nucleophiles present on AHAT.

Carcinogenic aryl amides are N-hydroxylated to form arylhydroxamic acids in vivo and in vitro by the polysubstrate monooxygenases (P-450). N-Hydroxylation is an essential step in the sequence of enzymatic transformations of aryl amides to their ultimate carcinogenic forms, which are postulated to be electrophilic reactants. Further metabolic activation of the arylhydroxamic acids, such as *N*-hydroxy-4-acetamidobiphenyl (*N*-OH-AABP; **1**), is required to generate the electrophilic species that are capable of reacting with biological macromolecules.[1,2]

Several cytosolic and microsomal enzymes catalyze the conversion of arylhydroxamic acids to reactive products. One such cytosolic enzyme is *N*-arylhydroxamic acid *N*,*O*-acyltransferase (AHAT), which catalyzes the incorporation of arylamine residues from arylhydroxamic acids into nucleic acids.[3] The electrophilic products generated from AHAT activation of arylhydroxamic acids not only react with biological nucleophiles but also irreversibly inactivate AHAT (paths c and d, Scheme I). In this regard, both *N*-hydroxy-2-acetamidofluorene and *N*-OH-AABP (**1**) function as suicide inactivators of AHAT.[4]

It is postulated that the electrophilic products generated from AHAT activation of arylhydroxamic acids are arylnitrenium ions which arise from the decomposition of the unstable *N*-acetoxyarene intermediate (I, Scheme I).[5] AHAT also catalyzes the transfer of acetyl groups from arylhydroxamic acids to aromatic amines (path a, Scheme I).[3b] Extensive studies of the tissue distribution and biochemical properties of AHAT have been reported.[3b,6-10]

Known substrates for AHAT are arylhydroxamic acids derived from 2-acetamidofluorene, 4-acetamidobiphenyl, diacetylbenzidine, 4-acetamidostilbene, 2-acetamidonaphthalene, and 2-acetamidophenanthrene.[5] All of these substrates are relatively planar molecules containing more than one aromatic ring. Because of the limited amount of information available regarding the types of arylhydroxamic acids that are capable of serving as substrates and/or irreversible inhibitors of AHAT, a series of *N*-hydroxyacetanilides was designed in which the *p*-phenyl ring of *N*-OH-AABP has been replaced with alkyl, alkenyl,

Scheme I

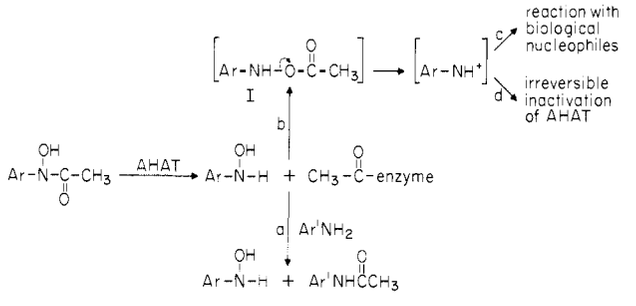

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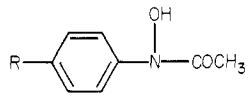
Table I. Physical Properties of *N*-Hydroxy-*N*-phenylacetamides



no.	R	mp, °C	yield, %	formula[a]
1	C_6H_5	150–151[b]	74	$C_{14}H_{13}NO_2$
2	H	65–67.5[c]	20	$C_8H_9NO_2$
3	CH_3	72–73[c]	18	$C_9H_{11}NO_2$
4	C_2H_5	67–69	12	$C_{10}H_{13}NO_2$
5	*n*-C_3H_7	80.5–82	24	$C_{11}H_{15}NO_2$
6	*n*-C_4H_9	45.5–48	39	$C_{12}H_{17}NO_2$
7	*n*-C_5H_{11}	60.5–62	13	$C_{13}H_{19}NO_2$
8	cyclohexyl	100–101.5[d]	68	$C_{14}H_{19}NO_2$
9	CH_2=CH	93–94.5	43	$C_{10}H_{11}NO_2$
10	CH_3CH=CH	124.5–126.5	46	$C_{11}H_{13}NO_2$
11	$(CH_3)_2C$=CH	118–119.5	61	$C_{12}H_{15}NO_2$

[a] Analyses for C, H, and N were within 0.4% of the theoretical values. [b] Miller, J. A.; Wyatt, C. S.; Miller, E. C.; Hartmann, H. A. *Cancer Res.* 1961, *21*, 1465–1473. [c] Kalinin, V. N.; Franchuk, I. F. *J. Appl. Spectrosc.* 1972, *16*, 514. [d] Fries, W.; Kiese, M.; Lenk, W. *Xenobiotica* 1973, *3*, 525–540.

and cycloalkyl substituents. It was anticipated that the relative AHAT activities of this series of compounds, each of which contains a hydrophobic para substituent, would provide useful insight into the structure–activity requirements of this enzyme system.

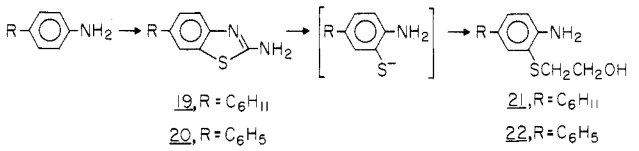
Synthesis. The hydroxamic acids **1–11** (Table I) were formed from the corresponding nitrobenzenes by a modification of the method of Smissman and Corbett.[11] This method involves reduction of the nitro compounds to hydroxylamines in the presence of zinc and ammonium chloride, followed by acetylation with acetyl chloride.

The 4-alkylnitrobenzenes **12–14**, which were not commercially available, were synthesized according to the procedure of Stock and Wasielewski, which involves nitration of the alkylbenzenes in nitromethane.[12] The ortho and para isomers were separated by spinning-band distillation, and the isomeric purity of the distillate fractions was determined by gas chromatography.

4-Cyclohexylnitrobenzene **15** was prepared by nitrating cyclohexylbenzene according to the procedures of Mayes and Turner[13] and Baas and Wepster.[14] The ortho and para isomers were separated by crystallization.

4-Vinylnitrobenzene **16** was obtained by a modification of the copper-catalyzed decarboxylation of *p*-nitrocinnamic acid described by Wiley and Smith.[15] 4-Propenylnitrobenzene **17** and 4-(2-methylpropenyl)nitrobenzene **18** were synthesized by the benzylphosphonate modification of the Wittig reaction in which diethyl *p*-nitrobenzylphosphonate was condensed with either acetaldehyde or acetone.[16] The trans configuration of the double bond in **17** was verified by comparison of the IR spectrum of the synthetic material to published spectral data for both the trans and cis isomers.[17]

Scheme II



19, R = C_6H_{11}
20, R = C_6H_5
21, R = C_6H_{11}
22, R = C_6H_5

Table II. Arylhydroxamic Acid *N,O*-Acyltransferase Catalyzed Transacetylation of Aminoazobenzene by *N*-Hydroxy-*N*-phenylacetamides

no.	rate,[a] nmol (mg of protein)$^{-1}$ min^{-1} rat	hamster
1	5.8 ± 0.1	22.4 ± 0.4
2	0.2 ± 0.1	0.6 ± 0.1
3	1.1 ± 0.1	2.1 ± 0.3
4	2.1 ± 0.1	3.5 ± 0.3
5	3.1 ± 0.1	5.3 ± 0.2
6	4.7 ± 0.6	8.8 ± 0.6
7	5.2 ± 0.1	13.8 ± 0.4
8	4.7 ± 0.1	9.4 ± 0.3
9	3.3 ± 0.1	6.6 ± 0.3
10	5.9 ± 0.8	13.0 ± 1.1
11	5.7 ± 0.3	13.1 ± 1.2

[a] Activity was measured as the AAB transacetylation rate (mean ± SE; N = 3–4) with partially purified hepatic enzyme. The procedure is described under Experimental Section.

The 2-aminobenzothiazoles **19** and **20** were prepared by the addition of ammonium thiocyanate to the 4-substituted anilines as described by Wood.[18] Compounds **19** and **20** were hydrolyzed in base according to the procedure of Horwitz and Clark,[19] and the resulting intermediates were S-alkylated with 2-bromoethanol to yield the 2-[(2-hydroxyethyl)thio]anilines **21** and **22** (Scheme II).

4-Cyclohexylphenylhydroxylamine **23** and 4-biphenylhydroxylamine **24** were prepared by reduction of the nitro compounds to the hydroxylamines in the presence of zinc and ammonium chloride.

Transacetylation of Aminoazobenzene (AAB). In order to determine whether the monophenylacetohydroxamic acids **2–11** could function as acetyl donors in the AHAT-catalyzed transacetylation of aromatic amines (path a, Scheme I), the relative rates of the enzymatic transacetylation of AAB by the hydroxamic acids were determined. Rat and hamster hepatic AHAT, partially purified by ammonium sulfate fractionation, were used for this purpose, as well as for the succeeding experiments which will be described. The transacetylation rates of the monophenylacetohydroxamic acids **2–11** were determined under conditions of zero-order kinetics and were compared with the transacetylation rate of *N*-OH-4-AABP (**1**), a known substrate for AHAT (Table II).

Comparison of the activity of the biphenyl compound **1** with that of *N*-hydroxyacetanilide (**2**) indicates that a single unsubstituted phenyl ring is not sufficient for *N*-arylhydroxamic acids to function as efficient acetyl donors with either rat or hamster hepatic AHAT. Several of the *N*-(4-substituted-phenyl)hydroxamic acids, however, were more effective acetyl donors than the unsubstituted compound **2**. As the length of the 4-alkyl chain increased from methyl (compound **3**) to pentyl (compound **7**), the compounds became increasingly more effective substrates for

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Table III. Effect of Inhibitors on Arylhydroxamic Acid N,O-Acyltransferase Catalyzed Transacetylation of Aminoazobenzene

		% inhibition			
		rat[a]		hamster[a]	
inhibitor	substrate:	1[b]	8[c]	1[b]	8[c]
iodoacetamide		95	99	100	100
N-ethylmaleimide		92	100	100	96
4-chloromercuri-benzenesulfonate		100	100	100	100

[a] Results are expressed as percent inhibition of AAB transacetylation measured after a 5-min incubation of partially purified hepatic enzyme with inhibitor (1.0 mM). The assay procedure is described under Experimental Section. The results are the means of two to three experiments, each done in triplicate. Control activities: rat = 6.1 and 4.3 nmol (mg of protein)$^{-1}$ min^{-1} for 1 and 8, respectively; hamster = 19.4 and 8.3 nmol (mg of protein)$^{-1}$ min^{-1} for 1 and 8, respectively. [b] Compound 1 = N-hydroxy-4-acetamidobiphenyl. [c] Compound 8 = N-hydroxy-3-cyclohexylacetanilide.

rat and hamster AHAT. Compound 7 was 89% as active as the standard 1 with rat AHAT and 62% as active as 1 with hamster AHAT.

Compounds containing unsaturated 4-substituents (9–11) were also found to be effective acetyl donors for the AHAT-catalyzed transacetylation of AAB (Table II). The greater activity of the 4-propenyl compound 10 compared to the 4-vinyl compound 9 indicates that the length of the alkenyl chain is a determinant of activity for compounds containing unsaturated substituents. Comparison of the activities of the compounds containing unsaturated substituents with the activities of those containing saturated substituents with the same number of carbon atoms reveals that the compound with the unsaturated substituent is always the better substrate. This point is illustrated by examining the AHAT activity of the paired compounds containing the following 4-substituents: vinyl (compound 9) vs. ethyl (compound 4), propenyl (compound 10) vs. propyl (compound 5), and phenyl (compound 1) vs. cyclohexyl (compound 8).

Although the structure–activity trends were the same for enzyme preparations from both species, the three most active AHAT substrates containing a single phenyl ring (7, 10, and 11) were approximately as active as the standard 1 with rat hepatic AHAT, whereas with hamster hepatic AHAT these three substrates were only about 60% as active as 1. For all the substrates investigated, the partially purified hamster hepatic AHAT preparation was more active than the partially purified rat enzyme (Table II).

Effect of Sulfhydryl Reagents on AHAT Activity. The sulfhydryl reagents iodoacetamide, N-ethylmaleimide, and p-chloromercuribenzenesulfonate inhibit AHAT-catalyzed transacetylation of AAB.[3b,6] Experiments were conducted to determine whether these reagents would inhibit the transacetylation of AAB when the monophenylhydroxamic acids were used as substrates. Transacetylation of AAB was inhibited by the sulfhydryl reagents to approximately the same extent when either the cyclohexylhydroxamic acid 8 or the biphenylhydroxamic acid 1 was used as the substrate (Table III). Although the sulfhydryl reagents are relatively nonspecific enzyme inhibitors, these results are supportive of the proposal that AHAT is responsible for the observed enzyme-catalyzed acetyl transfer from monophenylhydroxamic acids to AAB.

Adduct Formation and Identification. For the purpose of determining whether monophenyl-N-arylhydroxamic acids could be enzymatically converted to electrophilic reactants by AHAT, a nucleophile was added to the incubation mixture in order to trap reactive intermediates. The 4-cyclohexylphenylhydroxamic acid 8 was incubated with AHAT, N-hydroxy-4-cyclohexylaniline (23), and 2-mercaptoethanol. The hydroxylamine 23 was added to increase the rate of formation of the presumed N-acetoxyaniline intermediate (I, Scheme I). Bartsch et al. reported a 2- to 3-fold increase in the amount of adduct isolated as 3-(methylthio)-2-aminofluorene when N-hydroxy-2-aminofluorene was added to AHAT incubations containing methionine and N-hydroxy-2-acetamidofluorene.[3b]

In the presence of hamster hepatic AHAT, 2-mercaptoethanol, and the appropriate N-arylhydroxylamine, incubation of the 4-cyclohexylphenylhydroxamic acid 8 or the biphenylhydroxamic acid 1 led to the formation of the corresponding 2-mercaptoethanol adducts, 4-cyclohexyl-2-[(2-hydroxyethyl)thio]aniline (21) and 4-phenyl-2-[(2-hydroxyethyl)thio]aniline (22). Large-scale incubation mixtures were used in these experiments, and the mercaptoethanol adducts 21 and 22 were isolated by extraction of the incubation mixtures. The organic extracts were divided into acidic, basic, and neutral components. Preparative TLC of the basic components afforded the mercaptoethanol adducts, which were analyzed by mass spectrometry.

The mass spectrum of the 4-cyclohexyl adduct 21 exhibited two major peaks of approximately equal intensity, the molecular ion at m/e 251 (90.8% relative intensity) and a fragment peak at m/e 131 (100%). The mass spectrum of the 4-phenyl adduct 22 exhibited the molecular ion at m/e 245 (100%) and fragmentation peaks at m/e 201 (53.5%) and 200 (56.5%). The adducts 21 and 22 had mass spectral and chromatographic properties that were identical with those of the synthetically prepared compounds. Since the method of chemical synthesis unambigously determined the position of the 2-hydroxyethylthio side chain, the adducts isolated from the incubation mixtures also contain this group in the ortho position.

No mercaptoethanol adducts were detected by TLC when heat-denatured enzyme was used instead of active AHAT. Incubation of the hydroxylamines with AHAT and 2-mercaptoethanol in the absence of the substrate hydroxamic acids did not afford adducts 21 or 22.

Inactivation of AHAT. Hamster hepatic AHAT activated the 4-cyclohexylphenylhydroxamic acid 8 and the biphenylhydroxamic acid 1 to intermediates capable of reacting with mercaptoethanol to yield adducts 21 and 22. The reactive intermediates are believed to be N-acetoxyaniline derivatves which undergo heterolytic cleavage to generate arylnitrenium ions.[5] Conceivably, a nucleophilic group at or near the enzyme active site could react with these electrophiles, resulting in irreversible inactivation of AHAT (paths b and d, Scheme I). To test this hypothesis, we preincubated 8 and 1 with partially purified hamster hepatic AHAT for various lengths of time, after which the percent of AHAT activity remaining was determined using the AAB transacetylation assay. Both 8 and 1 (0.25 mM) produced a time-dependent loss of AHAT activity (Figure 1). This process exhibited apparent first-order kinetics when 1 was the inhibitor, as previously reported by Banks and Hanna.[4a] Inactivation by 8, however, did not display first-order kinetics. At the longer preincubation times, the rate of inactivation by 8 was faster than at the shorter preincubation times (Figure 1). Rat hepatic AHAT was not measurably inactivated by 8 or 1,

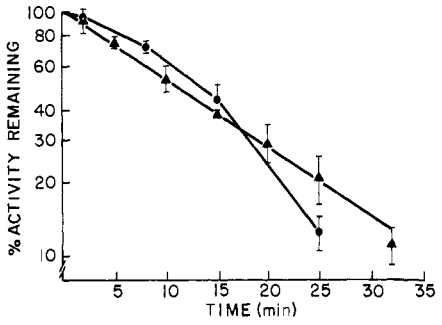

Figure 1. Inactivation of hamster hepatic AHAT by *N*-hydroxy-4-acetamidobiphenyl (1; 0.25 mM) (▲) and *N*-hydroxy-*N*-(4-cyclohexylphenyl)acetamide (8; 0.25 mM) (●). Activity is expressed as AAB transacetylation rate (percent of control; mean ± SE of four experiments with 1 and eight experiments with 8) measured after preincubation with inhibitor. Incubations and assays were carried out as described under Experimental Section. Control activities were 24.3 ± 0.2 and 11.6 ± 0.1 nmol (mg of protein)$^{-1}$ min^{-1} for 1 and 8, respectively.

Table IV. Inactivation of Hamster Hepatic Arylhydroxamic Acid *N*,*O*-Acyltransferase by *N*-Hydroxy-*N*-phenylacetamides

inhibitor	activity [a] before dialysis	activity [a] after dialysis
1 [b]	6.3 (18.1)	6.9 (18.3)
8 [c]	3.2 (7.6)	1.7 (6.1)

[a] Activities are expressed as AAB transacetylation rate [nmol (mg of protein)$^{-1}$ min^{-1}] following a 20-min preincubation of AHAT with 1 or 8. The results represent the mean of two experiments conducted in triplicate. The numbers in parentheses are values obtained in control experiments in which AHAT was not preincubated with inhibitors. The procedure is described under Experimental Section. [b] Compound 1 = *N*-hydroxy-4-acetamidobiphenyl. [c] Compound 8 = *N*-hydroxy-4-cyclohexylacetanilide.

even after preincubation for 1 h, as previously reported by Banks and Hanna.[4a]

To determine whether the inhibitors (8 or 1) formed a saturable enzyme–substrate complex before inactivating the enzyme, we preincubated various concentrations of inhibitor with AHAT for 10 min. The rate of AHAT inhibition was proportional to low concentrations of either the 4-cyclohexylphenylhydroxamic acid 8 or the biphenylhydroxamic acid 1, but it was independent of concentration at high concentrations of the inhibitors (data not presented). A concentration of 0.1 mM of either 8 or 1 was the minimal concentration which would produce complete inactivation of AHAT. Since the rate of inactivation was saturable with increasing concentrations of inhibitors, the enzyme active site appears to be involved in the inactivation process.[20] The inactivation of hamster hepatic AHAT by 8 or 1 was irreversible, as dialysis for 6 h did not restore activity (Table IV).

Inactivation in the Presence of Nucleophiles. The inclusion of a nucleophile in the incubation mixture is often useful in determining whether enzyme inactivation occurs before the reactive product is released from the enzyme active site or whether inactivation is caused by a reactive product that is released from the enzyme into solution. Nucleophiles do not decrease the rate of inactivation, and therefore do not protect the enzyme against inactivation, occuring from a reactive product remaining at the active site. On the other hand, nucleophiles should decrease the rate of inactivation and protect against inactivation occurring from reactive species in solution.[21] The protection of enzyme activity arises from the ability of the nucleophiles to scavenge the reactive products before they can react with the enzyme.[22]

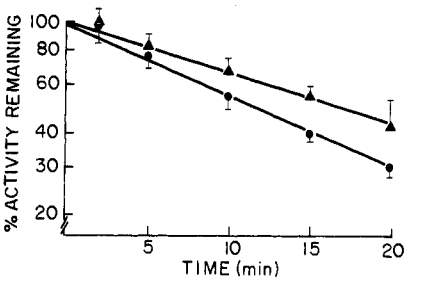

Figure 2. Inactivation of hamster hepatic AHAT by *N*-hydroxy-4-acetamidobiphenyl (1; 0.25 mM) in the presence (▲) or absence (●) of 2-mercaptoethanol (2.5 mM). Rates and activities are expressed as described under Figure 1. Incubations and assays were carried out as described under Experimental Section. Each point represents the mean plus or minus the range of two experiments, each done in triplicate. Control activities were 27.6 ± 0.2 and 22.6 ± 0.3 nmol (mg of protein)$^{-1}$ min^{-1} in the absence and presence of 2-mercaptoethanol, respectively.

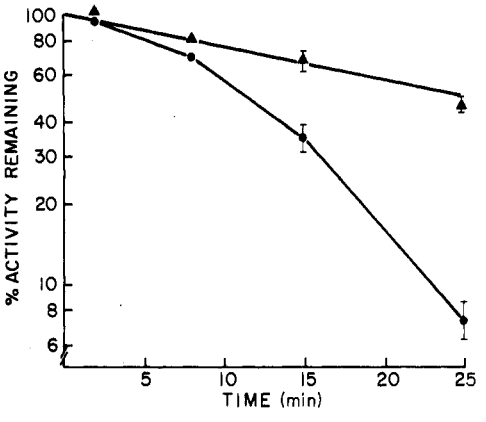

Figure 3. Inactivation of hamster hepatic AHAT by *N*-hydroxy-*N*-(4-cyclohexylphenyl)acetamide (8; 0.25 mM) in the presence (▲) or absence (●) of 2-mercaptoethanol (2.5 mM). Rates and activities are expressed as described under Figure 1. Incubations and assays were carried out as described under Experimental Section. Each point represents the mean ± SE of three experiments. Control activities were 11.3 ± 0.1 and 10.6 ± 0.1 nmol (mg of protein)$^{-1}$ min^{-1} in the absence and presence of 2-mercaptoethanol, respectively.

Inactivation of hamster hepatic AHAT by the biphenylhydroxamic acid 1 (0.25 mM) in the presence of 2.5 mM 2-mercaptoethanol occurred with little change in the inactivation rate (Figure 2). 2-Mercaptoethanol afforded minimal protection (12% at 20 min) for AHAT when 1 was the inhibitor. These results suggest that inactivation of hamster hepatic AHAT by 1 occurs primarily from a reactive intermediate which is complexed with the active site. This is in contrast to the result obtained when 0.25 mM 8 was the inhibitor. In the presence of 2.5 mM 2-mercaptoethanol, the rate of AHAT inactivation decreased, and the inactivation became a time-dependent process exhibiting apparent first-order kinetics (Figure 3). 2-Mercaptoethanol provided substantial protection (approximately 40% at 20 min) against the inactivation of AHAT by 8. These results suggest that a significant

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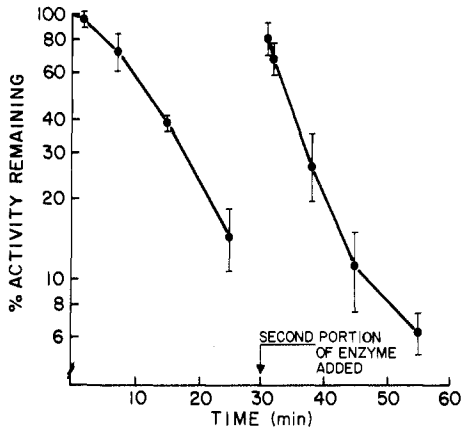

Figure 4. Inactivation of hamster hepatic AHAT by *N*-hydroxy-*N*-(4-cyclohexylphenyl)acetamide (8; 0.25 mM) as originally observed and after addition of fresh enzyme. Activities and rates are expressed as described under Figure 1. Incubations and assays were carried out as described under Experimental Section. Each point represents the mean ± SE of three experiments. Control activity was 11.6 ± 0.2 nmol (mg of protein)$^{-1}$ min^{-1}.

portion of the inactivation of hamster hepatic AHAT by 8 is produced by a reactive species in solution. However, the fact that some inactivation occurred in the presence of the nucleophile and the fact that the kinetics of inactivation in the presence of 2-mercaptoethanol appeared to be first order indicate that a portion of the inactivation occurred from a product remaining at the active site and not released into the medium. Both cysteine and glutathione (2.5 mM) provided protection (40 and 47%, respectively, at 25 min) whereas methionine (2.5 mM) provided little protection (10% at 25 min) against inactivation of hamster hepatic AHAT by 8 (0.25 mM).

Effect of Addition of a Second Portion of Enzyme. When fresh enzyme is added to a solution containing inactivated enzyme and excess inhibitor, the rate of inactivation of the second enzyme pulse should be the same as the first if a product that is complexed with the active site is responsible for the inactivation. However, if the rate of inactivation is faster after the addition of fresh enzyme, it may be concluded that an inhibitor is being formed and released into solution.[21] Further information regarding the inactivation mechanisms exhibited by the 4-cyclohexylphenylhydroxamic acid 8 and the biphenylhydroxamic acid 1 is provided from the evidence presented by Banks and Hanna[4a] and by the results shown in Figure 4. Banks and Hanna reported that enzyme, added to incubation mixtures after nearly complete inactivation of hamster hepatic AHAT activity by 1, was inactivated at the same rate as originally observed.[4a] The analogous experiment conducted with 8, however, resulted in a faster rate of inactivation for the added enzyme as compared to the original enzyme (Figure 4). This result provides additional evidence that reactive products accumulate in the medium when 8 is activated by AHAT.

Discussion

Most of the AHAT substrates that have been described in the literature contain an extended aromatic ring system such as that found in *N*-OH-AABP (1). It has been reported that *N*-hydroxyacetanilide is poor acetyl donor in the AHAT-catalyzed transacetylation of AAB and that *N*-hydroxyphenacetin is both activated to a nucleic acid binding metabolite by AHAT and serves as an efficient acetyl donor in the transacetylation of AAB.[5,23a,b] In the present study, a series of compounds was synthesized in which the *p*-phenyl ring of *N*-OH-AABP (1) was replaced by various alkyl and alkenyl substituents, as well as by a cyclohexyl group. The data in Table II indicate that the nature of the 4-substituent in compounds 1–11 markedly influences their ability to serve as acetyl donors in the AHAT-catalyzed transacetylation of AAB.

Although this series compounds was not designed for the purpose of conducting a quantitative structure–activity analysis, a preliminary study indicates that the transacetylation rates obtained with both the rat and hamster enzyme preparations can be correlated with either the molar refractivity or the length of the 4-substituent. Electronic properties may not be a major factor in determining the ability of these compounds to serve as acetyl donors, since the electronic properties of the alkyl substituents of compounds 1–7 are very similar, while transacetylation rates change dramatically as the length of the alkyl chain increases.

The introduction of unsaturation into the *p*-alkyl groups of these monophenylhydroxamic acids results in a considerable enhancement of their transacetylation rates. Indeed, compounds 10 and 11 are equal in effectiveness to *N*-OH-AABP (1) as an acetyl donor in the rat hepatic AHAT-catalyzed transacetylation of AAB (Table II). Although the enhanced activities of compounds 9 and 10 relative to that of the saturated analogues 4 and 5 might conceivably be attributed to the lower electron-donating capacity (as indicated by their relative σ values) of the olefinic substituents present in 9 and 10, the greater activity of 10 compared to 9 would argue against such a conclusion because the vinyl substituent (compound 9) and the propenyl substituent (compound 10) have similar electronic characteristics. Thus, substituent size and/or hydrophobicity appear to play an important role in the transacetylation activity of compounds containing either saturated or unsaturated alkyl substituents.

One of the objectives of the present study was to determine whether monophenyl-*N*-arylhydroxamic acids could be converted to reactive electrophilic intermediates by AHAT. Incubation of the 4-cyclohexyl compound 8 with hamster hepatic AHAT in the presence of 2-mercaptoethanol resulted in the formation of the mercaptoethanol adduct 21. An analogous product (22) was obtained with *N*-OH-AABP (1) as the substrate. The structures of the mercaptoethanol adducts 21 and 22 are consistent with those expected from an AHAT-catalyzed activation of *N*-arylhydroxamic acids.[5] It has previously been shown that activation of either *N*-OH-AABP (1) or *N*-hydroxy-2-acetamidofluorene by AHAT in the presence of methionine results in the formation of *o*-(methylthio)-4-aminobiphenyl and *o*-(methylthio)-2-aminofluorene, respectively.[3b] These ortho-substituted, primary arylamines are the same types of adducts produced when 1 and 8 were converted to electrophilic reactants by AHAT in the presence of 2-mercaptoethanol.

Recently, it was reported from this laboratory that the carcinogenic *N*-arylhydroxamic acids *N*-OH-AABP (1) and *N*-hydroxy-2-acetamidofluorene irreversibly inactivate AHAT by a suicide mechanism.[4] Therefore, experiments were conducted in order to determine the effects of the 4-cyclohexyl compound 8 on AHAT activity. The irreversible inactivation of AHAT by 8 provides further evidence that monophenylhydroxamic acids are converted to

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electrophilic reactants by this enzyme system (Table IV).

The kinetic characteristics of the inactivation of AHAT by 8 were different from those of *N*-OH-AABP (1), which exhibited apparent first-order kinetics (Figure 1).[4] The increased inactivation rate exhibited by 8 during the longer preincubation times indicates that the activation of 8 by AHAT may result in the accumulation of an electrophilic product in the medium. This conclusion is supported by the results obtained upon inclusion of a nucleophile, 2-mercaptoethanol, in the preincubation mixture (Figure 3). 2-Mercaptoethanol drastically reduced the rate of AHAT inactivation by the 4-cyclohexyl compound 8 and caused the inactivation to become an apparent first-order process. Thus, compound 8 appears to inactivate AHAT by a mechanism which includes a major pathway in which electrophiles are released into the medium and subsequently react with nucleophiles present on AHAT, as well as a minor pathway in which the activated species remains complexed with the active site and reacts with an active-site nucleophile in a suicide-like process. In contrast, even though 2-mercaptoethanol adducts could be isolated from large-scale incubations of *N*-OH-AABP (1) with AHAT, the nucleophile has little influence upon the rate of inactivation of AHAT by 1, which appears to inactivate the enzyme primarily by a suicide mechanism (Figure 2).[4]

In addition to 2-mercaptoethanol, other sulfhydryl nucleophiles, such as glutathione and cysteine, retarded the rate of hamster hepatic AHAT inactivation by 8, but methionine was ineffective. Although methionine provides a small amount of protection of the enzyme from inactivation by *N*-OH-AABP (1) (data not presented) and is an effective trapping agent for electrophiles generated from 1 by AHAT,[3b] other workers have also observed that methionine does not prevent the in vitro covalent binding to protein by activated forms of monophenyl-*N*-aryl compounds, such as acetaminophen and phenacetin.[24-26]

It was previously reported that *N*-OH-AABP (1) did not inactivate rat hepatic AHAT.[4a] A similar species selectivity was observed in the present study with 8 which irreversibly inhibited hamster hepatic AHAT but not the enzyme obtained from rat liver. It is not known whether the difference observed with the hamster and rat liver enzyme preparations is due primarily to differences in turnover rate or to differences in substrate specificity. The rat liver enzyme is inactivated by *N*-hydroxy-2-acetamidofluorene, but at a much lower rate than hamster hepatic AHAT.[4a] Although it has been reported that multiple species of AHAT may be present in hamster liver, it cannot be concluded from the data presented here whether more than one form of the enzyme is responsible for the transacetylation activities of compounds 1–11 and for the bioactivation of 1 and 8.[5]

The results reported in this paper clearly demonstrate that monophenylacetohydroxamic acids can be converted to electrophilic products by AHAT, a soluble enzyme system with wide species and tissue distribution. Thus, activation by AHAT may represent a potential toxification pathway for this class of compounds, as well as for those *N*-aryl amides which are metabolically converted to *N*-arylhydroxamic acids in vivo. The fact that activation of compounds such as 8 by AHAT results in the irreversible inhibition of the enzyme is of particular interest with regard to the recent report of Glowinski et al. who presented evidence that rabbit liver AHAT appears to be identical with the acetylcoenzyme A dependent *N*-acetyltransferase which is responsible for the metabolic acetylation of isoniazid, sulfamethazine, and other arylamines.[27] It is possible that prolonged exposure to monophenylhydroxamic acids of the type described in the present study could compromise the acetylation capacity of susceptible mammalian species and thus inhibit their ability to metabolize arylamines.

Experimental Section

Synthesis. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra (KBr) were obtained with a Beckman 33 or a Perkin-Elmer 281 recording spectrophotometer. NMR spectra were obtained with a Varian A-60A or a CFT-20 (modified for ^{1}H NMR) spectrometer; the samples were dissolved in $CDCl_3$ with tetramethylsilane as the internal standard. All compounds gave IR and NMR spectra consistent with their assigned structures. Mass spectra were obtained with an Associated Electronic Industries (AEI) MS-30 (electron impact) or a Finnigan 4000 (chemical ionization) mass spectrometer in the University of Minnesota Mass Spectrometry Laboratory, Department of Chemistry; samples were introduced by direct inlet. The gas chromatograph used was a Perkin-Elmer 3920 B equipped with a 6-ft OV-1 column. Analytical TLC was carried out with either plastic-backed plates (Eastman 13181 silica gel with fluorescent indicator, no. 6060) or glass-backed plates (Analtech, 250 μm, silica gel GF); compounds were visualized with UV light and with I_2 or the indicated spray reagent. Column chromatographic separations were carried out on silica gel 60, particle size 0.063–0.200 mm (70–230 mesh ASTM, EM reagents). Elemental analyses were performed by M-H-W Laboratories, Garden City, MI, or Phoenix, AZ; all analytical results were within 0.4% of the theoretical values. Distillations were performed on Nester Faust Annular Teflon spinning-band columns. References to −10 °C refer to the use of an ice–MeOH bath.

4-Cyclohexylnitrobenzene (15). A solution of cyclohexylbenzene (100 g, 0.62 mol) in 190 mL of glacial HOAc was added dropwise over 7.5 h to a cold (−10 °C) solution of 375 mL of fuming HNO_3 in 190 mL of glacial HOAc. After stirring for 30 min, the reaction mixture was poured into 1 L of ice. The mixture was extracted with CH_2Cl_2 (3 × 200 mL), which was then washed successively with H_2O (200 mL), 10% NaOH (3 × 200 mL), and saturated NaCl (100 mL) and dried ($MgSO_4$). The CH_2Cl_2 was evaporated in vacuo to yield a mixture of crystals and an oil. The solid material was collected by filtration and was recrystallized from absolute EtOH to afford **15**: yield 73 g (57%); mp 57–58 °C (lit.[14] mp 57–58 °C).

4-Alkylnitrobenzenes (12–14). The nitrating reagent was prepared by the dropwise addition of 10 mL of 70% nitric acid (0.17 mol) to 18 mL of cold concentrated sulfuric acid in an ice bath, and the solution was stirred for 15 min. The nitrating reagent was added dropwise over 30 min to a solution of the alkylbenzene (0.17 mol) in 150 mL of nitromethane at temperatures maintained below 20 °C. After stirring for 1.75 h, the reaction mixture was poured over 250 mL of ice and was extracted with $CHCl_3$ (2 × 100 mL), which was then washed successively with saturated $NaHCO_3$ (3 × 100 mL) and saturated NaCl (100 mL) and dried ($MgSO_4$). The $CHCl_3$ was evaporated in vacuo to yield an oil containing ortho and para isomers. The isomers were separated by spinning-band distillation, and the isomeric purity was determined by GC.

4-Propylnitrobenzene (12): bp 107–107.5 °C (1.0 torr) [lit.[28] bp 154 °C (20 torr)]; column temp 120 °C; R_t = 2.75 (para) and 1.83 min (ortho); yield 15%.

4-Butylnitrobenzene (13): bp 113 °C (2.6 torr) [lit.[29] bp 143–145 °C (15 torr); lit.[30] bp 113 °C (1 torr)]; column temp 130

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°C; R_t = 4.5 (para) and 3.0 min (ortho); yield 31%.

4-Pentylnitrobenzene (14): bp 126.5–127.2 °C (2.75 torr) [lit.[31] bp 158–159 °C (less than 14 torr)]; column temp 135 °C; R_t = 4.0 (para) and 2.75 min (ortho); yield 19%.

4-Vinylnitrobenzene (16). *p*-Nitrocinnamic acid (25 g, 0.13 mol), 70 mL of quinoline, and copper metal (1.66 g, 0.026 mol) were heated to 185 °C for 6 h. The reaction mixture was poured into a solution of 200 g of ice and 75 mL of concentrated HCl. The diluted reaction mixture was extracted with $CHCl_3$ (3 × 200 mL), which was then washed successively with saturated NaCl (200 mL) and 10% HCl (2 × 150 mL) and dried (Na_2SO_4). The $CHCl_3$ was evaporated in vacuo to give a red liquid: yield 12.3 g (64%). The liquid was dissolved in $CHCl_3$ and purified by chromatography on a silica gel (200 g) column which was eluted with $CHCl_3$ to afford 5.2 g (27 %) of **16**.

4-Propenylnitrobenzene (17). Diethyl *p*-nitrobenzylphosphonate[32] (12 g, 0.044 mol) was added dropwise to a solution of sodium metal (1 g, 0.04 mol) dissolved in 100 mL of absolute EtOH, and the reaction mixture was stirred for 25 min. Acetaldehyde (3 mL, 0.053 mol) was added, and the reaction mixture was stirred overnight. Water (100 mL) was added and the resulting precipitate was collected by filtration, washed with water, and recrystallized from EtOH/H_2O to give **17**: yield 4.0 g (56%); mp 90–92 °C (lit.[33] mp 93–93.5 °C).

4-(2-Methylpropenyl)nitrobenzene (18). Following the procedure of Tanida et al.,[34] diethyl *p*-nitrobenzylphosphonate[32] (14.2 g, 0.05 mol) was added dropwise to a solution of NaH (3.1 g, 0.06 mol) in 125 mL of dry dimethoxyethane, and the solution was stirred until H_2 evolution ceased. Acetone (12 mL, 0.26 mol) that had been distilled over 4Å molecular sieves was added, and the reaction mixture was stirred overnight. A gummy precipitate formed. The solvent was decanted from the precipitate. Warm benzene (3 × 50 mL) was added to the precipitate, the mixture was heated under reflux, and the benzene was decanted. The benzene decantations were combined with the previously decanted solution, and the combined solution was then washed sucessively with H_2O (3 × 200 mL), 10% HCl (2 × 100 mL), saturated $NaHCO_3$ (100 mL) and dried ($MgSO_4$). The solvent was evaporated in vacuo to give a red oil: yield 4.3 g (47%). The oil was purified on a silica gel (90 g) column. The elution solvent consisted of CH_2Cl_2/petroleum ether (60–70 °C): 750 mL, 10:90; 400 mL, 15:85; 200 mL, 20:80. Compound **18** was obtained as a yellow oil: yield 1.76 g (19%).

***N*-Arylhydroxamic Acids (1–11; Table I)**. The nitrobenzenes (0.10 mol) were dissolved in 120 mL of DMF, 60 mL of ethanol, and 30 mL of H_2O. Ammonium chloride (21.4 g, 0.40 mol) and zinc dust (26.0 g, 0.40 mol) were added, and the reaction mixture was stirred for 1 h at room temperature under a N_2 atmosphere. The reaction mixture was filtered, and the filter cake was washed with 200 mL of Et_2O. The Et_2O washes and the filtrate were combined and poured into 75 mL of cold H_2O, and the mixture was extracted with Et_2O (4 × 150 mL). The Et_2O fraction, which contained the hydroxylamine, was filtered and cooled to −10 °C, and 100 mL of saturated $NaHCO_3$ was added. Acetyl chloride (6.67 g, 0.085 mol) in 60 mL of ether was added dropwise over 30 min to the reaction mixture. The mixture was stirred at −10 °C for 30 min, and the Et_2O was removed in vacuo. A cold saturated solution of $NaHCO_3$ (250 mL) was added to the residue, and the mixture was stirred for 1 h at −10 °C. The solid material was collected by filtration, washed with 1 L of H_2O, and dissolved in $CHCl_3$. The $CHCl_3$ was extracted with 1% NaOH (3 × 100 mL); the basic extract was acidified (pH 1–2) with concentrated HCl, and the acidic solution was extracted with Et_2O (3 × 100 mL), which was then dried ($MgSO_4$) and filtered, and the filtrate was evaporated in vacuo. The compounds were recrystallized from petroleum ether (60–70 °C)/benzene. The hydroxamic acids were visualized as red spots on silica gel TLC with a 2.5% $FeCl_3$ in 0.5 N HCl spray reagent.

4-Cyclohexylphenylhydroxylamine (23). 4-Cyclohexylnitrobenzene (**15**; 1.54 g, 7.5 mmol) was dissolved in a solution of 15 mL of DMF, 6.0 mL of absolute EtOH, and 3.5 mL of H_2O. Ammonium chloride (1.6 g, 30 mmol) and zinc dust (2.0 g, 30 mmol) were added. The reaction mixture was stirred at room temperature under N_2 for 1.5 h and then filtered. The filter cake was washed with EtOH. The ethanol washes and the filtrate were combined and diluted with 250 mL of saturated NaCl. This aqueous layer was extracted with $CHCl_3$ (2 × 50 mL), which was then washed with H_2O (3 × 60 mL), dried ($MgSO_4$), and evaporated in vacuo to yield a solid. The solid was purified by dissolving it in $CHCl_3$ at room temperature. The mixture was cooled, and the crystals (0.24 g, 17%) were collected by filtration: mp_1 115–118 °C; mp_2 195–200 °C. Anal. ($C_{12}H_{17}NO$) C, H, N.

4-Biphenylhydroxylamine (24). Compound **24** was synthesized by the same procedure as described above for **23** with 5 g (0.025 mol) of 4-nitrobiphenyl, 220 mL of DMF, 70 mL of H_2O, 5.46 g (0.10 mol) of ammonium chloride, and 6.5 g (0.099 mol) of zinc dust. The dried $CHCl_3$ extract was concentrated in vacuo until a precipitate was evident. The mixture was cooled, and the precipitate was collected. The solid was recrystallized by dissolving it in $CHCl_3$ at room temperature and concentrating the solvent in vacuo until crystals appeared. The mixture was cooled, and the crystals were collected by filtration to afford 2.64 g (57%) of **24**, mp 150–152 °C (lit.[35] mp_1 150–153 °C; mp_2 188–200 °C).

6-Cyclohexyl-2-aminobenzothiazole (19). Bromine (0.64 mL, 11.7 mmol) was dissolved in 10 mL of absolute EtOH saturated with KBr. This solution was added dropwise over 0.5–1.0 h to a cooled mixture of 4-cyclohexylaniline (2.0 g, 11.6 mmol), 10 mL of absolute EtOH saturated with KBr, and ammonium thiocyanate (2.83 g, 37 mmol). The reaction mixture was stirred at room temperature for 1 h, poured into 120 mL of H_2O, neutralized (solid Na_2CO_3), and extracted with $CHCl_3$ (3 × 50 mL). The organic extract was dried ($MgSO_4$) and evaporated in vacuo. After recrystallization from petroleum ether (60–70 °C)/$CHCl_3$, the yield of **19** was 0.92 g (34%), mp 219–221 °C. Anal. ($C_{13}H_{16}N_2S$) C, H, N, S.

6-Phenyl-2-aminobenzothiazole (20). A solution of bromine (0.8 g, 5.0 mmol) in 15 mL of glacial HOAc was added dropwise over 1.5 h to a mixture of 4-aminobiphenyl (1.0 g, 5.9 mmol), 30 mL of glacial HOAc, and ammonium thiocyanate (0.9 g, 12 mmol). After an additional 15 min, the reaction mixture was diluted with H_2O and made basic with solid NaOH. The precipitate was collected and suspended in water, which was then made basic (10% NaOH). The basic mixture was extracted with $CHCl_3$ and then dried ($MgSO_4$) and evaporated in vacuo. The product was obtained in 49% yield (0.66 g) after recrystallization from $CHCl_3$/EtOAc, mp 227–229 °C (lit.[36] mp 227–228 °C).

4-Cyclohexyl-2-[(2-hydroxyethyl)thio]aniline (21). A mixture of KOH (0.28 g, 5 mmol), NaOH (0.3 g, 7.5 mmol), and $Na_2S·5H_2O$ (0.084 g, 0.5 mmol) was melted in a culture test tube over an open flame. The 2-aminobenzothiazole (**19**; 0.3 mmol) was added to the molten base. The reaction mixture was agitated by shaking for 1–2 min near the flame. The test tube was then immersed in a cold solution of 2-bromoethanol (1.87 g, 15 mmol) in 10 mL of H_2O, which released the reaction mixture by breakage of the test tube. After 1 h, the reaction mixture was extracted with Et_2O (4 × 20 mL). The organic fraction was extracted with 10% HCl (4 × 30 mL); the acidic extract was made basic with 10% NaOH; the basic solution was extracted with Et_2O, which was dried ($MgSO_4$) and then evaporated in vacuo to yield the crude product containing unreacted 2-aminobenzothiazole. The crude product pooled from two to three reactions was suspended in $CHCl_3$ and filtered to remove the more insoluble 2-aminobenzothiazole. Purification was achieved on a silica gel (10 g) column with $CHCl_3$ as the eluent. After recrystallization from petroleum ether (60–70 °C)/$CHCl_3$, the yield of **21** was 0.055 g (10%): mp 44–45 °C; NMR ($CDCl_3$) δ 1.18–1.83 (br d, 10 H, 5

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cyclohexyl CH_2's), 2.32 (br s, 1 H, CH), 2.82 (t, 2 H, SCH_2), 3.57 (t over br band, 5 H, CH_2OH, NH_2), 6.43–7.08 (m, 3 H, Ar H); EIMS (70 eV), *m/e* (relative intensity) 251 (M^+, 88), 131 (100); CIMS (NH_3), *m/e* 252. Anal. ($C_{14}H_{21}NOS$) C, H, N, S.

4-Phenyl-2-[(2-hydroxyethyl)thio]aniline (22). Compound 24 was synthesized by the method described above for **23**. Purification was achieved on a silica gel (13–15 g) column. The elution solvent was $CCl_4/CHCl_3$: 50 mL, 100:0; 25 mL, 90:10; 25 mL, 80:20; 25 mL, 70:30; 25 mL, 60:40; 25 mL, 50:50; 25 mL, 40:60; 25 mL, 30:70; 50 mL, 20:80; 50 mL, 10:90; 100 mL, 0:100. After recrystallization from petroleum ether (60–70 °C)/benzene, the yield of 24 was 0.051 g (11%); mp 65–66 °C; NMR ($CDCl_3$) δ 2.93 (t, 2 H, SCH_2), 3.6 (t over br band, 4 H, OCH_2, NH_2), 6.75–7.68 (m, 8 H, Ar H); EIMS (70 eV), *m/e* (relative intensity) 245 (M^+, 100), 212 (29), 201 (48), 200 (57); CIMS (NH_3), *m/e* 246 (29). Anal. ($C_{14}H_{15}NOS$) C, H, N, S.

Enzymatic Studies. Male golden syrian hamsters were purchased from Charles River (Wilmington, MA), and male Sprague-Dawley rats were purchased from Bio-Lab (White Bear Lake, MN). Ultracentrifugation was performed on a Beckman L5-65 ultracentrifuge and low-spin centrifugation on a Beckman J-21B or a J2-21 centrifuge. A Beckman 24 or 24/25 spectrophotometer was used. Incubations were performed in a Dubnoff, Blue M, or an Eberbach shaker bath. Cellulose dialysis sacks (10 × 0.62 in., washed in H_2O and packed in 0.2% benzoic acid), dithiothreitol (DTT), and methionine were purchased from Sigma Chemical Co. Preparative TLC was performed on 500-μm silica gel plates (Analtech, silica gel GF).

Tissue Preparation. Animals were lightly etherized before decapitation. Livers were excised and placed in cold 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT. Livers were blotted dry, weighed, minced, and homogenized with 1 mL of cold buffer per gram of liver in a Potter-type homogenizer with a motor-driven pestle. This 50% homogenate was centrifuged at 105000*g* for 60 min in a refrigerated Beckman preparative ultracentrifuge. The resultant supernatant was diluted with an equal volume of cold pyrophosphate buffer to afford a 25% solution.

Enzyme Preparation. Arylhydroxamic acid *N,O*-acyltransferase (AHAT) was partially purified (2- to 3-fold) from rat liver cytosol (25% solution of 105000*g* supernatant) by ammonium sulfate fractionation as described by King.[6] Modifications of this procedure were used to obtain partially purified hamster hepatic AHAT. The 25% solution of the 105000*g* supernatant from hamster liver was placed in an ice bath and brought to 35% saturation with ammonium sulfate by addition, with stirring, of an ice-cold saturated solution of ammonium sulfate in 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT. The cold 35% ammonium sulfate solution was stirred on ice for an additional 20–30 min before the precipitate was removed by centrifugation at 9500*g* for 10–15 min. The supernatant was then brought to 50% saturation by further addition of the cold saturated ammonium sulfate solution. The 50% saturated solution was stirred an additional 20–30 min at ice-bath temperatures before centrifugation. The precipitate, which contained most of the AHAT activity, was washed twice with cold 50% saturated ammonium sulfate.

The rat and hamster enzyme pellets obtained from the fractionation procedure were stored at −70 °C and were reconstituted in enough 0.05 M pyrophosphate buffer (pH 7.0, 1 mM DTT) to give approximately 30 mg/mL of protein. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.[37]

AAB Transacetylation Assay. Standard incubation mixtures consisted of 1 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, enzyme solution (2.5 mg of protein), 0.1 mL of 95% ethanol containing the substrates [2.5 μmol of hydroxamic acid (final concentration = 1.0 mM) and 0.375 μmol of AAB (final concentration = 0.15 mM)], and enough 1.15% KCl to bring the final volume to 2.5 mL. A 2- to 5-min temperature equilibration period was initiated by addition of the enzyme solution to 25-mL Erlenmeyer flasks, containing KCl and buffer, in a 37 °C shaker bath. Reactions were initiated by the addition of the substrate. Incubations were performed in air at 37 °C for various lengths of time. The enzymatic reaction was terminated by the addition of 2.5 mL of 20% (w/v) trichloroacetic acid in 50% (v/v) $EtOH/H_2O$, and the mixture was centrifuged in a desk-top centrifuge (20 min). The supernatants were analyzed spectrophotometrically at 497 nm.[23] Control experiments were carried out with heat-denatured enzyme. Incubations were performed in triplicate.

The incubation time for each compound was chosen from the linear portion of the product vs. time curves for fixed protein (1.0 mg/mL) and substrate concentrations (1.0 mM hydroxamic acid; 0.15 mM AAB). An incubation time of 5 min was used for all compounds tested with rat hepatic AHAT, whereas the incubation times used in the hamster hepatic AHAT assays ranged from 2 to 10 min.

Adduct Formation and Identification. Incubations were run on a scale 100 times that of a standard incubation. Incubation mixtures consisted of 100 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 2.5 mmol of 2-mercaptoethanol, AHAT enzyme solution (187.5 mg of protein), 250 μmol of hydroxamic acid and 25 μmol of the corresponding hydroxylamine in 10 mL of EtOH, and enough 1.15% KCl to bring the volume to 250 mL. The reaction flasks were slowly agitated for 1 h at 37 °C under a N_2 atmosphere. The reaction mixture was cooled and extracted with Et_2O (3 × 70 mL). The ethereal extracts were centrifuged to disperse the emulsion and were combined. The components in the combined Et_2O extract were divided into acidic, basic, and neutral fractions as follows. The Et_2O was first extracted with 10% NaOH (4 × 20 mL) and then with 10% HCl (3 × 20 mL); the Et_2O was washed with saturated NaCl (100 mL), dried ($MgSO_4$), and evaporated in vacuo to yield the neutral fraction (1 as substrate, 0.058 g; 8 as substrate, 0.062 g).

The 10% NaOH extracts were combined and extracted with Et_2O, which was then discarded. The basic solution was acidified with concentrated HCl (pH 1–2) and again extracted with Et_2O (3 × 25 mL), which was dried ($MgSO_4$) and evaporated in vacuo to yield the acidic fraction (1, 0.036 g; 8, 0.043 g).

The 10% HCl extracts were combined and extracted with Et_2O, which was discarded. The acidic solution was made basic with concentrated NH_4OH to pH 9.0 and extracted with Et_2O (3 × 20 mL), which was dried ($MgSO_4$) and evaporated in vacuo to afford the basic fraction (1, 0.028 g; 8, 0.0008 g).

The acidic, basic, and neutral residues were spotted on analytical silica TLC plates and compared to synthetic standards to help identify the components in each fraction.

The basic fraction containing the mercaptoethanol adduct was streaked on a 10 × 20 cm, 500-μm silica gel preparative TLC plate and eluted with $CHCl_3$/EtOAc (4:1). The region corresponding to the adduct was visualized under UV light and was scraped off the plate. The product was extracted by stirring the silica gel with $CHCl_3$ or EtOAc. The silica gel was filtered from the solvent, which was then dried ($MgSO_4$) and evaporated in vacuo. The residue was dissolved in $CHCl_3$ and streaked on a 5 × 20 cm, 500-μm silica gel TLC plate and eluted with $CHCl_3$/EtOAc (4:1). The region containing the mercaptoethanol adduct was scraped and treated as before to afford the adduct, which was then analyzed by MS. The spectrum was corrected for the TLC plate background and was compared to the spectrum obtained with synthetic material.

Other compounds isolated from the large-scale incubations of hamster hepatic AHAT with 2-mercaptoethanol, the hydroxamic acid (1 or 8), and the hydroxylamine (23 or 24) were the corresponding amines, amides and azoxy derivatives. Preliminary evidence indicates that the amines and azoxy compounds form nonenzymatically from the hydroxylamines, while the amides may be the product of an enzymatic transacetylation (data not presented). In addition, trace quantities of a metabolite that had a lower R_f value than 21 but exhibited the same molecular ion (*m/e* 251, 44% relative intensity) as 21 were detected; insufficient quantities of the adduct were obtained to permit its characterization.

Large-scale incubations were conducted as described above with heat-denatured enzyme instead of active AHAT. Heat-denatured AHAT solution was prepared by heating the enzyme solution (80–90 °C) for 20–30 min. If the compounds of interest were not

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visualized on TLC after appropriate workup of the incubation mixture containing heat-denatured enzyme, no attempt was made to extract the silica gel.

For the purpose of determining whether AHAT would catalyze adduct formation with the *N*-arylhydroxylamines as substrates, mixtures containing AHAT (170 mg of protein), 72 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 1.8 mmol of 2-mercaptoethanol, 180 μmol of *N*-hydroxy-4-aminobiphenyl or *N*-hydroxy-4-cyclohexylaniline in 7 mL of 95% EtOH, and enough 1.15% KCl to bring the volume to 180 mL were incubated at 37 °C for 1 h under N_2. The incubation mixture was treated as previously described. The basic fraction was streaked on TLC plates. If there was no compound visible at the region of interest, no attempt was made to extract the silica gel.

AHAT Inactivation Experiments. Standard preincubation mixtures consisted of 1 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 0.625 μmol of hydroxamic acid in 0.05 mL of 95% EtOH, hamster hepatic enzyme solution (1.875 mg of protein), and enough 1.15% KCl to bring the final volume to 2.4 mL. Preincubation for various lengths of time at 37 °C in air was initiated by the addition of enzyme. At the end of the preincubation time, the amount of AHAT activity remaining was assayed by the AAB transacetylation assay described earlier. Substrates (0.375 μmol of AAB and 2.5 μmol of the same hydroxamic acid as used in the preincubation) in 0.1 mL of 95% EtOH were added to initiate the assay. The incubation time for the assay was 2 (compound 1) or 4 min (compound 8). Control flasks contained 0.05 mL of 95% EtOH in place of the hydroxamic acid in the preincubation mixtures and 3.13 μmol (0.625 μmol + 2.5 μmol) of hydroxamic acid in the substrate solution used in the AAB transacetylation assay.

Dialysis Experiments. The preincubations were run on a scale of 8–16 times that of a standard preincubation. The 16× standard preincubation mixtures consisted of 16 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 10 μmol of hydroxamic acid in 0.8 mL of 95% EtOH, hamster hepatic enzyme solution (30 mg of protein), and enough 1.15% KCl to bring the final volume to 38.4 mL. The control preincubation solution contained 0.8 mL of 95% EtOH instead of the hydroxamic acid. Preincubation was carried out at 37 °C in air for 20 min. Portions (2.4 mL) were removed for the determination of AHAT activity by the AAB transacetylation assay. The remaining preincubated solutions were dialyzed at 4 °C against 330 mL of cold 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT and 2% EtOH. Nitrogen was bubbled through the buffers before and during dialysis. The buffers were changed three times during the 6-h dialysis period. At the end of the dialysis period, portions (2.4 mL) of the dialyzed solutions were assayed for AHAT activity by the AAB transacetylation assay. The substrate solution used to initiate the transacetylation assay before and after dialysis contained both 0.375 μmol of AAB and 2.5 μmol of the same hydroxamic acid as used in the preincubation in 0.1 mL of 95% EtOH. The incubation time was 8 (compound 8) or 5 min (compound 1).

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Species- or Isozyme-Specific Enzyme Inhibitors. 4.[1] Design of a Two-Site Inhibitor of Adenylate Kinase with Isozyme Selectivity

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The ATP analogues 6-(*n*-butylamino)-, 6-(di-*n*-butylamino)-, and 6-(*n*-butylthio)-9-β-D-ribofuranosylpurine 5′-triphosphate have been synthesized and studied as inhibitors and/or substrates of the rat muscle adenylate kinase isozyme (AK M) and the rat liver isozymes AK II and III. The 6-NH(*n*-Bu) and 6-S(*n*-Bu) analogues were substrates (V_{max} relative to ATP, 13–190%) of the three AK isozymes, whereas the 6-N(*n*-Bu)$_2$ analogue was a weak substrate and a competitive inhibitor of AK M and AK III. The affinities of the analogues relative to ATP [K_M (ATP)/K_M or K_i] were 0.03–0.075 for AK III and 0.14–0.28 for AK M, and affinities for AK M exceeded those for AK III by factors of 2.3–7.0. P^1,P^6-Di(adenosine-5′) pentaphosphate (Ap_5A) was synthesized by an improved method and was found to be a potent two-site inhibitor (K_i = 0.28 μM), competitive toward AMP or ATP, for the three AK isozymes. 8-SEt-Ap_5A also behaved as a two-site inhibitor; the 8-SEt group reduced the affinity for AK M 12-fold but increased the affinity for AK II and III 4-fold, resulting in ca. 45-fold more effective inhibition of AK II and III (K_i = 0.07 μM) than of AK M (K_i = 3.25 μM). The 8-SEt group of 8-SEt-ATP likewise reduced affinity for the ATP site of AK M but enhanced affinity for the ATP sites of AK II and III, resulting in at least 30-fold more effective inhibition of AK II and III. 8-SEt-AMP inhibited AK II and III noncompetitively (K_i = 21–24 mM) with respect to AMP, indicating that the 8-(ethylthio)adenosine moiety of 8-SEt-Ap_5A probably binds to the ATP sites of these isozymes. 8-SEt-Ap_5A had ca. 1000-fold more affinity for AK II or III than did 8-SEt-ATP. The findings indicate that isozyme-selective inhibitory effects of a substrate derivative can be imparted to a two-site inhibitor, leading to significant enhancement of inhibitory potency.

Studies with species and isozyme variants of thymidine kinase[2] and adenylate kinase (AK)[1] have shown that attachment of single substituents at various atoms of a substrate frequently influences affinity for the substrate site of these enzymes in a species- or isozyme-selective manner. Among the results obtained was the finding[1] that attachment to adenosine 5′-triphosphate (ATP) of ω-(acylamino)alkyl groups at N^6 or of alkylthio groups at C-8 gives rise to isozyme-selective effects involving affinity for the ATP sites of the rat muscle AK isozyme, the AK II isozyme predominant in rapidly growing rat hepatomas, and the rat liver isozyme AK III.[3,4] The magnitude of these selective effects could not be determined from the kinetic data in the case of the N^6-substituted ATP derivatives but was found to be greater than 30-fold in the case of the 8-alkylthio derivatives, which were moderately strong, competitive inhibitors [e.g., K_M (ATP)/K_i (8-SPr-ATP) = 1.5] of AK II and III and were weak, non-

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