9-(5′,6′-Dideoxy-β-D-*ribo*-hex-5′-ynofuranosyl)adenine, a Novel Irreversible Inhibitor of *S*-Adenosylhomocysteine Hydrolase†

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ABSTRACT: The acetylenic analogue of adenosine 9-(5′,6′-dideoxy-β-D-*ribo*-hex-5′-ynofuranosyl)adenine has been synthesized, and its behavior as an inhibitor of bovine *S*-adenosylhomocysteine hydrolase has been examined. Incubation of the enzyme with excess inhibitor caused a time-dependent, irreversible inactivation of the enzyme that was accompanied by the reduction of two equivalents of NAD^+ to NADH and the loss of the two remaining equivalents of NAD^+. With use of radiolabeled inhibitor, it was established that 4 equiv of the acetylenic analog bind irreversibly to the enzyme and that 4 equiv were required to inactivate the enzyme completely. The inactivated enzyme could not be reactivated by incubation with NAD^+. Denaturation studies revealed that 2 equiv of the inhibitor are bound more tightly to the enzyme than the remainder, suggesting the formation of a covalent linkage between the oxidized inhibitor and the enzyme. The putative covalent linkage was found to be acid sensitive but stable to mild base. The linkage could not be stabilized by treatment of the enzyme–inhibitor complex with either borohydride or cyanoborohydride. A K_I of 173 nM was measured for the inhibitor, making it one of the more potent inhibitors that have been reported. The enzyme used in these studies was isolated by modification of an affinity chromatography method reported by Narayanan and Borchardt [(1988) *Biochim. Biophys. Acta 965*, 22–28]. The affinity chromatography unexpectedly led to the isolation of two forms of the enzyme. The major form contained 4.0 mol of nucleotide cofactor/mol of enzyme tetramer, while the minor form carried only 2.0 mol/tetramer.

The enzyme *S*-adenosylhomocysteine hydrolase (SAH hydrolase) (E.C. 3.3.1.1) catalyzes the reversible hydrolysis of *S*-adenosylhomocysteine (**1**) (SAH) to adenosine (**2**) and homocysteine (**3**) in the absence of additional cofactors (Scheme I). In recent years considerable interest has emerged in pharmacological agents that inhibit SAH hydrolase (Ueland, 1982; Hershfield, 1983; Chiang, 1984). This interest has been generated because SAH acts as a potent inhibitor of most methyltransferases and because adenosine is cytotoxic to humans lacking adenosine deaminase (Hershfield et al., 1979). SAH hydrolase was initially isolated from rat liver (de la Haba & Cantoni, 1959) and subsequently found to occur in a number of eukaryotes (Knudsen & Yall, 1972; Walker & Duerre, 1975; Guranowski & Pawelkiewicz, 1977; Richards et al., 1978; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Hohman et al., 1984; Sebestova et al., 1984; Hershfield et al., 1985) and prokaryotes (Shimizu et al., 1984). The enzyme from mammalian tissues is a tetramer with a molecular weight of ca. 190000 and generally carries 1 molecule of nucleotide cofactor/subunit. However, Narayanan and Borchardt (1988) were able to isolate SAH hydrolase from beef liver, which contained only 2 molecules of nucleotide cofactor/tetramer. Regardless of the total nucleotide cofactor content, between 10 and 50% of the cofactor bound to the enzyme is present in the reduced form (Hershfield et al., 1985; de la Haba et al., 1986; Narayanan & Borchardt, 1988). Gomi et al. (1989) demonstrated that the four subunits of SAH hydrolase are identical by expressing the rat liver SAH hydrolase cDNA in *Escherichia coli*. The cloned enzyme is a tetramer containing 4 mol of tightly bound NAD^+, and its kinetic properties are indistinguishable from the rat liver enzyme. SAH hydrolase has also been cloned from *Dictyostelium discoideum* (Kasir et al., 1988), and the deduced amino acid sequence has been compared with that of the rat liver enzyme. Interestingly, 74% of the amino acids were in identical positions, and if conservative changes were taken into account, the homology was 84%.

Elegant mechanistic studies with SAH hydrolase isolated from beef liver have established that the conversion of SAH to adenosine and homocysteine is a multistep process (Palmer & Abeles, 1979). The enzyme-bound NAD^+ oxidizes the 3′-hydroxy group of SAH to give the 3′-keto derivative. β-Elimination of homocysteine from the keto derivative yields 3′-keto-4′,5′-didehydro-5′-deoxyadenosine, which then undergoes a Michael addition of water to form 3′-ketoadenosine. Finally, the 3′-ketoadenosine is reduced to adenosine by enzyme-bound NADH. Both the elimination and addition steps of the hydrolysis have been shown to proceed with syn geometry (Parry & Askonas, 1985). This stereochemistry is in accord with the generalization that enzyme-catalyzed β-dehydrations, where the abstracted proton is α to a ketone, proceed with syn geometry (Widlanski et al., 1987).

The mechanism of the reaction catalyzed by SAH hydrolase suggests that 9-(5′,6′-dideoxy-β-D-*ribo*-hex-5′-ynofuranosyl)-adenine (**4**) (acetylenic adenosine) might serve as a novel, irreversible inhibitor of the enzyme. Enzymatic oxidation of acetylenic adenosine to the 3′-keto derivative (**5**) could be followed by isomerization to the allenic ketone (**6**) (Scheme II). The ketone **6** should be a potent electrophile capable of irreversible reaction with an active site nucleophile. The process outlined in Scheme II bears a close analogy to the classic example of "suicide" enzyme inactivation, the reaction of the *N*-acetylcysteamine thioester of 3-decynoic acid with β-hydroxydecanoyl thioester dehydrase (Walsh, 1982; Schwab et al., 1986).

In this paper, we report a synthesis of the acetylenic adenosine analogue **4** and show that it behaves as an irreversible mechanism-based inactivator of SAH hydrolase. The

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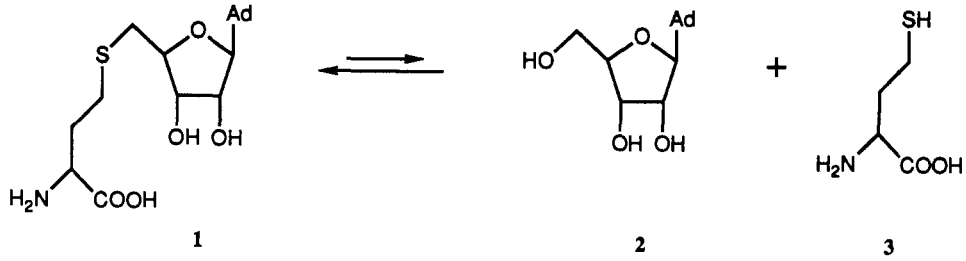
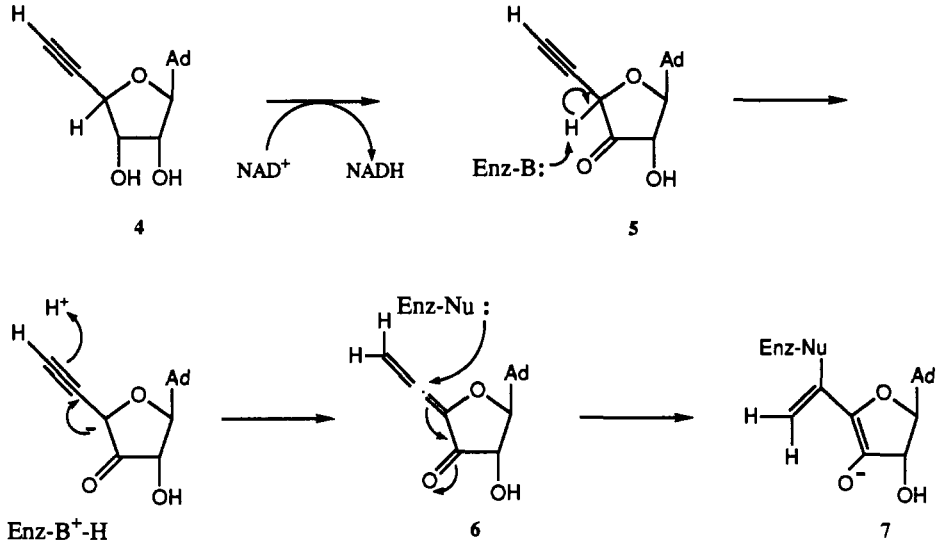
Scheme I: Hydrolysis of SAH by SAH hydrolase



1 2 3

Scheme II: Mechanism of Inactivation of SAH Hydrolase by Acetylenic Adenosine



4 5 6 7

adenosine analogue **4** has also been prepared and studied as an SAH hydrolase inhibitor by Edwards et al. (1991) (J. R. McCarthy, private communication, 1990).

Materials and Methods

Materials. Tritium-labeled compounds were obtained from Amersham Corp. and from New England Nuclear. The bovine liver was from a local slaughterhouse. Blue dextran–agarose, aprotinin, DTT, and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. Adenosine deaminase, NAD^+, and horse liver alcohol dehydrogenase were obtained from Boehringer Mannheim. DEAE-cellulose was obtained from Bio-Rad Laboratories and Sigma Chemical Co. All other reagents were purchased from Aldrich Chemical Co. and Fluka and were used without further purification. All solvents were dried and redistilled. Dimethyl diazomethylphosphonate and N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde were prepared by literature procedures (Gilbert & Weerasooriya, 1979; Colvin & Hamill, 1977; Ranganathan et al., 1974). [2-^{3}H]-*S*-Adenosyl-L-homocysteine was prepared by a modification of the method by Sakami and Stevens (1958).

SAH Hydrolase Assay. SAH hydrolase activity was assayed in the hydrolytic direction by use of either the Narayanan and Borchardt modification (1988) of the radioactive assay method of Richards et al. (1978) or by use of the spectrophotometric procedure of Palmer and Abeles (1979). The activities obtained from either assay fell within 10% of each other.

Protein Determination. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. The homogeneity of purified SAH hydrolase was confirmed by SDS–polyacrylamide discontinuous gel electrophoresis with a 4% stacking gel, pH 8.8, and a 10% separating gel, pH 6.8, according to the method of King and Laemmli (1971). The gels were stained with Coomassie brilliant blue and scanned on a transmission densitometer (E/C Apparatus Corporation, Model EC 910).

Isolation of S-Adenosylhomocysteine Hydrolase. All manipulations were conducted at 4 °C unless otherwise noted. The purification procedure is based on the method of Hershfield et al. (1985) with modifications by Narayanan and Borchardt (1988). The later stages of the purification did not work *in our hands*, so extensive modifications were necessary.

Bovine liver (480 g) was minced in 200 mL of a solution containing 1 mM DTT, 1 mM EDTA, 3 mL of aprotinin, and 100 mg of phenylmethanesulfonyl fluoride. The suspension was homogenized 10 times in a Waring blender for periods of 1 min. During homogenization the temperature was kept below 6–7 °C by cooling with an ice bath. The homogenate was centrifuged at 10000*g* for 1 h. About 180 mL of brown supernatant was collected, and KCl was added to a final concentration of 75 mM. DEAE-cellulose (300 mL) was then added, and the mixture allowed to stand for 15 min. The suspension was filtered with a Buchner funnel, with use of Whatman paper (No. 41). The filtrate was collected in a suction flask containing 100 mg of phenylmethanesulfonyl fluoride. The cellulose was washed with an additional 60 mL of 25 mM Tris buffer, pH 7.4, containing 1 mM DTT and EDTA (buffer A). Ammonium sulfate was added to the combined filtrate (460 mL) to achieve 71% saturation, and the mixture was allowed to stand for an hour. After the suspension was centrifuged for 30 min at 10000*g*, the clear red supernatant was carefully discarded and the brown precipitate was redissolved in 60 mL of buffer A. The solution was dialyzed against 4 L of buffer A for 18 h with five to six

changes of buffer.

The dialyzed material was applied to a Q-Sepharose column (5 cm × 28 cm) equilibrated with buffer A containing 25 mM KCl. The column was eluted at a rate of 8 mL/min with 1 L of buffer A that was 50 mM KCl, followed by another liter that was 175 mM KCl. Twenty-milliliter fractions were collected, and the dark green fractions containing the activity were combined and concentrated by pressure ultrafiltration to a volume of 15 mL with use of an Amicon ultrafiltration cell and a membrane with a 30 000 molecular weight cutoff.

One-third of the concentrated enzyme solution (5 mL) was applied to a blue dextran–agarose column (1.5 cm × 70 cm) previously equilibrated with 10 mM Tris buffer, pH 6.8, containing 1 mM DTT and EDTA. The enzyme was eluted with 800 mL of the equilibration buffer (eluant 1) and 15-mL fractions were collected at a rate of 1 mL/min. The active fractions were combined and concentrated by ultrafiltration to a volume of 5–10 mL. The column was further eluted at a rate of 2 mL/min with 300 mL of the same buffer having adenosine present at a concentration of 0.5 mM (eluant 2). The total collected volume was concentrated to 5 mL. From this dye-affinity column, two types of *S*-adenosylhomocysteine hydrolase were isolated. Eluant 1 contained enzyme having 4 mol of cofactor bound per mole of tetramer (type A), while eluant 2 contained enzyme that carried out only half the number of cofactors (type B). The remaining two-thirds (10 mL) of enzyme solution was processed by carrying out two additional runs on the blue dextran–agarose column.

The concentrated enzyme solution was further purified by FPLC on an AX Synchropak column (10 mm × 250 mm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 100 mM sodium chloride. The enzyme was injected in 8-mg fractions and eluted at a rate of 3 mL/min with a linear gradient to a final concentration of 550 mM sodium chloride over 20 min. The pure enzyme was collected between 9 and 11 min. These fractions were combined and concentrated by ultrafiltration to a volume of 0.5 mL. Sodium chloride was removed by repeated dilution with 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and EDTA, followed by reconcentration using ultrafiltration.

The isolated enzyme was more than 95% pure as determined by SDS–PAGE. The final specific activities varied between 0.4 and 0.5 IU.

Mechanistic Studies

(*1*) *Irreversible Inactivation Procedure.* SAH hydrolase (18 μL, 400 μg) was diluted with 50 mM potassium phosphate buffer, pH 7.6, to a total volume of 70 μL. Acetylenic adenosine (1 μM, 261.3 μg) was added to the reaction mixture, and the solution was incubated in a shaking water bath at 37 °C for 30 min. The reaction mixture was either dialyzed against 200 mL of the incubation buffer with four changes or applied to a Sephadex G-25 column (1 cm × 30 cm) and eluted with 100 mM potassium phosphate buffer, pH 7.6, at a rate of 20 mL/h. The enzyme was collected in the void volume. The residual enzyme activity was determined and compared to a control containing no inhibitor.

(*2*) *Time- and Concentration-Dependent Inactivation.* Acetylenic adenosine was preincubated at 37 °C at various concentrations (225 nM, 452 nM, 586 nM and 1.3 μM) in 5 mL of 20 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA. The reaction was started by the addition of SAH hydrolase (72.3 μg, 0.43 IU). Aliquots (0.975 mL) were removed every 5 min for a total of 30 min and added to 25 μL of a solution containing SAH (108 μM) and ADA (4 IU) to assay for residual activity.

(*3*) *Determination of the K_I.* Various concentrations of SAH hydrolase were preincubated for 5 min at 37 °C with the indicated concentrations of acetylenic adenosine in 975 μL of 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA. An assay to determine the residual SAH hydrolase activity was started by the addition of SAH (25 μL, 88.6 μM) and ADA (4 IU).

(*4*) *HPLC Synthesis Study.* The assays were set up at 37 °C in 1 mL of 10 mM potassium phosphate buffer, pH 7.6, containing 20 mM DL-homocysteine and 1 mM EDTA. The assay consisted of either acetylenic adenosine (0.5 mM), adenosine (0.5 mM), or adenosine (0.25 mM) plus acetylenic adenosine (0.25 mM) and was started by the addition of SAH hydrolase (14.69 μg, 0.15 IU). The reactions were monitored every 30 min by HPLC with use of the system of Chabannes et al. (1979).

(*5*) *Stoichiometry of the Acetylenic Adenosine Binding.* Enzyme–inhibitor complex was prepared in section 1 using [6′-^{3}H]acetylenic adenosine. The reaction mixture was dialyzed against 200 mL of the incubation buffer with four buffer changes. The residual enzyme activity was determined and compared to a control containing no inhibitor. The sample was then analyzed for its protein and tritium content.

(*6*) *Determination of the Minimum Number of Equivalents of Acetylenic Adenosine Needed To Inactivate the Enzyme.* SAH hydrolase (1.01 nM, 0.2 mg) was incubated with various concentrations of acetylenic adenosine in 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. Aliquots were removed after 1, 2, and 3 days and assayed for enzyme activity. The activity was expressed relative to a control incubation carried out in the absence of the inhibitor. Simultaneously, the binding stoichiometries were determined as in section 5.

(*7*) *Effect of Urea Treatment on the Enzyme–Inhibitor Complex.* Tritiated enzyme–inhibitor complex was prepared as described in section 1 and subsequently dialyzed against four portions of 200 mL of 20 mM potassium phosphate buffer, pH 7.6, containing 8 M urea at 4 °C. Samples were taken after 1, 2, 3, 4, and 5 days. The final work-up consisted of removal of urea by exhaustive dialysis against 200 mL of 20 mM potassium phosphate buffer, pH 7.6, with three buffer changes. Protein content and radioactivity were then determined. The same experiments were repeated at room temperature.

(*8*) *Reduction of the Enzyme–Inhibitor Complex with $NaBH_4$.* Labeled enzyme–inhibitor complex (133 μg) was prepared as described in section 1 and was diluted 1:1 with 20 mM potassium phosphate buffer, pH 7.6, containing 8 M urea. $NaBH_4$ (10 μL of a 0.1 M solution) was added, and the solution was incubated for 9 h at room temperature. The final work-up is described in section 7. The same experiment was repeated under nondenaturating conditions.

(*9*) *Reduction of the Enzyme–Inhibitor Complex with $NaCNBH_3$.* Labeled enzyme–inhibitor complex (525 μg in 525 μL of 50 mM potassium phosphate buffer, pH 7.6) was prepared as in section 1 and diluted 1:1 with 0.1 M citrate buffer, pH 3.55, containing 8 M urea. $NaCNBH_3$ (2 mg) was added to the reaction mixture, and the incubation was carried out for 21 h. The solution had a final pH of 3.9. The final work-up is described in section 7. The same experiment was repeated under nondenaturating conditions.

(*10*) *Reduction of the Native Enzyme and the Enzyme–Inhibitor Complex with [^{3}H]$NaBH_4$.* Unlabeled enzyme–inhibitor complex (271 μg) was diluted to a total volume of 240 μL with 20 mM potassium phosphate buffer, pH 7.6, containing 8 M urea. [^{3}H]$NaBH_4$ (1 μM, sp act. 3.5 mCi/mmol)

was added, and the mixture was allowed to incubate for 22 or 33 h at room temperature. The final work-up is described in section 7. The native enzyme was treated in an identical fashion.

(*11*) *Denaturation of the Reduced Enzyme–Inhibitor Complex.* Labeled enzyme–inhibitor complex (67 μg in 90 μL of 20 mM potassium phosphate buffer, pH 7.6) was reduced with $NaBH_4$ or $NaCNBH_3$ as described in sections 8 and 9 and then dialyzed against either 200 mL of an aqueous solution of 1% SDS and 0.1% TFA, pH 2.2, for 15 h at room temperature or 200 mL of 20 mM potassium phosphate buffer, pH 7.6, containing 8 M urea for 20 h at 4 °C. The final work-up is described in section 7.

(*12*) *Determination of NAD^+ and NADH.* The assay is based on a private communication from R. Abeles (1990) and uses a procedure that is a minor modification of that reported by Palmer and Abeles (1979). $HClO_4$ was added to the enzyme solution to a final concentration of 0.2 M, and the mixture was incubated for 5 min at 4 °C. It was then neutralized with 1 N KOH and centrifuged. The supernatant was removed, and the precipitate was washed two additional times with 20 μL of H_2O. All the fractions were combined and used in the following NAD^+ assay.

Glycine hydrochloride (0.2 mL of a 0.25 M solution) containing 0.9 mM glutathione was mixed with 0.2 mL of a 46.5 mM semicarbazide hydrochloride solution, pH 7.0, and 20 μL of 95% EtOH in a 1-mL cuvette. The NAD^+-containing solution (76 μL) was added. The solution was mixed well and used as the blank to measure background absorption at 340 nm. Horse liver alcohol dehydrogenase (0.04 mg) was then added to start the reaction, and the mixture was incubated for 15 min at room temperature. The absorbance at 340 nm was determined, and the absolute NAD^+ content was calculated in comparison to a standard curve (2–10 nM NAD^+).

(*13*) *Synthesis of 9-(5′,6′-Dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine* (**4**)

Synthesis of N^6-Benzoyl-2′,3′-O-isopropylidene-9-(5′,6′-dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine (**9**). The procedure utilizes a synthetic method reported by Gilbert and Weerasooriya (1979). In a dry 25-mL three-necked flask equipped with a magnetic stirbar, a gas inlet tube, and a rubber stopper, potassium *tert*-butoxide (1.22 mM) was suspended in dry THF (1.5 mL), and the flask was flushed with nitrogen and cooled to −78 °C. A solution of dimethyl diazomethylphosphonate (Colvin & Hamill, 1977) (180 mg) in THF (3 mL) was added dropwise and allowed to react for 5 min. N^6-Benzoyl-2′,3′-O-isopropylideneadenosine-5′-aldehyde (**8**) (Ranganathan et al., 1974) (450 mg, 1.10 mM) in THF (3 mL) was added, and the reaction was kept stirring at −78 °C for 12 h. The reaction was then allowed to warm to room temperature and was stirred overnight. The reaction mixture was poured into saturated aqueous ammonium chloride (22 mL), the flask was rinsed with dichloromethane (20 mL) and water (10 mL), and the aqueous solution was concentrated to a final volume of 32 mL. The concentrate was then poured into a separatory funnel and extracted three times with dichloromethane (40 mL). The organic extracts were combined and washed twice with brine, dried over sodium sulfate, and evaporated. The residue was applied to a flash chromatography column and eluted with 10% acetone in chloroform. The fractions containing the acetylenic adenosine derivative **9** were combined and stripped, leaving a white crystalline solid (190.1 mg, 0.469 mM, 43% yield). mp: 215–218 °C (d). ^1H NMR (300 MHz, $CDCl_3$): δ 1.45 (s, 3 H, CMe), 1.62 (s, 3 H, CMe), 2.47 (d, 1 H, C-6′), 5.10 (s, 1 H, C-4′), 5.17 (d, 1 H, C-3′), 5.81 (d, 1 H, C-2′), 6.32 (d, 1 H, C-1′), 7.56, 8.05 (m, 5 H, ArH), 8.33 (s, 1 H, C-2), 8.87 (s, 1 H, C-8), 8.93 (br s, 1 H, NH). ^{13}C NMR (77.45 MHz, $CDCl_3$, MeOH reference): δ 164.23, 151.49, 148.62, 140.98, 132.41, 131.49, 127.43, 126.83, 113.12, 92.58, 91.33, 84.77, 83.31. IR ($CHCl_3$): 3360 (s), 2950, 2100 (w), 1680, 1582, 1550 cm^{-1}. HRMS: observed ion 1, M^+, $C_{21}H_{19}N_5O_4$; expected mass (amu) = 405.143665; observed mass (amu) = 405.1426, intensity ratio of M^+ to base peak = 0.059; observed ion 2, $(M + 1)^+$, $^{13}CC_{20}H_{19}N_5O_4$; expected mass (amu) = 406.147020; observed mass (amu) = 406.1467, intensity ratio of $(M + 1)^+$ to base peak = 0.019.

Synthesis of 2′,3′-O-Isopropylidene-9-(5′,6′-dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine (**10**). The protected acetylene **9** (11.0 mg, 0.027 mM) was dissolved in ammonia-saturated methanol (15 mL), and the reaction mixture was allowed to stand overnight. Volatiles were then removed in vacuo. The residue was applied to a silica gel column and eluted with 40% acetone in chloroform. The fractions containing the pure product **10** were combined, and the solvent was removed in vacuo. A white solid (7.13 mg, 0.0237 mM) was obtained (87%). mp: 172–174 °C (d). UV λ_{max} (MeOH): 259 nm (ε 10000). ^1H NMR (D_2O): δ 1.50 (s, 3 H, CMe), 1.67 (s, 3 H, CMe), 2.75 (s, 1 H, C-6′), 5.23 (d, 1 H, C-4′, J = 3.6 Hz), 5.40 (m, 1 H, C-3′), 5.68 (d, 1 H, C-2′, J = 5.7 Hz), 6.32 (s, 1 H, C-1′), 8.23 (s, 1 H, C-2), 8.26 (s, 1 H, C-8). IR (KBr): 3300 (br), 3100 (br), 2075 (s), 1650, 1575 cm^{-1}. HRMS: observed ion 1, M^+, $C_{14}H_{15}N_5O_3$; expected mass (amu) = 301.117455; observed mass (amu) = 301.1172, intensity ratio of M^+ to base peak = 0.066; observed ion 2, $(M + 1)^+$, $^{13}CC_{13}H_{15}N_5O_3$; expected mass (amu) = 302.120810; observed mass (amu) = 302.1206, intensity ratio of $(M + 1)^+$ to base peak = 0.014.

Synthesis of 9-(5′,6′-Dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine (**4**). Trifluoroacetic acid (5 mL, 90%) was added to 2′,3′-O-isopropylidene-9-(5′,6′-dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine (**10**) (18.3 mg, 0.061 mM) contained in a 25-mL round-bottom flask equipped with a stirbar, and the mixture was allowed to react for 40 min. The solvent was then removed in vacuo, and the residue was dissolved in a small volume of water. The solution was then loaded onto an AG 50W-X8 cation-exchange column, H^+ form, (1 cm × 14 cm), and the column was washed with 50 mL of water. Acetylenic adenosine was eluted with 750 mL of 1 N ammonium hydroxide. The solvent was removed from the eluant in vacuo, and the residue was applied to a silica gel flash column and eluted with EtOAc, $CHCl_3$, and acetone in a ratio of 3:1:3. White crystals (15 mg) of **4** were obtained (94%). mp: 208–210 °C (d). UV λ_{max} (H_2O): 259 nm (ε 11600). ^1H NMR (300 MHz) (D_2O): δ 1.94 (s, 1 H, C-6′), 4.61 (s, 1 H, C-4′), 4.89 (m, 1 H, C-3′), 5.06 (m, 1 H, C-2′), 6.16 (d, 1 H, C-1′, J = 5.6 Hz), 8.25 (s, 1 H, C-2), 8.41 (s, 1 H, C-8). ^{13}C NMR (77.45 MHz, DMSO-d_6): δ 156.05, 152.02, 149.55, 139.22, 118.93, 87.26, 81.17, 78.50, 75.15, 73.44, 72.92. IR (KBr): 3200 (br), 2075 (w), 1625, 1575 cm^{-1}. HRMS: observed ion 1, M^+, $C_{11}H_{11}N_5O_3$; expected mass (amu) = 261.086155; observed mass (amu) = 261.0856, intensity ratio of M^+ to base peak = 0.382; observed ion 2, $(M + 1)^+$, $^{13}CC_{10}H_{11}N_5O_3$; expected mass (amu) = 262.08951; observed mass (amu) = 262.0891, intensity ratio of $(M + 1)^+$ to base peak = 0.059.

Synthesis of [6′-^3H]-9-(5′,6′-Dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine (**4a**). A dry 15-mL three-necked flask equipped with reflux condenser connected with a gas inlet tube, stirbar, and rubber septum was flushed with nitrogen for 10

min. The protected acetylenic adenosine derivative **9** (25 mg, 0.062 mM) was added to the flask, and the flask was flushed for another 15 min. Dry THF (0.5 mL) was added followed by a solution of EtMgBr in THF (125 μL, 0.248 mM). The reaction mixture was heated to 50–55 °C for 5 h. After 5 h, an additional 2 equiv (63 μL) of EtMgBr was added, and heating was continued for another 2 h at 60 °C. The reaction mixture was cooled to room temperature, and 25 μL of $[^3H]H_2O$ (10 Ci/mL) was added, followed by overnight stirring. Excess solvent was removed in vacuo, and the residue was taken up in water and methylene chloride. The water layer was extracted three times with methylene chloride. The combined organic layers were washed twice with brine and dried over Na_2SO_4, and the solvent was evaporated in vacuo. The residue was applied to a silica gel flash column and eluted with 10% acetone in chloroform. The purified **9a** was deprotected in two steps as described for the unlabeled compound to yield 3.3 mg of **4a** (21% overall yield, sp act. 0.607 mCi/mmol). By carrying out an analogous reaction using deuterated water and examination of the product by ^1H NMR, it was established that the isotopic label was introduced only at C-6′.

(14) [2-^3H]-S-Adenosyl-L-homocysteine (**15**)

[2-^{3}H]-*S*-Adenosyl-L-homocysteine (**15**) was synthesized by a modification of the procedure of Sakami and Stevens (1958).

[2-^3H]-2′,3′-O-Isopropylidene-5′-(p-toluenesulfonyl)-adenosine (**17**). [2-^{3}H]-2′,3′-*O*-Isopropylideneadenosine (**16**) (306.2 mg, 0.996 mM), prepared from commercially available [2-^{3}H]adenosine by the method of Hampton (1961), was added to a dry 50-mL round-bottom flask equipped with a rubber septum and a stirbar. The flask was flushed with nitrogen, and pyridine (7 mL) was added. The solution was cooled to 0 °C, *p*-TsCl (220 mg, 1.154 mM) was added, and the flask was flushed with nitrogen. The reaction mixture was stirred for an additional 5 min to dissolve the *p*-TsCl, during which time the clear solution turned orange. The flask was transferred to a −20 °C refrigerator and allowed to stand for 24 h. After 24 h an additional 200 mg of TsCl was added with stirring, and the reaction was then allowed to continue at 0 °C for 24 h. The reaction mixture was then poured into 50 mL of ice water, and the suspension was stirred for 30 min. The crystals were collected by filtration and thoroughly washed with ice water. The light yellow solid was dried in vacuo and recrystallized from a mixture of hexane and chloroform. The white crystals were collected by filtration and dried in vacuo yielding 370.4 mg of tosylate (81%). The mother liquor from the recrystallization was evaporated in vacuo, and the residue was applied to a silica gel flash column with use of EtOAc as an eluant. An additional 30 mg of tosylate was recovered, resulting in an overall yield of 87.4% (401.8 mg). The spectral data agreed with the assigned structure.

[2-^3H]-2′,3′-O-Isopropylidene-S-adenosyl-L-homocysteine (**18**). A 15-mL three-necked flask equipped with a liquid ammonia condenser protected by a potassium hydroxide tube, a magnetic stirbar, and two gas inlet tubes was flushed with dry nitrogen, and fresh liquid ammonia (5 mL) was distilled into it. L-Homocystine (58.2 mg, 0.217 mM) and sodium metal (20 mg, 0.87 mM) were added under a stream of nitrogen. The reaction was stirred, and additional sodium was added until the blue color persisted for 15 min. Sufficient solid NH_4Cl was added to just discharge the blue color, and this was followed by addition of the tritiated tosylate **17** (200 mg, 0.433 mM). The reaction was refluxed for 10 h and then allowed to warm to room temperature overnight. The last traces of ammonia were removed in vacuo. The residue was dissolved in water, the solution was extracted two times with chloroform, and the aqueous phase was taken to dryness in vacuo. The residue was applied to a Sephadex G-10 column (1.5 cm × 100 cm) and eluted with water containing thiodiglycol (50 μL/L) at a rate of 25 mL/h. Fractions containing the product were combined, and the solvent was once again removed in vacuo. The residue was applied to a silica gel flash column and eluted with butanol/AcOH/MeOH (4:1.5:4.5). The tritiated homocysteine derivative **18** was obtained as a white crystalline solid (133.6 mg, 72.7% yield). The spectral data agreed with the assigned structure.

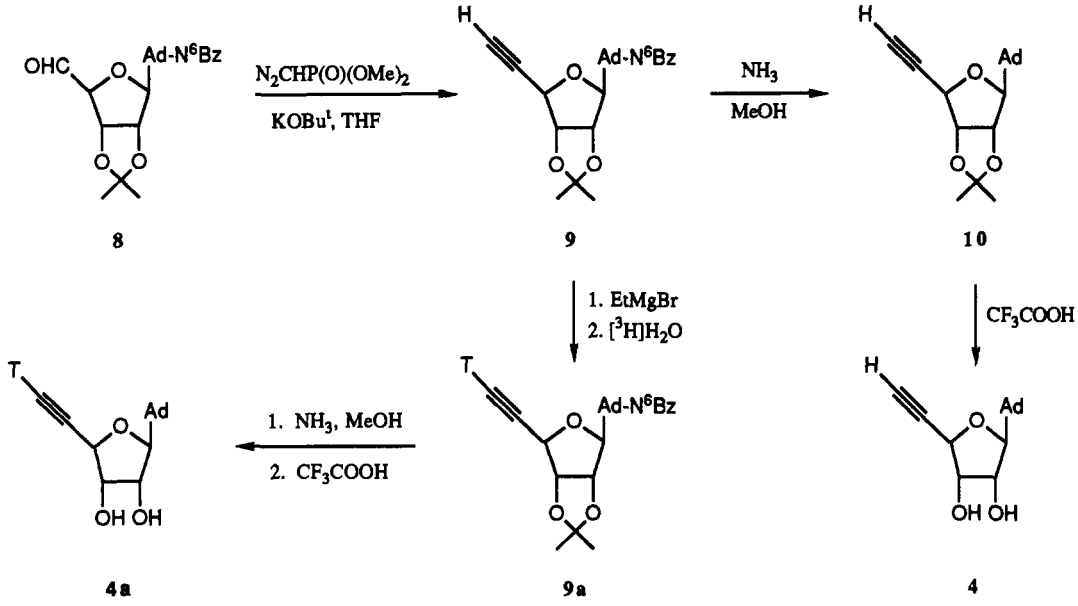
[2-^3H]-S-Adenosyl-L-homocysteine (**15**). Dithiothreitol (2.5 mg) and **18** (20.27 mg, 0.0478 mM) were added to a dry 5-mL pear-shaped flask equipped with a magnetic stirbar, and the flask was flushed with argon. The flask was sealed with a rubber stopper, and flushing was continued for 10 min. H_2SO_4 (1 N, 0.15 mL) was then added, and the flask was flushed with argon for an additional 15 min. The reaction was stirred at room temperature for 40 h, at the end of which time the solution was neutralized with $Ba(OH)_2$ (0.2 N, 0.75 mL). The suspension was centrifuged, the supernatant was removed, and the $BaSO_4$ precipitate was washed with warm water (5 mL) and recentrifuged. This process was repeated two times. The aqueous extracts were combined and lyophilized. The residue from lyophilization was applied to a Sephadex G-10 column and eluted with water containing thiodiglycol (50 μL/L) at a rate of 20 mL/h. The fractions containing SAH were pooled, and the solvent was removed in vacuo. The residue was triturated with acetone to remove traces of thiodiglycol. If necessary, the SAH was recrystallized from hot water in a Craig tube. The SAH was dried over P_2O_5 in vacuo for 2 h at 100 °C and overnight at 45 °C to yield 14.6 mg of white crystals (80% yield). The spectral data agreed with the assigned structure.

Results

Synthesis of Inhibitor and of Labeled SAH. The synthetic methodology used to prepare acetylenic adenosine (**4**) and [6′-^{3}H]acetylenic adenosine (**4a**) is outlined in Scheme III. The key steps include the conversion of N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde (**8**) (Raganathan et al., 1974) to N^6-benzoyl-2′,3′-*O*-isopropylidene acetylenic adenosine (**9**) in 43% yield with use of dimethyl diazomethylphosphonate (Colvin & Hamill, 1977) and the exchange of the 6′-hydrogen with tritiated water. The site of proton exchange was established by synthesizing the corresponding deuterated form of acetylenic adenosine. Analysis of the deuterated compound by ^1H NMR revealed a 65% replacement of the acetylenic proton by deuterium. A modified synthesis of SAH and [2-^{3}H]SAH (**15**) was developed on the basis of the method of Sakami and Stevens (1958); our improved procedure increased the overall yield from 21 to 51%.

Enzyme Purification. SAH hydrolase was purified by a procedure that involves extensive modification of that reported by Narayanan and Borchardt (1988). Surprisingly, our procedure led to the isolation of two forms of SAH hydrolase. These two forms (type A and type B) are resolved by chromatography on a blue dextran–agarose column with use of the conditions reported by Narayanan and Borchardt (1988). Type A was loosely bound to the column and was eluted after washing with 150–200 mL of buffer (Figure 1). Type B was *very* tightly bound to the column and was eluted only in the presence of 0.5 mM adenosine. Type A proved to be the predominant form of the enzyme. The nucleotide cofactor content of the type A enzyme was found to be 4.0 mol of cofactor/mol of SAH hydrolase, consistent with the earlier

Scheme III: Synthesis of Acetylenic Adenosine and [6′-^{3}H]-Acetylenic Adenosine



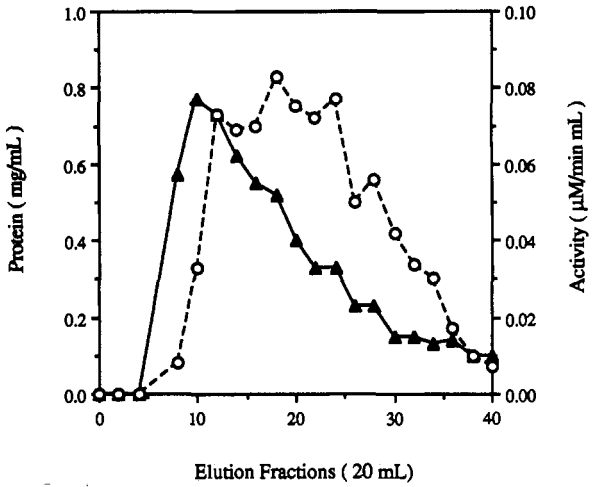


FIGURE 1: Elution profile of type A enzyme from the blue dextran–agarose column. The enzyme activity (○) and the protein content (▲) were determined.

Table I: Purification of SAH Hydrolase from Bovine Liver

	volume (mL)	protein (mg/mL)	total protein (mg)	total units (μM/min)	sp act. (μM/(min·mg))
DEAE-cellulose	490	43.3	21,217	57.3	0.0027
$(NH_4)_2SO_4$ fractionation	530	27.5	14,575	50.8	0.0035
Q-Sepharose	34	102.5	3,485	17.9	0.005
blue dextran–agarose					
type A	4.3	43.1	185.4	33	0.1–0.13
type B	2.55	51	130.05	3.89	0.03
FPLC, AX 300					
type A	2.07	12.3	25.39	5.54	0.3–0.5
type B	0.69	2.02	1.4	0.21	0.3

results of Palmer and Abeles (1979). On the other hand, the nucleotide content of type B SAH hydrolase was only half that of type A. In some instances, up to 50% of the cofactors found in both forms of the enzyme were in a reduced form. For reasons that are unclear, only type B SAH hydrolase was isolated by Narayanan and Borchardt (1988).

By using FPLC for the final purification step and omitting gel filtration, we were able to isolate SAH hydrolase in greater than 95% purity. The purified SAH hydrolase appeared as a single band on SDS–PAGE with a MW of 49 500, and the overall yield of type A enzyme was 12.5% (Table I). The final specific activity of the purified enzyme varied between 0.4 and 0.5 IU in the direction of hydrolysis. This result agrees well with those of Palmer and Abeles (1976) and Richards et al. (1978), who reported 0.64 IU and 0.5 IU–0.65 IU, respectively.

Inactivation Kinetics. Our experiments demonstrate that the inactivation of SAH hydrolase by acetylenic adenosine depends on both the concentration of the inhibitor and the time of incubation. At high concentrations of inhibitor (greater than 1 μM), the inactivation appears to follow first-order kinetics; however, at lower concentrations, a more complicated kinetic pattern predominates. The curvilinear time dependence of the inhibition at lower concentrations is characteristic of class B inactivators of SAH hydrolase as described by Chiang et al. (1981). We determined the K_I and the catalytic turnover number using the method of Cha et al. (1975) and Cha (1975) by plotting v_0/vi versus inhibitor concentration at varying enzyme concentrations and determined a K_I for acetylenic adenosine of 173 nM (Figure 2). We obtained a catalytic turnover number of 16.4 min^{-1} from the slope of a plot of inhibitor concentration versus the velocity intercepts of an Ackerman–Potter plot. These two findings indicate that acetylenic adenosine is one of the more potent inhibitors of SAH hydrolase.

Inactivation of SAH Hydrolase upon Incubation with Acetylenic Adenosine. After incubation of the enzyme with saturating concentrations of inhibitor followed either by extensive dialysis or by gel filtration on a Sephadex G-75 column, less than 5% of the enzyme activity was recovered as compared to a control containing no inhibitor. A titration of SAH hydrolase with varying concentrations of acetylenic adenosine in three different runs showed that approximately 4.0–4.5 equiv of acetylenic adenosine are required to inactivate the enzyme (Figure 3). In all cases the reaction was complete after 24 h, and no further loss of activity was observed after a prolonged incubation period of 48–72 h.

Binding Stoichiometry. The binding stoichiometry was determined with the aid of [6′-^{3}H]acetylenic adenosine. Excess inactivator was incubated with SAH hydrolase for 30 min under standard conditions. The stoichiometry of the enzyme–inhibitor complex was determined after the removal of free ligands by either extensive dialysis or gel filtration on a

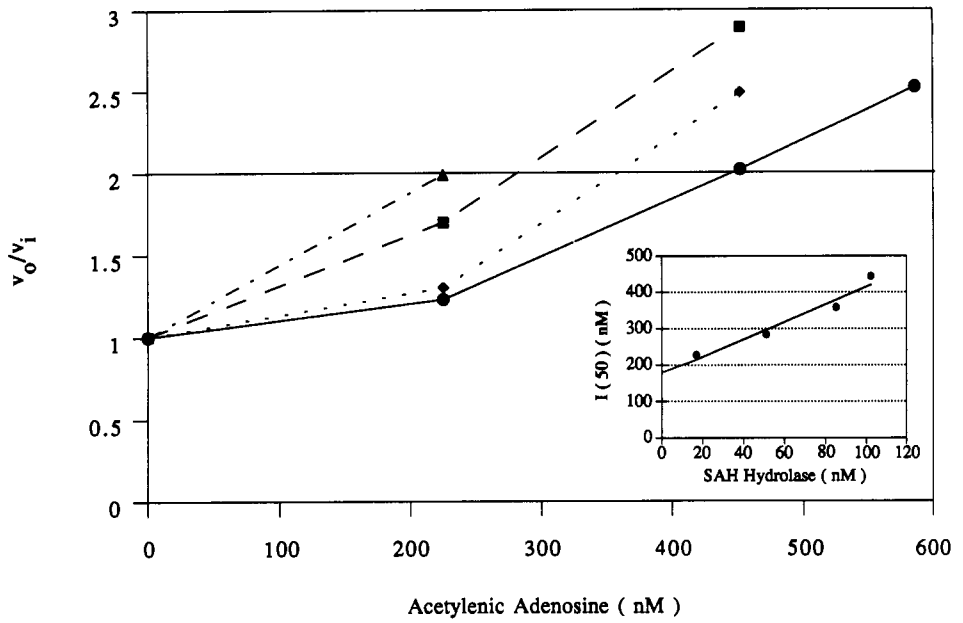


FIGURE 2: Determination of K_I. Plot of v_0/v_i versus inhibitor concentrations with the following amounts of SAH hydrolase: (▲) 17.05 nM; (■) 51.51nM; (◆) 85.25nM; (●) 102.3 nM. (Inset) Replot of I_{50} values versus the amount of SAH hydrolase.

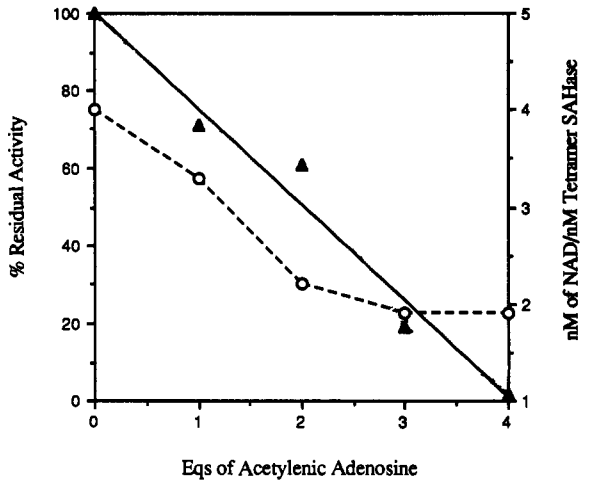


FIGURE 3: Titration of SAH hydrolase with acetylenic adenosine. The decrease in enzyme activity (▲) and the nucleotide cofactor content (○) were monitored.

Sephadex G-25 column. A ratio of 3.68 mol of [6′-^{3}H]-acetylenic adenosine/mol of enzyme was determined with type A SAH hydrolase of greater than 95% purity. With use of type B SAH hydrolase of similar purity, a binding stoichiometry of 3.2 mol of inhibitor/mol of enzyme was observed. Earlier experiments performed with SAH hydrolase with a purity greater than 75% yielded a binding stoichiometry of 4.47 mol of [6′-^{3}H]acetylenic adenosine/mol of enzyme. Overall, these results indicate that 4 mol of inhibitor bind/mol of enzyme.

In order to determine the minimum number of equivalents of inhibitor required to inactivate SAH hydrolase, the type A enzyme was incubated with limiting amounts of [6′-^{3}H]-acetylenic adenosine under standard conditions. The incorporation of tritium into the enzyme was found to be proportional to the loss of enzyme activity, and the binding of 4 mol of acetylenic adenosine per enzyme tetramer was found to be required for complete inactivation; the binding of 2 mol of acetylene adenosine/mol of enzyme caused only 50% inhibition (Figure 3).

Reduction of Enzyme-Bound NAD$^+$ with Acetylenic Adenosine. Upon incubation of type A SAH hydrolase with excess inhibitor under standard conditions (Table II), approximately 50% of the NAD$^+$ was reduced to NADH, while the remaining half of the NAD$^+$ was released from the enzyme. In a different set of experiments, type A enzyme was incubated with either 1 or 2 equiv of acetylenic adenosine for 24 h at 25 °C in the presence of 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 3 mM DTT. The reduction of 1 and 2 mol of NAD$^+$/mol of enzyme tetramer was observed, respectively. These results are summarized in Table II. The stability of NAD$^+$ under these conditions was confirmed by means of a control experiment that contained no inhibitor. The amount of NAD$^+$ associated with the native and inactivated enzyme was determined after denaturation of the enzyme with 0.2 M $HClO_4$. The amount of free NAD$^+$ was measured after filtration of the incubation mixture with use of an ultrafree-MC unit (MW cutoff 10 000) to remove the protein. The filtrate was also analyzed for protein content to exclude possible leakage of the filter.

Table II: NAD$^+$ and NADH Content of SAH Hydrolase before and after Inactivation with Acetylenic Adenosine

	NAD$^+$ (μM/μM of tetramer)	NADH[a] (μM/μM of tetramer)
type A, native	4.0 (1.5)[b]	0 (2.02)[b]
type B, native	2.5 (0.92)[b]	0 (1.2)[b]
4-inactivated type A[c]	2.2	1.8
4-inactivated type A[d]	1.9	

[a] NADH values were obtained by subtracting the NAD$^+$ values from the total number of nucleotides bound per enzyme tetramer. [b] Nucleotide content from different preparations. [c] NAD$^+$/NADH determined after denaturation of the enzyme–inhibitor complex. [d] NAD$^+$ determined in the filtrate of the enzyme–inhibitor complex without denaturation.

Stability of the SAH Hydrolase–Acetylenic Adenosine Complex. SAH hydrolase–acetylenic adenosine complex, synthesized from type A enzyme and excess [6′-^{3}H]acetylenic adenosine, was denatured by dialysis against 8 M urea at 4 °C and at room temperature for up to 5 days (Figure 4). The urea denaturation caused stepwise release of unidentified labeled material from the enzyme. After 48 h at 4 °C, nearly 1 equiv of inhibitor was still bound to the enzyme; however, complete release of all labeled material was observed at room

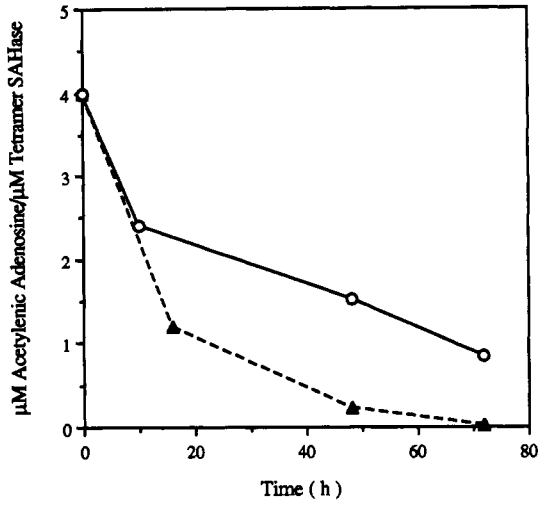


FIGURE 4: Denaturation of the enzyme–inhibitor complex with 8 M urea at 25 °C (▲) and 4 °C (○).

temperature after 48 h. Incubation of the enzyme–inhibitor complex with 0.2 N $HClO_4$ for 10 min at 4 °C or dialysis against 1% TFA/0.1% SDS (pH 2.4) at room temperature for 24 h released all the labeled material. Similar behavior was observed with type B SAH hydrolase.

Incubating the SAH hydrolase–acetylenic adenosine complex (1.4 nM) with 50 μM NAD^+ for 2.5 h at 25 °C did not reactivate the enzyme. The stability of the complex was further tested by incubation of [6′-^{3}H]acetylenic adenosine complex (0.3 mg) with 0.5 μM adenosine for 2 h. No tritiated material was released.

Since the proposed inactivation mechanism (Scheme II) could lead to the formation of an enamine or Schiff's base linkage between the inhibitor and an amino group at the active site of the enzyme, we attempted to stabilize the enzyme–inhibitor complex by reduction. However, no increase in stability was achieved by reduction of the enzyme–inhibitor complex with either $NaCNBH_3$ or $NaBH_4$ under denaturating or nondenaturating conditions. Furthermore, *no* incorporation of tritium was observed when SAH hydrolase–inhibitor complex was reduced with [^{3}H]$NaBH_4$ under denaturating conditions as compared to a control utilizing native enzyme.

Influence of Adenosine on the Inhibition of SAH Hydrolase by Inhibitor **4**. By incubating adenosine, acetylenic adenosine, and homocysteine with SAH hydrolase and monitoring the rate of SAH formation by HPLC, it was found that the enzyme could be protected from the inhibitory effects of acetylenic adenosine by the presence of adenosine. From this, we can conclude that adenosine and acetylenic adenosine compete for the same binding site. Incubation of acetylenic adenosine and homocysteine with SAH hydrolase under standard conditions followed by HPLC analysis after 30 min at 37 °C revealed that no SAH-like products were formed. This suggests that oxidized acetylenic adenosine does not serve as a substrate for the Michael addition reaction with homocysteine. Control assays were run with both adenosine and acetylenic adenosine.

Discussion

Enzyme Isolation. The purification of SAH hydrolase using a blue dextran–agarose column led to the isolation of two forms of the enzyme that we call type A and type B. Type A contains 4 mol of nucleotide cofactor/mol of enzyme tetramer, while type B contains only 2 mol/mol of tetramer. The affinity ligand bound to blue dextran–agarose is a semi-specific high molecular weight compound substituted with the monochlorotriazinyl dye Cibacron blue F3GA. Presumably, the blue chromophore binds to the nucleotide binding sites of the enzyme by mimicking a nucleotide (Lowe & Pearson, 1983; Naranayan & Borchardt, 1988). Consistent with this theory, type A enzyme, which has all its nucleotide binding sites occupied, binds only loosely to the column and is eluted by buffer that does not contain adenosine. In contrast, elution of type B enzyme, which has only half of its nucleotide binding sites filled, requires the presence of 0.5 mM adenosine in the buffer.

To our knowledge this is the first report of the co-occurrence of these two forms of SAH hydrolase. Type A enzyme has been previously isolated from bovine liver and other mammalian sources by a number of groups (Richards et al., 1978; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Ueland, 1982; Hershfield et al., 1985; de la Haba et al., 1986), while type B SAH hydrolase has been isolated from bovine liver in the apparent absence of type A enzyme (Matuszewska & Borchardt, 1987; Narayanan & Borchardt, 1988). It must therefore be concluded that SAH hydrolase can exist in two forms in bovine liver and possibly in other mammalian sources. This is an intriguing observation since there is evidence that these two forms are not interconvertible in vitro. Matuszewska and Borchardt (1987) reported that exogenous NAD^+ cannot bind tightly to type B enzyme, although type B SAH hydrolase exhibited up to a 30% increase in enzyme activity in the present of exogenous NAD^+. Furthermore, no exchange of free NAD^+ is observed with type A enzyme, nor are any cofactors released during the normal catalytic cycle (Palmer & Abeles, 1979). In our hands, the activity of type A enzyme was unaltered by the addition of exogenous NAD^+, an observation consistent with previous reports (Palmer & Abeles, 1979; Fujioka & Takata, 1981).

In some cases both the type A and type B SAH hydrolase that we isolated carried partially reduced NAD^+. This phenomenon has previously been observed (Hershfield et al., 1985; de la Haba et al., 1986; Narayanan & Borchardt, 1988), though the reasons for the behavior exhibited by the Type A enzyme remain unclear. In the case of the type B enzyme, a possible explanation may be the use of elution buffer that contains 0.5 mM adenosine, since it has been shown by several groups (Chiang et al., 1981; Abeles et al., 1982; Hershfield et al., 1985) that adenosine, which is an inhibitor of SAH hydrolase at concentrations of greater than 0.1 mM, can cause reduction of NAD^+ to NADH. For example, Abeles et al. (1982) observed that adenosine causes up to 50% reduction of the bound cofactor of bovine liver SAH hydrolase and that the enzyme can bind up to two equivalents of adenosine per tetramer with no apparent inactivation.

Enzyme Inactivation. The results of our experiments demonstrate that incubation of SAH hydrolase with saturating concentrations of the acetylenic adenosine analogue **4** causes complete and irreversible inactivation of the enzyme. This inactivation is accompanied by the reduction of 2 mol of NAD^+/mol of SAH hydrolase and the release of the remaining 2 mol of NAD^+ from the enzyme. More detailed studies of the inactivation process indicate that it occurs by two different mechanisms. If the enzyme is titrated with the inhibitor, the first 2 equiv cause the formation of 2 equiv of NADH and reduce the enzyme activity to 50% of the value for the native enzyme. The addition of another 2 equiv results in complete inactivation of the enzyme and release of the remaining 2 equiv of NAD^+. The inactivated enzyme could not be reactivated by incubation with exogenous NAD^+. On the basis of these observations, we conclude that the first 2 equiv of acetylenic

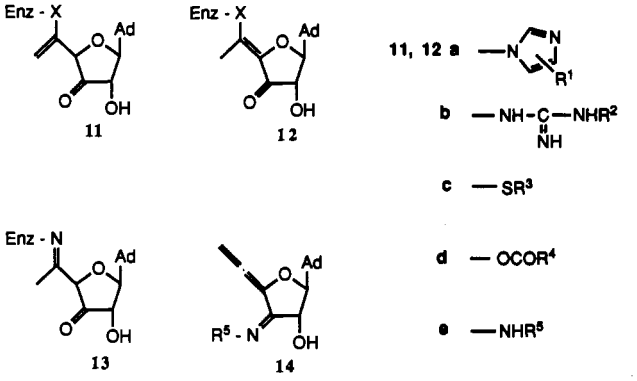


FIGURE 5: The structures of putative enzyme–inhibitor adducts.

adenosine act as mechanism-based inhibitors (Silverman, 1988) that may be forming covalent bonds with the enzyme in the manner shown in Scheme II, while the remaining 2 equiv act as tight binding inhibitors that inactivate the enzyme by causing the loss of NAD^+.

Denaturation experiments provided additional support for the notion that the inactivation of SAH hydrolase by acetylenic adenosine involves two different mechanisms. SAH hydrolase inactivated with [6′-^{3}H]acetylenic adenosine was denatured with 8 M urea either at 4 °C or at room temperature, and the release of tritiated material was monitored versus time. In both sets of experiments, approximately 2 equiv of labeled material were released from the enzyme much faster than the remaining 2 equiv. However, all of the radioactivity was eventually released at room temperature, an observation that suggests that any covalent linkage formed between the enzyme and the oxidized form of the inhibitor must be quite labile. Labeled material was not released upon incubation of the enzyme–inhibitor complex with 0.5 mM adenosine for 2 h at 26 °C.

The putative enzyme–inhibitor linkage was found to be very acid labile but stable between pH 7 and 9. This observation provides some insight into the nature of the covalent linkage that may have formed between **4** and a nucleophilic residue at the active site of SAH hydrolase. A number of chemical modification studies have been carried out to determine the nature of the amino acid residues present at the active site of the rat liver SAH hydrolase. A histidine residue (Gomi & Fujioka, 1983), an arginine residue (Takata & Fujioka, 1983), and two cysteine residues (Gomi & Fujioka, 1982; Takata & Fujioka, 1984; Schatz et al., 1983) have been shown to be essential for the catalytic activity of the enzyme. Evidence for a carboxyl group at the active site has also been obtained (Takata et al., 1985). Finally, a lysine residue has been suggested to lie in or around the active site of the beef liver enzyme (Patel-Thombre & Borchadt, 1985). On the basis of the preceding information and the proposed mechanism of inactivation (Scheme II), 12 possible structures can be envisioned for the adduct between the enzyme and oxidized acetylenic adenosine (Figure 5). Most of these structures should be reducible by borohydride or cyanoborohydride unless shielded by the enzyme. However, those structures (**12b**, **12e**) that are vinylogous amides might be resistant to reduction (Borch et al., 1971). For this reason, we favor these as possible structures for the putative enzyme–inhibitor adduct.

A comparison of the inhibition behavior of acetylenic adenosine with the behavior reported for two other well-studied mechanism-based inactivators of SAH hydrolase, 2′-deoxyadenosine and neplanocin A, reveals some similarities, but unfortunately, no clear pattern emerges. 2′-Deoxyadenosine causes complete inactivation of SAH hydrolase with a binding stoichiometry of 2–4 equiv of inhibitor/mol of enzyme tetramer and by a mechanism that results in about 80–90% cleavage of the C–N glycosidic linkage of the inhibitor (Abeles et al., 1982). The inactivation is accompanied by reduction of NAD^+ to NADH, with the degree of reduction depending on experimental conditions, and by release of the remaining NAD^+ into the medium. Furthermore, SAH hydrolase inactivated by 2′-deoxyadenosine can be partially reactivated by incubation with exogenous NAD^+ (de la Haba et al., 1986).

Somewhat contradictory behavior has been observed for the inactivation of SAH hydrolase by neplanocin A. Wolfson et al. (1986) found that enzyme bearing 4 equiv of NAD^+/tetramer is completely inactivated after binding of 2 equiv of neplanocin A and that 50% of the enzyme-bound NAD^+ is reduced. On the other hand, Borchardt et al. (1984) reported that the same form of the enzyme is completely inactivated after only 1 equiv of inhibitor has bound. In another study Matuszewska and Borchardt (1987) report that SAH hydrolase bearing only 2 equiv of nucleotide cofactor (1 NAD^+, 1 NADH) is inactivated by 1 equiv of inhibitor with concomitant reduction of 1 equiv of NAD^+. The enzyme–inhibitor complex formed in these latter experiments could be almost entirely reactivated by incubation with exogenous NAD^+.

Overall, the inactivation behavior of SAH hydrolase is remarkably complex, and the particular mechanism is strongly influenced by the small differences in the structures of the inactivators. Our results show that acetylenic adenosine is one of the more potent of the known inhibitors of SAH hydrolase (K_I = 173 nM) (Hershfield, 1979; Houston et al., 1985; McCarthy et al., 1989; Matuszewska & Borchardt, 1987; Naranayan et al., 1988; Glazer et al., 1986; Montgomery et al., 1982; Patel-Thombre & Borchardt, 1985) and that it inactivates bovine liver SAH hydrolase by a combination of two mechanisms, one that is mechanism-based, and one that involves the loss of the nucleotide cofactors. Half-site reactivity is observed during the inactivation even though the four subunits of the enzyme are identical. Denaturation studies suggest that a labile covalent bond may be formed between the enzyme and those molecules of inhibitor that act in a mechanism-based fashion. Additional studies with new inactivators as well as structural information on the active site of the enzyme and on the nature of the interactions between subunits may be necessary before a complete understanding of the inactivation behavior of SAH hydrolase becomes possible.

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REFERENCES

Abeles, R. H., Fish, S., & Lapinskas, B. (1982) *Biochemistry 21*, 5557–5562.

Borch, R. F., Bernstein, M. D., & Durst, H. D. (1971) *J. Am. Chem. Soc. 93*, 2897–2904.

Borchardt, R. T., Keller, B. T., & Patel-Thombre, U. (1984) *J. Biol. Chem. 259*, 4353–4358.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177–2185.

Cha, S., Agarwal, R. P., & Parks, A. E., Jr. (1975) *Biochem. Pharmacol. 24*, 2187–2197.

Chabannes, B. E., Bidard, J. N., Sarda, N. N., & Cronenberger, L. A. (1979) *J. Chromatogr. 170*, 430–436.

Chiang, P. (1984) *Adv. Exp. Med. Biol. 165*, 199–203.

Chiang, P. K., Guranowski, A., & Segall, J. E. (1981) *Arch. Biochem. Biophys. 207*, 175–184.

Colvin, E. W., & Hamill, B. J. (1977) *J. Chem. Soc., Perkin Trans. 1*, 869–874.
de la Haba, G., & Cantoni, G. L. (1959) *J. Biol. Chem. 234*, 603–608.
de la Haba, G., Agostini, S., Bozzi, A., Merta, A., Unson, C., & Cantoni, G. L. (1986) *Biochemistry 25*, 8337–8342.
Edwards, M. L., Mehdi, S., Bowlan, T. L., & Kenny, M. T. (1991) *J. Med. Chem.* (submitted for publication).
Fujioka, M., & Takata, Y. (1981) *J. Biol. Chem. 256*, 1631–1635.
Gilbert, J. C., & O'Leary, M. (1975) *Biochemistry 14*, 5194–5199.
Gilbert, J. C., & Weerasooriya, U. (1979) *J. Org. Chem. 44*, 4997–4998.
Glazer, R. I., Hartman, K. D., Knode, M. C., Richard, M. M., Chiang, P. K., Tseng, C. K., & Marquez, V. E. (1986) *Biochem. Biophys. Res. Commun. 135*, 688–694.
Gomi, T., & Fujioka, M. (1982) *Biochemistry 21*, 4171–4176.
Gomi, T., & Fujioka, M. (1983) *Biochemistry 22*, 137–143.
Gomi, T., Date, T., Ogawa, H., Fujioka, M., Aksamit, R. R., Backlund, P. S., Jr. & Cantoni, G. L. (1989) *J. Biol. Chem. 26*, 16138–161342.
Guranowski, A., & Pawelkiewicz, J. (1977) *Eur. J. Biochem. 80*, 517–523.
Hampton, A. (1961) *J. Am. Chem. Soc. 83*, 3640–3645.
Hayman, S., & Colman, R. (1977) *Biochemistry 16*, 998–1005.
Hershfield, M. S. (1983) *Dev. Pharmacol. 2*, 171–180.
Hershfield, M., Kredich, N. M., Ownby, D. R., Ownby, H., & Buchley, R. (1979) *J. Clin. Invest. 63*, 807–811.
Hershfield, M. S., Aiyar, V. N., Premakumar, R., & Small, W. C. (1985) *Biochem. J. 230*, 43–52.
Hohman, R. J., Guitton, M. C., & Veron, M. (1984) *Arch. Biochem. Biophys. 233*, 785–795.
Houston, D. M., Dolence, E. K., Keller, B. T., Patel-Thombre, U., & Borchardt, R. T. (1985) *J. Med. Chem. 28*, 471–477.
Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology*, 490–496, McGraw Hill, New York.
Kasir, J., Aksamit, R. R., Backlund, P. S., Jr., & Cantoni, G. L. (1988) *Biochem. Biophys. Res. Com. 153*, 359–364.
King, J., & Laemmli, U. K. (1971) *J. Mol. Biol. 62*, 465–473.
Knudson, R. C., & Yall, I. (1972) *J. Bacteriol. 112*, 569–575.
Lowe, C. R., & Pearson, J. C. (1983) *Methods Enzymol. 104*, 97–113.
Matuszewska, B., & Borchardt, R. T. (1987) *J. Biol. Chem. 262*, 265–268.
McCarthy, J. R., Jarvi, E. T., Matthews, D. P., Edwards, M. L., Prakash, N. J., Bowlin, T. L., Mehdi, S., Sunkara, P. S., & Bey, P. (1989) *J. Am. Chem. Soc. 111*, 1127–1128.
Montgomery, J. A., Clayton, S. J., Thomas, H. J., Shannon, W. M., Arnett, G., Bodner, A. J., Kion, I., Cantoni, G. L., Chiang, P. K. (1982) *J. Med. Chem. 25*, 626–629.
Morisaki, M., & Bloch, K. (1972) *Biochemistry 11*, 309–314.
Narayanan, S. R., & Borchardt, R. T. (1988) *Biochim. Biophys. Acta 965*, 22–28.
Narayanan, S. R., Keller, B. T., Borcherding, D. R., Scholtz, S. A., & Borchardt, R. T. (1988) *J. Med. Chem. 31*, 500–503.
Palmer, J. L., & Abeles, R. H. (1976) *J. Biol. Chem. 251*, 5817–5819.
Palmer, J. L., & Abeles, R. H. (1979) *J. Biol. Chem. 254*, 1217–1226.
Parry, R. J., & Askonas, L. J. (1985) *J. Am. Chem. Soc. 107*, 1417–1418.
Patel-Thombre, U., & Borchardt, R. T. (1985) *Biochemistry 24*, 1130–1136.
Ranganathan, R. S., Jones, G. H., & Moffatt, J. G. (1974) *J. Org. Chem. 39*, 290–298.
Richards, H. H., Chiang, P. K., & Cantoni, G. L. (1978) *J. Biol. Chem. 253*, 4476–4480.
Sakami, W., & Stevens, A. (1958) *Bull. Soc. Chim. Biol. 40*, 1787–1793.
Schatz, R. A., Vunnam, C. R., & Sellinger, O. Z. (1983) in *Transmethylation* (Usdin, R., Borchardt, R., & Creveling, C., Eds.) pp 143–153, Elsevier, New York.
Schwab, J. M., Ho, C.-K., Li, W.-B., Townsend, C. A., & Salituro, G. M. (1986) *J. Am. Chem. Soc. 108*, 5309–5316.
Sebestowa, L., Votruba, I., & Holy, A. (1984) *Collect. Czech. Chem. Comm. 49*, 1543–1557.
Shimizu, S., Shiozaki, S., Ohshiro, T., & Yamada, H. (1984) *Eur. J. Biochim. 141*, 385–392.
Silverman, R. B. (1988) in *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, Vol. 1, pp 10–12, CRC Press, Inc., Boca Raton, FL.
Takata, Y., & Fujioka, M. (1983) *J. Biol. Chem. 258*, 7374–7378.
Takata, Y., & Fujioka, M. (1984) *Biochemistry 23*, 4357–4362.
Takata, Y., Gomi, T., & Fujioka, M. (1985) *Arch. Biochem. Biophys. 240*, 827–835.
Ueland, P. (1982) *Pharmacol. Rev. 34*, 223–253.
Walker, R. D., & Duerre, J. R. (1975) *Can. J. Biochem. 53*, 312–319.
Walsh, C. (1982) *Tetrahedron 38*, 871–909.
Widlanski, T. S., Bender, S. L., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 1873–1874.
Wolfson, G., Chisholm, J., Tashjian, A. H., Fish, S., & Abeles, R. H. (1986) *J. Biol. Chem. 261*, 4492–4498.