in the distal third of the tail with 0.1 mL of Freund's complete adjuvant (FCA) composed of a fine suspension of killed and dried *Mycobacterium butiricum* (Difco) in liquid paraffin at a concentration of 5 mg/mL. The day of FCA injection was designated as day 0 of the experiment. On day 16 after FCA, the volume of both hindpaws was measured by mercury displacement according to the method of Hall and Hallet.[18] Only the rats with well-established arthritis (mean increase in volume of both hindpaws between 1 and 2.5 mL) and comparable edema volumes in the left and right hindpaws were selected for the experiment. Arthritic animals were distributed into experimental groups of six rats so that all groups had comparable mean hindpaw edema volumes. Drug treatment was started on day 16 and administered daily until day 23. On the last day of the experiment, hindpaw volumes were measured, and changes from day 16 readings for the 12 hindlegs of each group were calculated. From these measurements, the relative potency and the 95% confidence limits of the drugs were established by analysis of variance.[19]

Inhibition of Prostaglandin Synthesis. The preparation of an acetone and diethyl ether washed powder of sheep vesicular glands has been described previously.[20] The powder was homogenized in 0.05 M KH_2PO_4–NaOH buffer (pH 7.4) in the presence of phenol (0.6 mM) as activator. The homogenate was centrifuged at 1000*g* at 2 °C for 5 min, and the resulting low-speed supernatant was used as enzyme source. The assay consisted of 4 mL of enzyme solution (equivalent to 5 mg of acetone powder), test drug (50–400 μg added in 40 μL of acetone), and arachidonic acid (20 μg containing 2×10^6 dpm $^3H_8$-labeled material) dissolved in 40 μL of acetone. The enzyme solution was preincubated for 1 min at 37 °C with test drug before substrate arachidonic acid was added and further incubated at 37 °C for 10 min. Control experiments consisted of enzyme solution and arachidonic acid in the absence of test drug. The incubation was terminated by the addition of 5 vol of ethanol. The precipitated protein was filtered, and the filtrate was evaporated to complete dryness in vacuo. The residue was dissolved in ethanol and analyzed by TLC (silica gel G; Brinkmann) using as developing solvent chloroform–methanol–acetic acid–water (90:9:1:0.65, v/v). After development, the radioactive zones were located with a Berthold radiochromatogram scanner and scraped with scintillation vials, and after the addition of methanol–water (1 mL, 1:1, v/v) and Bioflur (10 mL, New England Nuclear), the vials were counted in a Beckman scintillation spectrophotometer. Results (Figure 2) are expressed in terms of the amount (percent) of arachidonic acid present in the sample. In control experiments, the amount of unreacted arachidonic acid represented 12.4 ± 0.3% (n = 5), while PGE_2 and $PGF_{2\alpha}$ represented 58.9 ± 1.5% (n = 5) of the radioactivity in the sample. The rest of the radioactivity migrated in the region of monohydroxy fatty acids (probably 11- and 15-HETE, formed via cyclooxygenase). Results with drugs showed inhibition of both PGs, as well as the hydroxy fatty acids (seen also with indomethacin), indicating an inhibition at the level of cyclooxygenase.

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Registry No. (±)-I, 87226-38-8; (+)-I, 87249-11-4; (−)-I, 87226-41-3; (1*S*)-etodolac (*S*)-(−)-borneol ester, 87226-39-9; (1*R*)-etodolac (*S*)-(−)-borneol ester, 87226-40-2; prostaglandin synthetase, 9055-65-6.

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N-Arylhydroxamic Acid *N,O*-Acyltransferase. Positional Requirements for the Substrate Hydroxyl Group

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N-Arylhydroxamic acid *N,O*-acyltransferase (AHAT) is a cytosolic enzyme system that is capable of converting toxic and carcinogenic *N*-arylhydroxamic acids into electrophilic reactants and of catalyzing the transacetylation of arylamines. The role of the *N*-hydroxyl group in promoting AHAT-catalyzed transacetylation of arylamines was investigated by the synthesis and biochemical evaluation of a series of *o*-hydroxyaryl amides and *N*-arylglycolamides. Several of these compounds are metabolites of carcinogenic aryl amides in vivo. 3-Hydroxy-4-acetamidobiphenyl (**8**) was weakly effective as an acetyl donor when partially purified preparations of hamster or rat hepatic AHAT were used to catalyze the transacetylation of 4-aminoazobenzene. 1-Hydroxy-2-acetamidofluorene (**1**), 3-hydroxy-2-acetamidofluorene (**2**), 2-glycolamidofluorene (**3**), 4-glycolamidobiphenyl (**9**), and *trans*-4-glycolamidostilbene (**5**) were less effective acyl donors than 4-acetamidobiphenyl (**7**) itself. The compounds were also assayed for their abilities to participate in the AHAT-catalyzed conversion of *N*-arylhydroxylamines to electrophilic intermediates that form methylthio adducts upon reaction with *N*-acetylmethionine. None of the compounds exhibited more than 4% of the activity of the prototype compound, *N*-hydroxy-4-acetamidobiphenyl (**10**). These results indicate that the presence of an hydroxyl group on the ring position ortho to the amide group or on the α-position of the acyl group is not sufficient to confer significant acyltransferase activity with AHAT.

Carcinogenic aryl amides are N-hydroxylated by the cytochrome P-450 dependent polysubstrate monooxygenase system to form *N*-arylhydroxamic acids, a class of proximate carcinogens.[1] Arylhydroxamic acid *N,O*-acyltransferase (AHAT) catalyzes the rearrangement of these *N*-arylhydroxamic acids to reactive electrophiles, which form covalent adducts with nucleic acids and other cellular nucleophiles, including those present on AHAT itself (Scheme I).[2,3] Additional, or concurrent, biotransformations of *N*-arylhydroxamic acids by AHAT include deacylation to form mutagenic arylhydroxylamines and transfer of the acyl group to arylamines (Scheme I).[4,5]

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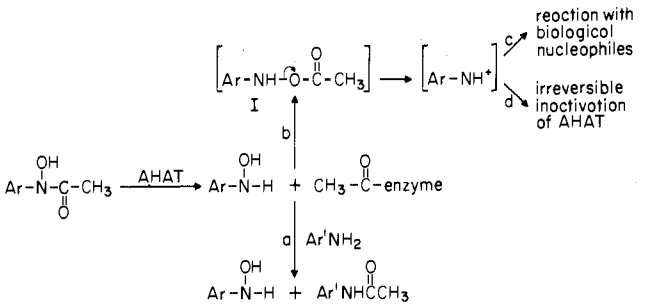
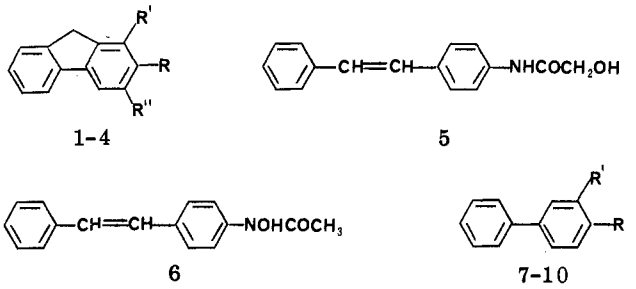
Scheme I



AHAT is widely distributed in tissues of man and other species susceptible to the induction of tumors by arylamines and arylamides.[6] Furthermore, the AHAT of rabbit liver has been shown to be indistinguishable from the genetically polymorphic acetylcoenzyme A dependent *N*-acetyltransferase (EC 2.3.1.5) (NAT) that acetylates sulfonamides, carcinogenic arylamines, and other xenobiotics.[7] The selective inactivation of NAT activities by mechanism-based inhibitors (suicide substrates) of AHAT provided evidence for the identity of these two enzyme systems in hamster liver.[8] Thus, it is important to gain an understanding of the relationships between substrate structure and AHAT activity in order to better define its role in xenobiotic metabolism and carcinogen activation.

Although a free *N*-hydroxyl group is required for the conversion of *N*-arylhydroxamic acids to electrophiles by AHAT, the presence of an *N*-hydroxyl group does not appear to be an absolute prerequisite for the formation of the initial acyl–enzyme complex. The AHAT of rat liver cytosol promotes the transfer of the acetyl group of *N*-methoxy-2-acetamidofluorene to 2-aminofluorene, albeit at a much slower rate than the corresponding transfer of the acetyl group from *N*-hydroxy-2-acetamidofluorene.[3] Presumably, the *N*-hydroxyl group renders the acyl group of an *N*-arylhydroxamic acid more susceptible to nucleophilic attack than the acyl group of an aryl amide by virtue of its electron-withdrawing inductive effect and/or by intramolecular hydrogen bonding with the adjacent carbonyl oxygen atom.[9] *N*-Arylhydroxamic acids, *o*-hydroxyaryl amides, and *N*-arylglycolamides share a common structural feature in that a hydroxyl group resides in close proximity to the amide carbonyl group. Molecular models of such compounds indicate that intramolecular hydrogen bonds can readily form between the hydroxyl and carbonyl groups, and it was recently demonstrated that this type of hydrogen bonding exists in the solid state form of 1-hydroxy-2-acetamidofluorene (**1**).[10] Thus, it is conceivable that *o*-hydroxyaryl amides and *N*-arylglycolamides might serve as acyl group donors in AHAT-catalyzed reactions. Such enzymatic activity would represent a possible mode of secondary metabolism of *o*-hydroxyaryl amides and *N*-arylglycolamides, both of which are products of the metabolism of aryl amides.[11,12] This report describes the results of experiments designed to compare the abilities of *N*-arylhydroxamic acids, *o*-hydroxyaryl amides, and *N*-arylglycolamides to serve as acyl donors in the transacylations catalyzed by partially purified preparations of rat and hamster AHAT.

Table I. *N*-Arylhydroxamic Acid *N*,*O*-Acyltransferase Catalyzed Transacylating Activities



no.	R	R′	R″	transacylation rate[a]
1	$NHCOCH_3$	OH	H	0.11 ± 0.05
2	$NHCOCH_3$	H	OH	0.05 ± 0.03
3	$NHCOCH_2OH$	H	H	0.02 ± 0.01
4	$NOHCOCH_3$	H	H	19.30 ± 0.60
5				0.18 ± 0.08
6				9.73 ± 1.01
7	$NHCOCH_3$	H		0.27 ± 0.06
8	$NHCOCH_3$	OH		0.69 ± 0.01
9	$NHCOCH_2OH$	H		0.03 ± 0.03
10	$NOHCOCH_3$	H		13.29 ± 0.39

[a] Rates are expressed as nanomoles of 4-aminoazobenzene acylated per milligram of mg protein per minute (mean ± SE, $N = 3$) with a partially purified hamster hepatic enzyme. The procedure is described under Experimental Section.

Synthesis. The 1- and 3-hydroxy-2-acetamidofluorenes (**1** and **2**, Table I) were prepared by acetylation of 1- and 3-hydroxy-2-aminofluorene, and the glycolamides (**3**, **5**, and **9**) were prepared by condensation of the appropriate aryl amines with ethyl glycolate as described by Shapiro et al.[13] 3-Hydroxy-4-acetamidobiphenyl (**8**) was obtained by reduction of 3-hydroxy-4-nitrobiphenyl with stannous chloride and subsequent acetylation of the amine with acetic anhydride.

Results

Transacylation Assay. A characteristic of AHAT (NAT) is the ability to catalyze the transfer of acyl groups from acetylcoenzyme A or from *N*-arylhydroxamic acids to aryl amides (Scheme I). The method of Booth was used to measure the abilities of the *o*-hydroxyaryl amides and glycolamides to serve as acyl donors in the hamster hepatic AHAT-catalyzed transacylation of 4-aminoazobenzene.[5] The results are shown in Table I. Only 3-hydroxy-4-acetamidobiphenyl (**8**) was more active than 4-acetamidobiphenyl itself, and none of the *o*-hydroxy or glycolamido derivatives was more than 5% as active as the hydroxamic acid, *N*-hydroxy-4-acetamidobiphenyl (**10**).

The formation of 4-acetamidoazobenzene, the product of transacetylation of the acetyl group of **8** to 4-aminoazobenzene, was confirmed by TLC analysis of ethyl acetate extracts of incubation mixtures containing **8**, 4-

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Table II. Effect of Inhibitors on Arylhydroxamic Acid N,O-Acyltransferase Catalyzed Transacetylation of 4-Aminoazobenzene

inhibitor	substrate:	% inhibn[a] 8[b]	10[c]
4-chloromercuri-benzenesulfonate		92	96
N-ethylmaleimide		77	86
iodoacetamide		99	99
2-acetamidofluorene		49	59

[a] Results are expressed as percent inhibition of 4-aminoazobenzene transacetylation measured after a 10-min incubation of partially purified hamster hepatic enzyme with inhibitor (1 mM). The procedure is described under Experimental Section. The results are expressed as the means of three experiments, each done in triplicate. Control activities: 0.84 ± 0.14 and 12.31 ± 1.20 nmol (mg of protein)$^{-1}$ min^{-1} (mean ± SE, N = 3) for 8 and 10, respectively. [b] Compound 8 = 3-hydroxy-4-acetamidobiphenyl. [c] Compound 10 = N-hydroxy-4-acetamidobiphenyl.

aminoazobenzene, and partially purified hamster hepatic AHAT; extracts of incubation mixtures containing 8, 4-aminoazobenzene, and heat-denatured enzyme did not contain detectable amounts of 4-acetamidoazobenzene. 4-Glycolamidoazobenzene could not be detected by TLC of ethyl acetate extracts from incubation mixtures of **3** or **9** with hamster hepatic AHAT and 4-aminoazobenzene. The rate of the AHAT-catalyzed transacetylation of 4-aminoazobenzene was dependent upon the concentration of **8** and was linear for incubation periods up to at least 15 min (data not presented).

The data shown in Table II demonstrate that the enzyme-catalyzed transacetylation of 4-aminoazobenzene by **8** was sensitive to the same sulfhydryl reagents and to the same reversible inhibitor (2-acetamidofluorene) that are known to decrease AHAT activity.[3] The similar extent of inhibition when either **8** or the biphenylhydroxamic acid **10** was used as the substrate supports the conclusion that both reactions are catalyzed by AHAT.

When compounds **8**, **7**, or **10** were incubated with partially purified rat hepatic AHAT in the presence of 4-aminoazobenzene, the transacetylation rates were 0.69 ± 0.04, 0.19 ± 0.07, and 5.62 ± 0.56 nmol (mg of protein)$^{-1}$ min^{-1} (mean ± SE, N = 3), respectively. Thus, the o-hydroxyamide **8** appears to be as good a substrate for rat hepatic AHAT as for the hamster enzyme, whereas the hydroxamic acid **10** exhibits considerably more activity in the presence of the hamster enzyme preparation.

Electrophile Generation Assay. As indicated in Scheme I, AHAT can catalyze the transfer of an acyl group to the oxygen atom of an arylhydroxylamine to produce a reactive N-acyloxyamino compound. Enzymatic O-acylation of 4-(hydroxylamino)biphenyl yields an [(acyloxy)amino]biphenyl, which can be captured by radiolabeled N-acetylmethione to produce a sulfonium intermediate; thermal decomposition of the sulfonium adduct affords radiolabeled 3-(methylthio)-4-aminobiphenyl, which can be extracted from the medium and quantified as described under the Experimental Section.[2] As expected, based on their low activities in the transacylation assay, none of the o-hydroxyaryl amides or glycolamides exhibited more than 4% of the activity of N-hydroxy-4-acetamidobiphenyl (**10**) when incubated in the presence of partially purified hamster hepatic AHAT. Compound **8**, which exhibited the highest rate of transacylation, supported methylthio adduct formation at a rate of only 1.05 ± 0.33 nmol (mg of protein)$^{-1}$ 30 min^{-1} (mean ± SD for triplicate incubations). The highest rate of methylthio adduct formation observed with any member of this group of compounds was 1.27 ± 0.17 nmol (mg of protein)$^{-1}$ 30 min^{-1} (mean ± SD for triplicate incubations) when the glycolamide **5** was used as substrate, whereas the rate for the biphenylhydroxamic acid **10** was 32.33 ± 4.43 nmol (mg of protein)$^{-1}$ 30 min^{-1} (mean ± SD, N = 4).[14]

Discussion

Previous studies have shown that AHAT activity is dependent upon certain molecular features of the N-arylhydroxamic acid, including acyl group structure, the structure and type of substitution of the aromatic ring, and the position of substitution of the aromatic ring by the N-hydroxyacyl amide moiety.[2,15,16-18] The compounds chosen for this study contain the biphenyl, fluorene, or stilbene ring systems, and although N-arylhydroxamic acids that contain these polycyclic aromatic ring systems are excellent substrates for rat and hamster hepatic AHAT, the o-hydroxyaryl amides and arylglycolamides were very poor substrates (Table I). The most active compound in this group, **8**, was almost 20 times less active than the hydroxamic acid **10** in the 4-aminoazobenzene transacylation assay. The low level of activity was not due to the depletion of substrates by deacylases. Glowinski et al. reported that deacylase activity was not detectable in hamster hepatic cytosol.[19] Also, it has been demonstrated that the deacylase inhibitor bis(p-nitrophenyl) phosphate[20] has no effect on the rate of the transacetylation of 4-aminoazobenzene by N-hydroxy-2-acetamidofluorene (**4**) in the presence of a partially purified hamster hepatic AHAT preparation.[21]

Several phenolic compounds are formed during metabolism of the carcinogen 2-acetamidofluorene. Although 1-hydroxy-2-acetamidofluorene (**1**) and 3-hydroxy-2-acetamidofluorene (**2**) are often minor metabolites, compared to 7-hydroxy-2-acetamidofluorene, the former compounds are reported to be selectively retained by isolated rat hepatocytes, whereas the 7-hydroxylated metabolite and other phenolic metabolites are excreted into the medium.[22a] It has been proposed that enzyme-catalyzed isomerization of the carcinogenic hydroxamic acid N-hydroxy-2-acetamidofluorene to **1** and **2** is a metabolic detoxification pathway.[22b] The results of the present study indicate that metabolites **1** and **2** are not likely to be further metabolized by the AHAT of hepatic cytosol. Similarly, the disposition of the N-arylglycolamides **3** and **9**, which are minor metabolites of the parent aryl amides, is not likely to be influenced by interaction with AHAT. Shirai et al. have reported that **9** does not induce tumors in female rats.[23] The ability of **8** to support a low level of transacylation

(14) The previously reported rate of methylthio adduct formation for N-hydroxy-4-acetamidobiphenyl was higher than the present value.[17] The rate given in the present paper is the correct value.

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activity in vitro with both rat and hamster enzyme preparations is of interest because 3-hydroxy-4-aminobiphenyl, the product of the deacylation of 8 by AHAT, is a potent carcinogen in newborn male mice.[24]

The results listed in Table I suggest that there is a rather strict positional requirement for the hydroxyl group of AHAT substrates. Movement of the hydroxyl group of *N*-arylhydroxamic acids to positions on the ring that are ortho to the amide or α to the carbonyl group dramatically diminishes the ability of the substrate to undergo AHAT-catalyzed acyl transfer. Therefore, neither the inductive effect of the hydroxyls in these positions nor any intramolecular hydrogen bonding that may occur is sufficient to cause the compounds to serve as substrates for AHAT. The poor substrate activity of the *o*-hydroxy amides and glycolamides may in part be due to unfavorable steric interactions with the enzyme. It is known that *N*-arylhydroxamic acids with acyl groups larger than acetyl are often poor substrates for AHAT.[15,16]

Experimental Section

Melting points were determined with a Mel-Temp apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 237B Grating infrared spectrophotometer. Mass spectra were recorded in the electron-impact mode at 70 eV with an AEI MS-30 spectrometer (probe temperature 175–200 °C). Elemental analyses were performed by Midwest Microlab (Indianapolis, IN) and were within ±0.4% of theoretical values.

2-Amino-3-hydroxyfluorene was purchased from K & K Rare and Fine Chemicals, and L-[*Me*-^{14}C]methionine was obtained from New England Nuclear. *N*-Acetyl-L-[*Me*-^{14}C]methionine (0.2–0.4 mCi/mmol) was prepared according to the procedure of Wheeler and Ingersoll.[25] Dr. H. T. Nagasawa (Veteran's Administration Hospital, Minneapolis, MN) provided a sample of 1-hydroxy-2-aminofluorene.

The following compounds were obtained according to published procedures: 3-hydroxy-4-nitrobiphenyl, mp 96 °C (lit.[26] mp 103 °C); 3-hydroxy-4-aminobiphenyl, mp 183–185 °C (lit.[27] mp 185–186 °C); 4-acetamidobiphenyl (**7**), mp 172–173 °C (lit.[28a] mp 171–172 °C); 4-acetamidoazobenzene, mp 159–160 °C (lit.[28b] mp 141 °C); 3-hydroxy-4-acetamidobiphenyl (**8**), mp 194–196 °C (lit.[27] mp 199–200 °C); 1-hydroxy-2-acetamidofluorene (**1**), mp 202–204 °C (lit.[29] mp 208 °C); 3-hydroxy-2-acetamidofluorene (**2**), mp 235–236 °C (lit.[29] mp 238 °C); 4-(hydroxyamino)biphenyl, mp 149–150 °C (lit.[30] mp 150–153 °C); *N*-hydroxy-4-acetamidobiphenyl (**10**), mp 149–150 °C (lit.[31] mp 150 °C); 4-glycolamidobiphenyl (**9**), mp 179–180 °C (lit.[12a] mp 177–178 °C); and 2-glycolamidofluorene (**3**), mp 187–188 °C (lit.[12a] mp 188–189 °C). The synthesis of **4** and **6** have been described.[8,32]

***trans*-4-Glycolamidostilbene (4).** A mixture of 0.23 g (1.2 mmol) of *trans*-4-aminostilbene,[33] 3.5 g (33.6 mmol) of ethyl glycolate, and 10 mL of benzene was heated under reflux for 24 h. Petroleum ether (15 mL) was added to the cooled mixture, and the yellow crystals were collected by filtration (0.11 g) and recrystallized from ethyl acetate: yield 0.08 g (28%); mp 215–216 °C; IR (KBr) 3425, 3340, 3020, 1675, 1600 cm^{-1}; EIMS, *m/e* (relative intensity) 253 (M^+). Anal. ($C_{16}H_{15}NO_2$) C, H, N.

4-Glycolamidoazobenzene. A mixture of 2 g (0.01 mol) of 4-aminoazobenzene, 20 g (0.20 mol) of ethyl glycolate, and 100 mL of benzene was heated under reflux for 6 days. The solvent was removed under reduced pressure, and the residue was triturated with 100 mL of ethyl acetate–cyclohexane (1:1, v/v). The dark orange crystals were collected by filtration (1.1 g) and recrystallized from ethyl acetate: yield 0.80 g (32%); mp 166–167 °C; IR (KBr) 3375, 3325, 1660, 1515 cm^{-1}; EIMS, *m/e* (relative intensity) 255 (M^+). Anal. ($C_{14}H_{13}N_3O_2$) C, H, N.

Enzymatic Studies. Male Sprague–Dawley rats were purchased from Bio-Lab (White Bear Lake, MN), and male Golden Syrian hamsters were obtained from Charles River (Wilmington, MA). Ultracentrifugation was performed on a Beckman L5-65 ultracentrifuge, and low-spin centrifugation was performed on a Beckman J-21B or a J2-21 centrifuge. A Beckman 34 spectrophotometer was used. Incubations were performed in a Dubnoff shaker bath. Dithiothreitol (DTT), L-methionine, and Grade III NAD^+ were obtained from Sigma Chemical Co.

Tissue Preparation. The details of the tissue preparation have been reported recently.[16,18]

Enzyme Preparation. The details of the enzyme preparation have been reported.[3,16,18]

AAB Transacylation Assay. Incubation mixtures contained sodium pyrophosphate buffer (50 μmol, pH 7), DTT (1 μmol), 0.07–0.125 mL of enzyme preparation (2.5–3.75 mg of protein from partially purified AHAT), 4-aminoazobenzene (0.375 μmol), substrate (2.5 μmol), and sufficient 1.15% KCl to give a final volume of 2.5 mL. In experiments with **6**, 0.25 μmol of **6** and 0.1 μmol of 4-aminoazobenzene were used. Reactions were started by the addition of a mixture of substrate and 4-aminoazobenzene. The following solvents were used for the test compounds: 95% ethanol (0.1 mL), for **8**, **1**, **4**, **3**, and **10**; Me_2SO (0.05 mL), for **2**, **9**, and **7**; ethanol–Me_2SO (3:1, 0.1 mL), for **5**; and Me_2SO–$MeCH_2CH_2OH$ (1:1, 0.05 mL), for **6**. The mixtures were incubated in air at 37 °C for 15 min. Incubation time was 5 min for **10**, 2 min for **4**, and 1 min for **6**. The reactions were terminated by the addition of 2.5 mL of 20% (w/v) trichloroacetic acid in 50% (v/v) EtOH–H_2O, followed by centrifugation of the mixture in a desk-top centrifuge. The supernatants were analyzed spectrophotometrically as described by Booth.[5] Control experiments were carried out with heat-denatured enzyme.

In the inhibition experiments, the inhibitor (2.5 μmol) in 0.05 mL of EtOH was incubated with the enzyme for 5 min prior to the addition of the substrate and 4-aminoazobenzene. Incubations were then carried out as described above.

For TLC analysis of the incubation mixtures, the reactions were terminated by the addition of ethyl acetate (5 mL). The mixtures were saturated with solid NaCl and then centrifuged, and the organic phases were separated. The extracts from three or more incubation mixtures were combined, dried (Na_2SO_4), and concentrated under reduced pressure. Portions of the extracts were then chromatographed along with authentic samples of the substrate, 4-aminoazobenzene, and the expected 4-(acylamido)-azobenzene. Compound **8** and 4-acetamidoazobenzene were separated on plastic-backed silica strips (Eastman, no. 6060) with either acetic acid–benzene (95:5) or chloroform–ethyl acetate (3:1) eluents. Brinkman precoated silica gel plates (60F 254) were used to separate **9** and 4-glycolamidoazobenzene (benzene–methanol; 6:1), as well as **5** and 4-glycolamidoazobenzene (petroleum ether–acetone; 2:1). The compounds were visualized under ultraviolet light.

Electrophile Generation Assay. Incubations mixtures contained sodium phosphate buffer (41 μmol, pH 6.8), 0.03–0.075 mL of AHAT preparation (1.0–1.5 mg of protein), *N*-acetyl-L-[*Me*-^{14}C]methionine (10 μmol, 0.2–0.4 mCi/mmol), 4-(hydroxyamino)biphenyl (0.1 μmol, dissolved in 0.05 mL of 95% ethanol), NAD^+ (0.8 μmol), substrate (1.0 μmol dissolved in 0.1 mL of 95% ethanol or other solvents as described under AAB transacylation assay), and sufficient H_2O to give a final volume of 1.0 mL. Reactions were begun by the addition of enzyme or substrate and were terminated after 30 min of shaking in air at 37 °C by the addition of 5 mL of cold diethyl ether. The amount of benz-

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ene–hexane extractable radiolabeled methylthio adduct formed was measured by the procedure of Bartsch et al.[2,16] Control experiments were carried out with heat-denatured enzyme.

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S-Carbobenzoxyglutathione: A Competitive Inhibitor of Mammalian Glyoxalase II

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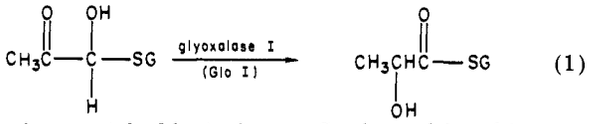
An effective competitive inhibitor of mammalian glyoxalase II has been synthesized and studied. The compound, *S*-carbobenzoxyglutathione, is almost totally inactive as an inhibitor of mammalian glyoxalase I. This is in marked contrast to other glyoxalase II competitive inhibitors, which in general are even more effective against glyoxalase I. *S*-Carbobenzoxyglutathione has found utility as an affinity ligand for the purification of rat liver glyoxalase II, and it may well have use in the study of the glyoxalase enzymes in vivo.

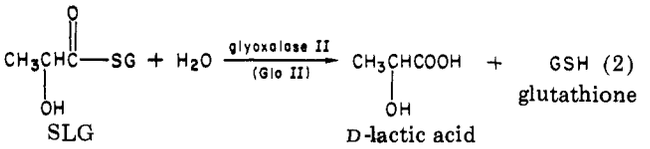
The widely distributed glyoxalase system[1-6] catalyzes the following reactions:



(1)

hemimercaptal adduct of methylglyoxal and glutathione

S-D-lactoylglutathione (SLG)

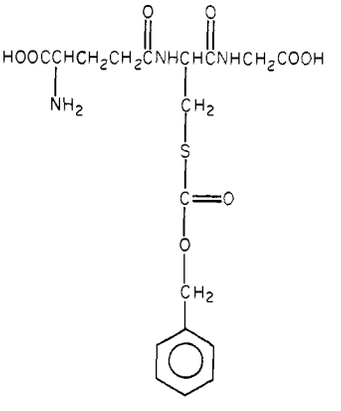


SLG

D-lactic acid

Over the many years of study of the enzymes of the glyoxalase system, Glo I and Glo II (EC 4.4.1.5 and 3.1.2.6, respectively), a number of potential inhibitors of these enzymes have been synthesized and studied in vivo and in vitro.[7-12] Some of these inhibitors have antitumor activity.[8,9,12] Most of the compounds that serve as effective inhibitors have a structural relationship to the substrates of Glo I and Glo II; consequently, they generally act as competitive inhibitors of both of these enzymes. Thus, the most effective inhibitory compounds have been various alkyl, aryl, and phenacyl thioethers of GSH. An example, *S*-(*p*-chlorophenacyl)glutathione is an excellent inhibitor of both Glo I and Glo II;[7,13] it has been utilized in Glo II active-site mapping studies and as an affinity ligand in the purification of Glo II.[13,14]

While it might be anticipated that various alkyl and aryl thioesters of GSH might serve as inhibitors of the glyoxalase enzymes, the thioesters often serve as substrates for Glo II and, in general, are ineffective inhibitors of Glo I in crude systems or in vivo.[15] While searching for better ligands for the affinity chromatographic purification of rat liver Glo II, we synthesized a new inhibitor of Glo II. The compound, *S*-carbobenzoxyglutathione (CBG), is not hydrolyzed by Glo II, does not inhibit Glo I, and is a potent competitive inhibitor of Glo II. This is the first report, to our knowledge, of a competitive inhibitor of Glo II that is inactive toward Glo I from various sources. Further, the compound has been used effectively as an affinity ligand in a two-step total purification of rat liver Glo II.[16]



CBG

Results and Discussion

The synthesis of CBG was carried out under conditions that preclude condensation of carbobenzoxy chloride with the single free amino group of glutathione; thus, the pH of the aqueous phase of the reaction mixture was kept at pH 7.8 or lower. Oxidation of glutathione to oxidized glutathione could be largely prevented by carrying out the

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