Affinity Labeling of Histamine *N*-Methyltransferase by 2′,3′-Dialdehyde Derivatives of *S*-Adenosylhomocysteine and *S*-Adenosylmethionine. Kinetics of Inactivation†

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ABSTRACT: *S*-Adenosyl-L-methionine (AdoMet), *S*-adenosyl-L-homocysteine (L-AdoHcy), and related ribonucleosides have been oxidized with periodic acid to the corresponding 2′,3′-dialdehydes. Both AdoMet dialdehyde and L-AdoHcy dialdehyde were observed to rapidly and irreversibly inactivate histamine *N*-methyltransferase (HMT). Equally active as an irreversible inhibitor was *S*-adenosyl-D-homocysteine dialdehyde (D-AdoHcy dialdehyde), which is consistent with the known affinity of HMT for *S*-adenosyl-D-homocysteine (D-AdoHcy). Other analogues of AdoHcy dialdehyde (*S*-adenosyl-L-cysteine dialdehyde, *S*-adenosyl-L-homocysteine sulfoxide dialdehyde, and adenosine dialdehyde) also produced irreversible inactivation of HMT, but at predictably slower rates. The corresponding acyclic 2′,3′-ribonucleosides, which were obtained by $NaBH_4$ reduction of the ribonucleosides dialdehydes, were found to be very weak, reversible inhibitors of HMT. Kinetic analysis of the inactivation of HMT produced by L-AdoHcy dialdehyde, AdoMet dialdehyde, and D-AdoHcy dialdehyde suggested mechanisms involving the formation of dissociable enzyme–inhibitor complexes prior to irreversible inactivation. Studies using L-[2,8-^{3}H]AdoHcy dialdehyde revealed that incorporation of radioactivity into HMT closely paralleled the loss of enzyme activity. The results of these studies indicate that L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, and AdoMet dialdehyde are affinity labeling reagents for HMT.

The inactivation of histamine in most mammalian tissues is dependent upon the enzyme histamine *N*-methyltransferase (HMT) (EC 2.1.1.8). HMT is a soluble enzyme which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (L-AdoMet)[1] to histamine resulting in the formation of 1-methylhistamine (Schayer, 1956; Brown et al., 1959). Similar to most AdoMet-dependent methyltransferases, HMT is sensitive to inhibition by the demethylated product *S*-adenosyl-L-homocysteine (L-AdoHcy) (Zappia et al., 1969; Baudry et al., 1973; Borchardt, 1977). In an effort to elucidate the relationship between the chemical structure and catalytic function of HMT, we have synthesized analogues of AdoHcy as reversible, product-type inhibitors (Borchardt & Wu, 1974, 1975, 1976a; Borchardt et al., 1974, 1976a; Borchardt, 1977) and analogues of AdoMet as alternate substrate type inhibitors (Borchardt & Wu, 1976b; Borchardt et al., 1976b). The data obtained from these studies yielded valuable information about those structural features of AdoHcy and AdoMet which are necessary to produce maximum binding of these ligands to HMT.

In an effort to further probe the active site of HMT, we have synthesized affinity labeling reagents for this enzyme by incorporating a chemically reactive functional group into the basic AdoHcy and AdoMet structures. Structurally related ribonucleosides have been converted to affinity labeling reagents by periodate oxidation of the 2′,3′-*cis*-diol functionality and the resulting dialdehyde derivatives have been shown to react with lysine residues of proteins to form Schiff base adducts (Kwan & Olcott, 1966; Crawford et al., 1967; Buttkus, 1967; Chio & Tappel, 1969). For example, the periodate oxidation product of 6-methylmercaptopurine ribonucleoside was shown to inhibit *E. coli* RNA polymerase (Wu & Goldthwait, 1969; Krakow & Frank, 1969; Wu & Wu, 1974; Wu et al., 1974) and *E. coli* DNA-dependent polymerase I (Salvo et al., 1976; Kimball, 1977) by covalently binding to an ϵ-amino group of a lysine residue at the enzymes' initiation sites. Based on these studies, we synthesized 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*R*)-homocysteinyl-3′-deoxy-(*S*)-glyceraldehyde (L-AdoHcy dialdehyde) by periodate oxidation of AdoHcy as a possible affinity labeling reagent for HMT. In preliminary studies (Borchardt et al., 1977), L-AdoHcy dialdehyde was shown to produce rapid and irreversible inactivation of HMT.

In this report we will provide evidence that L-AdoHcy dialdehyde, as well as 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*S*)-homocysteinyl-3′-deoxy-(*S*)-glyceraldehyde (D-AdoHcy dialdehyde) and 2′-*O*-[(*R*)-formyl(adenin-9-yl)-methyl]-3′-*S*-(*R*)-methioninyl-3′-deoxy-(*S*)-glyceraldehyde (L-AdoMet dialdehyde), serve as affinity labeling reagents for HMT. The evidence will include their kinetics of inactivation of HMT, substrate protection studies, and inhibitor incorporation studies.

Materials and Methods

Melting points were obtained on a calibrated Thomas-Hoover Uni-melt and were corrected. Microanalyses were conducted on a F&M Model 185 C, H, N analyzer, The University of Kansas, Lawrence, Kan.

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‡ Established Investigator of the American Heart Association.

[1] Abbreviations used: AdoCy, *S*-adenosyl-L-cysteine; AdoCy dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(R)-cysteinyl-3′-deoxy-(*S*)-glyceraldehyde; adenosine dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-(*R*)-glyceraldehyde; L-AdoHcy, *S*-adenosyl-L-homocysteine; D-AdoHcy, *S*-adenosyl-D-homocysteine; L-AdoHcy dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*R*)-homocysteinyl-3′-deoxy-(*S*)-glyceraldehyde; D-AdoHcy dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*S*)-homocysteinyl-3′-deoxy-(*S*)-glyceraldehyde; L-AdoHcy sulfoxide, *S*-adenosyl-L-homocysteine sulfoxide; AdoHcy sulfoxide dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*R*)-homocysteinylsulfoxide-3′-deoxy-(*S*)-glyceraldehyde; L-AdoMet, *S*-adenosyl-L-methionine; L-AdoMet dialdehyde; 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*-(*R*)-methioninyl-3′-deoxy-(*S*)-glyceraldehyde; HMT, histamine *N*-methyltransferase, TLC, thin-layer chromatography.

0006-2960/78/0417-4145$01.00/0

Unless otherwise stated, the infrared (IR), nuclear magnetic resonance (NMR), and ultraviolet (UV) data were consistent with the assigned structures. IR data were recorded on a Beckman IR-33 spectrophotometer, NMR data on a Perkin-Elmer R-24B spectrophotometer (Me_4Si), and UV data on a Cary Model 14 spectrophotometer. Thin-layer chromatography (TLC) was carried out using Analtech silica gel GF (250 μm), Analtech Avicel F (250 μm), or Brinkman polygram (0.1 mm) cellulose impregnated with polyethylenimine. Spots were detected by visual examination under UV light and/or by ninhydrin spray. Scintillation counting was done on Beckman LS-150 and LS-300 scintillation counters.

L-AdoMet-$^{14}CH_3$ (New England Nuclear, 55.0 Ci/mol) was diluted to a concentration of 10 μCi/mL and stored at −20 °F. L-AdoMet chloride (Sigma) was stored as 0.01 M aqueous stock solution. Adenosine and L-AdoHcy were purchased from Sigma. The AdoHcy analogues were synthesized according to published procedures as cited below: D-AdoHcy and L-AdoHcy sulfoxide (Borchardt & Wu, 1974) and L-AdoHcy (Borchardt et al., 1976a).

General Procedure for the Oxidation of Ribonucleosides to the Corresponding Ribonucleoside Dialdehydes. To an ice-cooled, magnetically stirred suspension of the ribonucleoside (e.g., L-AdoHcy, D-AdoHcy, etc.; 1 mmol) in 8 mL of H_2O was added paraperiodic acid (1 mmol) in one portion. The reaction mixture was kept in the dark at ambient temperature and the course of the reaction was monitored by TLC on cellulose (H_2O) or on silica gel (5% Na_2HPO_4). After all the ribonucleoside had reacted, the reaction mixture was extracted repeatedly with Et_2O. The aqueous layer was then diluted with 8 mL of H_2O and neutralized with lead carbonate to pH 5–6. Following filtration, the aqueous layer was lyophilized. Further purification of AdoHcy dialdehyde derivatives was accomplished by thick-layer chromatography on cellulose (Analtech Avicel, 1000 μm) eluting with H_2O. The desired product was recovered by extraction with H_2O from the cellulose followed by lyophilization. Yields generally ranged from 50 to 90%. For L-AdoMet dialdehyde, the solution obtained after neutralization with lead carbonate was chromatographed on a column of Dowex 2 (1 × 10 cm: chloride form; Baddiley et al., 1962). This replaced the periodate and iodate counterion with acetate. The ribonucleoside dialdehydes were not obtained in crystalline form, since their instability prohibited crystallization by classical techniques. The dialdehydes were characterized by their chromatographic properties (see Table I for the chromatographic systems used and the R_f values obtained), their spectral properties (UV, IR, and NMR) and their reducibility using $NaBH_4$ to the corresponding acyclic ribonucleosides (Borchardt et al., 1978). The KI–starch test was employed to check the dialdehyde samples for contamination by periodate and iodide. The NMR spectral data obtained for the dialdehyde derivatives are provided below as further evidence for the assigned structures.[2]

L-AdoHcy Dialdehyde and D-AdoHcy Dialdehyde: NMR (D_2O) δ 8.43, 8.35, 8.30, 8.27, 8.23, 8.20 (6 s, 2 H, H_2 and H_8), 6.23–5.70 (m, 1 H, $H_{1'}$), 5.70–5.13 (m, 2 H, $H_{2'}$ and $H_{3'}$), 3.83 (t, 1 H, H_α), 3.50–2.67 (m, 4 H, $H_{5'}$ and H_γ), 2.50–1.83 (m, 2 H, H_β).

L-AdoHcy Sulfoxide Dialdehyde. NMR (D_2O) δ 8.48, 8.41, 8.37, 8.30, 8.24, 8.19 (6 s, 2 H, H_2 and H_8), 6.33, 6.0, 5.82 (3 d, 1 H, $H_{1'}$), 5.80–5.20 (m, 2 H, $H_{2'}$ and $H_{3'}$), 4.0–3.20 (m, 5 H, $H_{5'}$, H_α and H_γ), 2.60–2.0 (m, 2 H, H_β).

L-AdoCy Dialdehyde: NMR (D_2O) δ 8.53, 8.47, 8.40, 8.34, 8.30, 8.23 (6 s, 2 H, H_2 and H_8), 6.25, 6.13, 5.80 (3 d, 1 H, $H_{1'}$), 5.75–5.07 (m, 2 H, $H_{2'}$ and $H_{3'}$), 4.20 (t, 1 H, H_α), 4.13–3.00 (m, 4 H, $H_{5'}$ and H_β).

Adenosine Dialdehyde: NMR (D_2O) δ 8.61–8.00 (m, 2 H, H_2 and H_8), 6.06, 5.95, 5.85, 5.65 (4 s, 1 H, $H_{1'}$), 5.60–4.95 (m, 2 H, $H_{2'}$ and $H_{3'}$), 4.90–3.40 (m, 3 H, $H_{4'}$ and $H_{5'}$).

AdoMet Dialdehyde: NMR (D_2O) δ 8.5–7.8 (m, 2 H, H_2 and H_8), 6.2–5.85 (m, 1 H, $H_{1'}$), 5.8–5.2 (m, 3 H, $H_{2'}$, $H_{3'}$ and $H_{4'}$), 3.95–3.25 (m, 5 H, H_α, H_γ, and $H_{5'}$), 2.89, 2.63 (2 s, 3 H, $-CH_3$), 2.55–1.90 (m, 2 H, H_β).

Preparation of L-[2,8-^{3}H]AdoHcy Dialdehyde. L-[2,8-^{3}H]AdoHcy was prepared by conversion of [2,8-^{3}H]adenosine (New England Nuclear; specific activity, 31 Ci/mmol) to [2,8-^{3}H]-5′-chloro-5′-deoxyadenosine, followed by condensation with L-homocysteine in Na/liquid NH_3 (Borchardt et al., 1976c). L-[2,8-^{3}H]AdoHcy (48 mg, 0.125 mmol; specific activity, 0.47 mCi/mmol) was oxidized with paraperiodic acid (30 mg, 0.130 mmol) in H_2O as described above. After purification by thick-layer chromatography on cellulose (Analtech, 1000 μm) eluting with H_2O, 5.5 mg (12%, specific activity = 0.47 mCi/mmol) of the desired L-[2,8-^{3}H]AdoHcy dialdehyde was isolated. Spectral and chromatographic properties were consistent with those of the unlabeled L-AdoHcy dialdehyde.

Conversion of L-AdoHcy Dialdehyde to L-AdoMet Dialdehyde. L-AdoHcy dialdehyde (9 mg, 0.025 mmol) was converted to L-AdoMet dialdehyde using methyl iodide (1 mL, 8 mmol) in a mixture of formic acid (0.5 mL) and acetic acid (0.5 mL) according to a general methylation procedure described earlier (Borchardt et al., 1976b). The reaction mixture was kept at ambient temperature for 7 days and the progress of the reaction monitored by TLC on silica gel [9 parts of EtOH/H_2O/HOAc (50:5:3) and 1 part of 0.2 M phosphate buffer, pH 7.0]. The reaction mixture was diluted with H_2O (2 mL), extracted three times with ethyl ether, and lyophilized. The product exhibited identical spectral (UV and NMR) and chromatographic properties with the sample of L-AdoMet dialdehyde obtained by direct oxidation of L-AdoMet.

Reduction of Adenosine Dialdehyde to 2′-O-[(R)-Hydroxymethyl(adenin-9-yl)methyl]glycerol. (a) Reduction with $NaBH_4$. To a stirred solution of adenosine dialdehyde (1.326 g, 5 mmol) in 20 mL of 0.1 M phosphate buffer, pH 8.4, was added $NaBH_4$ (740 mg, 20 mmol) in portions over a 30-min period. The reaction mixture was maintained at ambient

TABLE I: R_f Values for Ribonucleosides and Ribonucleoside Dialdehydes on Thin-Layer Chromatography.

compound	R_f values & chromatography system[a] A	B	C
L-AdoHcy (or D)	0.73	0.62	0.72
L-AdoHcy dialdehyde (or D)	0.83	0.05	0.78
L-AdoCy	0.74	0.55	0.79
L-AdoCy dialdehyde	0.86	0.09	0.82
L-AdoHcy sulfoxide	0.72	0.61	0.74
L-AdoHcy sulfoxide dialdehyde	0.89	0.12	0.77
L-AdoMet	0.34	0.67	0.28
L-AdoMet dialdehyde	0.43	0	0.20
adenosine	0.75	0.48	0.80
adenosine dialdehyde	0.82	0.07	0.85

[a] The chromatography systems had the following compositions: system A, 5% Na_2HPO_4 on silica gel GF; system B, 5% Na_2HPO_4 on polygram (0.1-mm cellulose impregnated with polyethylenimine); system C, 9 parts of EtOH/HOAc/H_2O (20:2:2) and 1 part of pH 7.4, 0.1 M phosphate buffer on silica gel GF.

[2] Peak assignments identified using the conventional nucleoside numbering system.

temperature for 4 h after which the solution was adjusted to pH 5 with 5% HCl and then readjusted to pH 7.0 with 0.2 N NaOH. The resulting solution was applied to a cellulose column and the product eluted with H_2O. Lyophilization afforded 240 mg of an amorphous powder. The product showed a single spot in TLC (silica gel, 5% Na_2HPO_4), which had an R_f value different than that for adenosine dialdehyde or adenine. Crystallization from EtOH–H_2O yielded the desired 2′-*O*-[(*R*)-hydroxymethyl(adenin-9-yl)methyl]glycerol, mp 137–139 °C (effervescence); NMR (D_2O) δ 8.30, 8.10 (2 s, 2 H, H_2 and H_8), 5.98 (t, 1 H, $J_{1'-2'}$ = 6.0 Hz, $H_{1'}$), 4.06 (d, 2 H, $J_{1'-2'}$ = 6.0 Hz, $H_{2'}$), 3.60 (d, 4 H, $J_{4'-3'}$ and $J_{4'-5'}$ = 15 Hz, $H_{3'}$, and $H_{5'}$), 3.40–3.90 (m, 1 H, $H_{4'}$, overlapping with doublet peaks of $H_{3'}$ and $H_{5'}$).

(b) Reduction with $NaBD_4$. Adenosine dialdehyde (265 mg, 1 mmol) in 5 mL of 0.0025 mM NaOD in D_2O (approximately pH 8.4) was preincubated at ambient temperature for 1 h and then reduced with $NaBD_4$ (164 mg, 4 mmol) according to the procedure described above for the reduction with $NaBH_4$. Crystallization from EtOH–H_2O yielded 259 mg (96%) of the desired [2′,3′-D]-2′-*O*-[(*R*)-hydroxymethyl(adenin-9-yl)-methyl]glycerol: NMR (D_2O) δ 8.19, 8.32 (2 s, 2 H, H_2 and H_8), 5.98 (d, 1 H, $H_{1'-2'}$ = 6 Hz, $H_{1'}$), 4.06 (d, 1 H, $H_{1'-2'}$ = 6 Hz, $H_{2'}$), 3.40–3.90 (m, 4 H, $H_{3'}$, $H_{4'}$, and $H_{5'}$).

Enzyme Purification and Assay. HMT was purified from guinea pig brain (Pel-Freez Biologicals) according to the methods previously described by Brown et al. (1959). The enzyme was purified through the dialysis step resulting in a preparation which contained 82 mg of protein per mL with a specific activity of 0.19 nmol of product (mg of protein)$^{-1}$ min^{-1} using histamine as the substrate. The enzyme was further purified by chromatography of the dialyzed preparation on Sephadex G-100. A 3-mL aliquot of the dialyzed preparation was layered onto a Sephadex G-100 column (3 × 53 cm), which had been preequilibrated with 10 mM phosphate buffer, pH 7.4. The protein was eluted with the same buffer and 2-mL fractions were collected. The fractions of highest HMT activity were pooled and concentrated yielding a preparation containing 2.6 mg of protein per mL with a specific activity of 3.74 nmol of product (mg of protein)$^{-1}$ min^{-1}. This represented a 19-fold increase in specific activity as compared with the dialyzed preparation (68% of the total HMT activity was recovered).

The HMT activity was determined using histamine and AdoMet-$^{14}CH_3$ as substrates according to a previously described radioassay (Borchardt et al., 1976a,b). For the experiments to determine kinetic inhibition patterns (Figure 1), the histamine concentration was held constant at 1.0 mM, while the AdoMet concentrations were varied from 24 to 210 μM.

The other methyltransferases used in this study were purified from the following sources according to previously described procedures (Borchardt et al., 1976a): catechol *O*-methyltransferase, rat liver (male, Sprague-Dawley, 180–200 g); phenylethanolamine *N*-methyltransferase, bovine adrenal medulla (Pel-Freez Biologicals); hydroxyindole *O*-methyltransferase, bovine pineal glands (Pel-Freez Biologicals). The enzyme activities were measured and the inhibitors evaluated using AdoMet-$^{14}CH_3$ and the appropriate acceptor substrates as described earlier (Borchardt et al., 1976a).

HMT Inactivation Experiments. A typical inactivation experiment was carried out in a total volume of 0.25 mL containing 40 mM phosphate buffer, pH 7.4, variable concentrations of the ribonucleoside dialdehyde derivatives, and the enzyme preparation (~100 μg). The preincubation step was started by the addition of enzyme and incubation was carried out at 37 °C. In the protection experiments varying amounts of AdoMet or histamine were included during the preincubation. After the appropriate preincubation time, the samples were assayed by addition of 0.05 μCi of AdoMet-$^{14}CH_3$, AdoMet, and/or histamine to give final concentrations of 1 mM for each substrate. The assay mixtures were incubated for 15 min at 37 °C and the reaction was stopped by addition of 0.25 mL of 0.25 M borate buffer (pH 10.0). The assay mixture was extracted with 10 mL of toluene–isoamyl alcohol (1:1) and after centrifugation a 5-mL aliquot of the organic phase was measured for radioactivity. The results were corrected using the appropriate histamine blank. The percent activity remaining at any given time was calculated relative to zero-time activity. The pseudo-first-order kinetic constants of inactivation, K_{app}, were calculated from the slopes of the plots of log percentage activity remaining vs. preincubation time (Kitz & Wilson, 1962; Petra, 1971; Borchardt et al., 1977).

Incorporation Studies Using [2,8-^{3}H]AdoHcy Dialdehyde. Reaction mixtures for the incorporation experiments consisted of the following components: H_2O so that the final volume was 0.25 mL; 40 mM phosphate buffer, pH 7.4; [2,8-^{3}H]AdoHcy dialdehyde (160 μM; specific activity, 0.47 mCi/mmol); and HMT (105 μg). The incubations were carried out at 37 °C. After the appropriate incubation times, the reaction mixtures were transferred to an ice bath and then filtered through a Millipore filter (type HAMK, 0.25 mm) under suction. The filter was washed with 20 mL of distilled water and dried, and the extent of radioactivity incorporated was determined by placing the filter in a vial containing 10 mL of Biofluor (New England Nuclear) and counting for radioactivity.

The [2,8-^{3}H]AdoHcy dialdehyde inactivated HMT used in the Sephadex G-100 experiment (Figure 6) was prepared in the following manner. HMT (1.3 mg; specific activity 3.74 nmol mg^{-1} min^{-1}) in 0.5 mL of 10 mM phosphate buffer, pH 7.4, was incubated with [2,8-^{3}H]-L-AdoHcy dialdehyde (22 μg; specific activity, 0.47 mCi/mmol) at 37 °C for 30 min. The excess inhibitor was removed by exhaustive dialysis against 10 mM phosphate buffer, pH 7.4. To the dialyzed, [2,8-^{3}H]-AdoHcy dialdehyde inactivated HMT was added active HMT (1.3 mg) and this mixture was applied to a Sephadex G-100 column (1 × 100 cm). The column was eluted with 10 mM phosphate buffer, pH 7.4, and 1.8-mL fractions were collected. An aliquot (0.22 mL) from each fraction was assayed for HMT activity. Another aliquot (1 mL) from each fraction was used to determine radioactivity by scintillation counting in 10 mL of Biofluor.

Results

Chemistry. The ribonucleoside dialdehydes which were prepared as potential affinity labeling reagents for HMT are listed in Figure 1. These dialdehydes were prepared by the oxidation of the corresponding ribonucleosides using 1 equiv of periodic acid in H_2O (Figure 2). In general, the dialdehydes were isolated in 50–90% using this oxidation procedure. These ribonucleoside dialdehydes were quite unstable under the acidic reaction conditions resulting in their slow hydrolysis to adenine. The chemical instability of these dialdehydes prohibited the usual crystallization and characterization by chemical analysis. Therefore, the compounds were characterized by their spectral (NMR, UV, and IR) properties, chromatographic properties (Table I), and reducibility to the corresponding acyclic ribonucleosides.

Surprisingly, the products isolated by periodate oxidation of the ribonucleosides did not exhibit the expected carbonyl stretching vibrations in the IR region (~1700 cm^{-1}); nor did they exhibit free aldehydic protons in the NMR (δ 8.5–10.0).

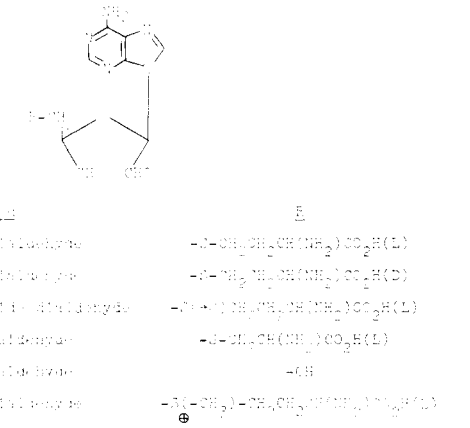


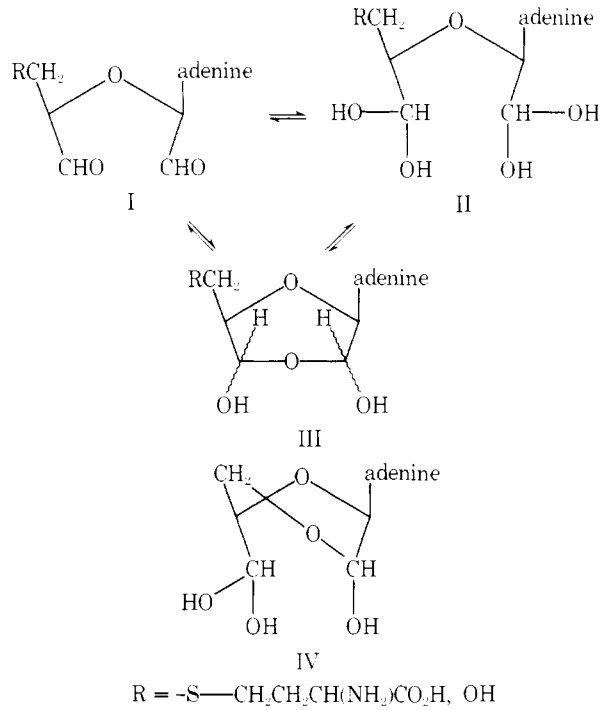
FIGURE 1: Ribonucleoside dialdehydes as potential affinity labeling reagents for HMT.

FIGURE 2: General route for the synthesis of ribonucleoside dialdehydes and acyclic 2′,3′-ribonucleosides.

Instead, we observed hydroxyl stretching vibrations (3300 cm^{-1}) in the IR, and the NMR showed the $C_{2'}$ and $C_{3'}$ protons upfield in the region of δ 5.2–6.0. These spectral data suggest that the dialdehydes probably exist in various hydrated forms rather than as the free aldehydes. Trichloroacetaldehyde, which also exists in hydrated forms in aqueous media, was found to exhibit similar IR and NMR spectral properties. In addition, Rowan et al. (1951) reported that, upon periodate oxidation of methyl-4,6-benzylidene-α-D-glucopyranoside, a hydrate form of the expected dialdehyde product was isolated.

Further evidence to support the assignment of an aldehyde oxidation state to the periodate oxidation products was obtained by reduction of these products with $NaBH_4$. Upon $NaBH_4$ reduction, quantitative yields of the corresponding acyclic ribonucleosides were isolated (Figure 2; Borchardt et al., 1978). To confirm that an equilibrium existed between the hydrated dialdehydes and the free aldehydes, the compounds were chromatographed on cellulose impregnated with polyethylenimine (5% Na_2HPO_4). Aldehydes, which are capable of forming a Schiff's base complex with this cellulose support, migrate very little in this chromatographic system. Consistent with our structural assignments, we observed that the products obtained from periodate oxidation of the ribonucleosides did not migrate from the origin in this TLC system, whereas the corresponding ribonucleosides and acyclic ribonucleosides migrate with definitive R_f values (Table I). These results suggest that the periodate oxidation products contain aldehydic (or "masked" aldehydic) functionalities.

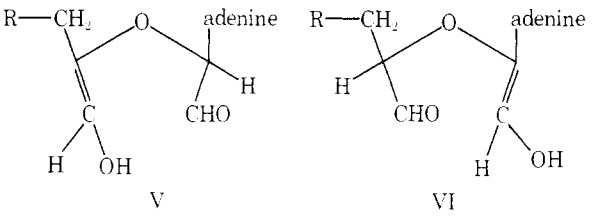
NMR data suggest that at least three forms of L-AdoHcy dialdehyde exist at equilibrium in aqueous solution. Possible structures include the hydrated form II and the various possible diastereomeric forms of hydrated species III. For adenosine dialdehyde, the hydrated structure IV is also possible because of the presence of the additional 5′-hydroxyl group. The existence of 4 doublets for the proton at $C_{1'}$ and 4 singlets each for



R = -S—$CH_2CH_2CH(NH_2)CO_2H$, OH

the protons at C_2 and C_8 in the NMR spectra of the adenosine dialdehyde provide further support to this conclusion.

To rule out the possibility that enolic structures V and VI



were involved in this equilibrium, adenosine dialdehyde was preincubated with NaOD in D_2O and then reduced with $NaBD_4$. It would be expected that deuterium exchange would be observed at $C_{1'}$ and $C_{4'}$, if the tautomeric structures V and VI existed. The NMR spectra of the product isolated after $NaBD_4$ reduction of adenosine dialdehyde showed no deuterium incorporation at $C_{1'}$ and $C_{4'}$; however, as expected, single deuterium atoms were incorporated at both $C_{2'}$ and $C_{3'}$. The absences of enolic structures such as V and VI indicate that the configurations at $C_{1'}$ and $C_{4'}$ are retained during periodate oxidation of the ribonucleoside. Based on these observations, the *R* configuration was assigned to $C_{1'}$ in all the dialdehyde derivatives; the *S* configuration was assigned to $C_{4'}$ in L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, L-AdoMet dialdehyde, L-AdoCy dialdehyde, and L-AdoHcy sulfoxide dialdehyde; and the *R* configuration was assigned to $C_{4'}$ in adenosine dialdehyde.

Since sulfide functionalities are known to be oxidized at ambient temperature by $NaIO_4$ to form sulfoxides (Leonard & Johnson, 1962, 1964; Johnson & McCant, 1964; Martin & Uekel, 1964), we determined whether AdoHcy sulfoxide and/or AdoHcy sulfoxide dialdehyde were possibly the products being generated upon periodate oxidation of L-AdoHcy. For comparison purposes, an authentic sample of L-AdoHcy sulfoxide dialdehyde was prepared by periodate oxidation of L-AdoHcy sulfoxide (Borchardt et al., 1974). In two TLC systems (Table I), neither L-AdoHcy sulfoxide or L-AdoHcy sulfoxide dialdehyde comigrated with the product isolated from periodate oxidation of L-ADOHcy (or D-Ad-

TABLE II: Effects of Ribonucleoside Dialdehydes on HMT Activity.[a]

compound	concn (mM)	% inhibition
L-AdoHcy	0.2	40
	2.0	89
L-AdoHcy dialdehyde	0.2	29
	2.0	90
D-AdoHcy	0.2	73
	2.0	99
D-AdoHcy dialdehyde	0.2	30
	2.0	89
L-AdoCy	0.2	14
	2.0	43
L-AdoCy dialdehyde	0.2	7
	2.0	12
L-AdoHcy sulfoxide	0.2	0
	2.0	9
L-AdoHcy sulfoxide dialdehyde	0.2	10
	2.0	36
adenosine dialdehyde	0.2	32
	2.0	46
L-AdoMet dialdehyde	0.2	46
	2.0	87

[a] The standard assay mixture contained the following components (in μmol): water, so that the final volume was 0.25 mL; histamine (0.5); inhibitor (variable); AdoMet (0.25); 0.05 μCi of AdoMet-$^{14}CH_3$; phosphate buffer, pH 7.40 (10); and the enzyme preparation. Incubation was carried out for 60 min at 37 °C after which the N-methylated product was isolated as described in Materials and Methods.

oHcy). These results suggest that treatment of L-AdoHcy (or D-AdoHcy) with 1 equiv of periodate results in oxidation of the *cis*-2′,3′-diol and not oxidation of the sulfide. Further proof of our structural assignments comes from the fact that L-AdoHcy dialdehyde can be methylated with methyl iodide in formic acid to yield the same product which is obtained from periodate oxidation of L-AdoMet, that being L-AdoMet dialdehyde.

HMT Inactivation Studies. In a preliminary communication (Borchardt et al., 1977), we provided evidence to suggest that L-AdoHcy dialdehyde was an affinity labeling reagent for HMT. To further examine the specificity of this protein–ligand interaction, we synthesized in this study dialdehyde derivatives of other ribonucleosides which are known to bind to HMT. In addition, we prepared dialdehyde derivatives of ribonucleosides which are known to have little or no affinity for HMT. In the former group were included D-AdoHcy, a known reversible inhibitor of HMT (Borchardt & Wu, 1974) and L-AdoMet, the methyl donor for the HMT catalyzed reaction. The latter group included L-AdoCy, L-AdoHcy sulfoxide, and adenosine which have been shown to exhibit little or no affinity for HMT (Borchardt & Wu, 1974; Borchardt et al., 1976a). Based on our earlier experiments (Borchardt et al., 1977), it would be predicted that D-AdoHcy dialdehyde and L-AdoMet dialdehyde would be potent irreversible inhibitors of HMT, whereas L-AdoCy dialdehyde, L-AdoHcy sulfoxide dialdehyde, and adenosine dialdehyde would be substantially less active. In Table II are listed the inhibitory activities of the ribonucleosides and their corresponding dialdehyde derivatives toward the HMT catalyzed transmethylation. These results are quite consistent with our prediction, since the most active dialdehydes were L-AdoHcy dialdehyde, L-AdoMet dialdehyde, and D-AdoHcy dialdehyde. It should be noted that the diols obtained by $NaBH_4$ reduction of L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, and L-AdoMet dialdehydes are inactive as inhibitors of HMT, suggesting a crucial role for the aldehydic functionalities in the mechanism of inhibition by these dialdehydes (Borchardt et al., 1978). The enzyme inhibitory activities of L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, and L-AdoMet dialdehyde are apparently limited to HMT, since none of these compounds showed inhibitory activity toward other methyltransferases which were tested [e.g., catechol *O*-methyltransferase (EC 2.1.1.6); phenylethanolamine *N*-methyltransferase (EC 2.1.1.28); hydroxyindole *O*-methyltransferase (EC 2.1.1.4)].

When the kinetics of L-AdoHcy dialdehyde inhibition of HMT were determined, a noncompetitive kinetic pattern was observed when L-AdoMet was the variable substrate (242.5 ± 59 μM). Similar results were also observed for inhibition by D-AdoHcy dialdehyde and L-AdoMet dialdehyde. The noncompetitive kinetics observed for L-AdoHcy dialdehyde and D-AdoHcy dialdehyde are in sharp contrast to the competitive kinetic patterns observed for inhibition by L-AdoHcy or D-AdoHcy (Borchardt & Wu, 1974). These noncompetitive kinetic patterns suggest a mechanism involving irreversible inactivation of HMT.

In order to determine the reversible or irreversible nature of this inhibition, HMT was preincubated for various times with L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, or L-AdoMet dialdehyde and residual enzyme activity determined. With all three dialdehydes, dramatic decreases in enzymatic activity as a function of increasing preincubation time were observed. In contrast to the rapid inactivation of HMT produced by L-AdoHcy dialdehyde, incubation with L-AdoHcy or acyclic AdoHcy resulted in no loss in enzyme activity. These results suggest an important role for the aldehydic functionality in the enzyme inactivation process. The inactivation of HMT produced by L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, or L-AdoMet dialdehyde was completely irreversible, since enzyme activity could not be recovered after dialysis or gel filtration on Sephadex G-25.

To establish whether the inactivation of HMT by D-AdoHcy dialdehyde and L-AdoMet dialdehyde proceeds by unimolecular reactions within dissociable complexes rather than via nonspecific bimolecular reactions, the rates of enzyme inactivation as a function of inhibitor concentrations were determined. Kinetic evidence for the existence of such a rate-limiting step during the inactivation of HMT by L-AdoHcy dialdehyde was previously reported (Borchardt et al., 1977). The model for this type of inactivation is shown in eq 1 and 2, where E·I is the reversible complex, E–I the inactive enzyme, k_2 the first-order rate constant, and K_I the steady-state constant of inactivation. $K_I = [E][I]/[E{\cdot}I]$ (See Kitz & Wilson, 1962; Petra, 1971 for the derivation of these equations.)

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E\text{–}I \qquad (1)$$

$$\frac{1}{k_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \qquad (2)$$

For various concentrations of D-AdoHcy dialdehyde and L-AdoMet dialdehyde, the time courses of HMT inactivation were determined and as expected pseudo-first-order kinetics were observed in all cases.

Therefore, for each inhibitor concentration an apparent first-order rate constant for inactivation (k_{app}) can be calculated. Plots of the reciprocals of the apparent first-order rate constants ($1/k_{app}$) vs. the reciprocals of the inhibitor concentrations ($1/[I]$), according to eq 2, gave linear relationships (Figure 3). The linearity and positive intercepts on the abscissa observed in these reciprocal plots provide evidence for the

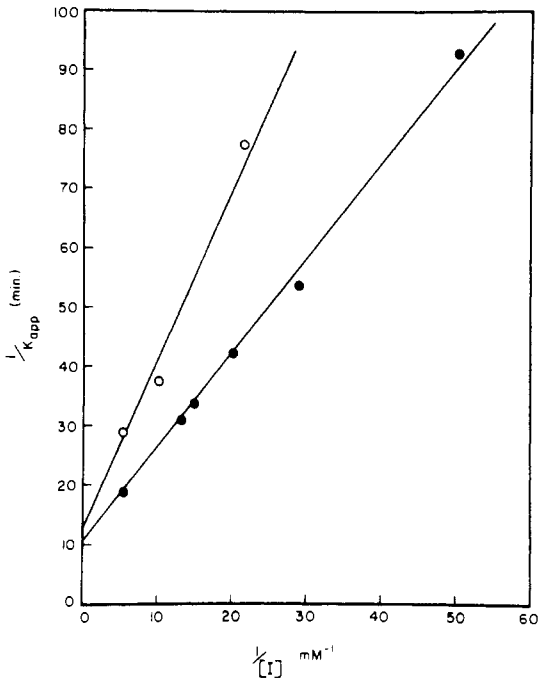


FIGURE 3: Double-reciprocal plots of the pseudo-first-order rate constants of inactivation, k_{app}, vs. inhibitor concentrations. (●—●) AdoMet dialdehyde; (○—○) D-AdoHcy dialdehyde. Kinetic constants, k_2 and K_I, were calculated from the Y intercept and the slope, respectively, using the least-squares method.

formation of a dissociable enzyme–inhibitor complex prior to enzyme inactivation. From the data shown in Figure 3, the limiting rate constant of inactivation, k_2, and the steady-state constant of inactivation, K_I, were calculated and are listed in Table III. Also shown in Table III for comparison's sake are the data obtained for L-AdoHcy dialdehyde and adenosine dialdehyde.

In an effort to further define the mechanism by which these dialdehydes inactivate HMT, the kinetic order of the enzyme–inhibitor reaction was determined using the approach described by Levy et al. (1963). The magnitude of the pseudo-first-order rate constant, k_{app}, depends upon the concentration of inhibitor as described in eq 3, where k' is a first-order constant, and N is a number equal to the average order of the reaction with respect to the concentration of the inhibitor ([I]). Using eq 4, the order of the reaction can be experimentally estimated by plotting the log k_{app} against log [I] with the slope equal to N, the kinetic order of the reaction.

$$k_{app} = k'[I]^N \quad (3)$$

$$\log k_{app} = \log k' + N \log [I] \quad (4)$$

In Figure 4 are shown the plots of log k_{app} vs. log [I] for the inactivation of HMT by L-AdoHcy dialdehyde and L-AdoMet dialdehyde. In all cases linear relationships were observed with the slopes equal to 0.84 ± 0.06 for L-AdoHcy dialdehyde, 0.96 ± 0.43 for D-AdoHcy dialdehyde, and 0.84 ± 0.04 for L-AdoMet dialdehyde. These slopes of approximate unity suggest that one molecule of the inhibitor is bound to one molecule of HMT when inactivation occurs.

In an attempt to further elucidate the nature of the interaction between these dialdehydes and the active site of HMT, substrate protection studies were carried out. Since these ribonucleoside dialdehydes would be expected to initially bind at the AdoMet binding site on HMT, AdoMet should protect the enzyme from inactivation by these agents. The data in Table IV show that AdoMet (or D-AdoHcy) in the preincubation mixture significantly protects the enzyme from inactivation by D-AdoHcy dialdehyde. Similar data were obtained for L-AdoHcy dialdehyde and L-AdoMet dialdehyde. In contrast, the presence of histamine provided only partial protection of the enzyme from inactivation.

TABLE III: Kinetic Constants for the Inactivation of HMT by L-AdoHcy Dialdehyde, D-AdoHcy Dialdehyde, L-AdoMet Dialdehyde, and Adenosine Dialdehyde.

compound	kinetic constants[a] $k_2 \pm$ SEM (min^{-1})	$K_I \pm$ SEM (μM)
L-AdoHcy dialdehyde[b]	0.079 ± 0.006	216 ± 1.6
D-AdoHcy dialdehyde[c]	0.100 ± 0.009	306 ± 2.6
L-AdoMet dialdehyde[c]	0.085 ± 0.006	126 ± 2.0
adenosine dialdehyde	0.641 ± 0.039	3768 ± 46

[a] Kinetic constants, k_2 and K_I, were calculated from the Y intercept and the slope, respectively, of double-reciprocal plots of the pseudo-first-order rate constants of inactivation, K_{app} vs. inhibitor concentration, using the least-squares method according to eq 2. [b] Data taken from Borchardt et al. (1977). [c] k_2 and K_I's were calculated from data shown in Figure 3.

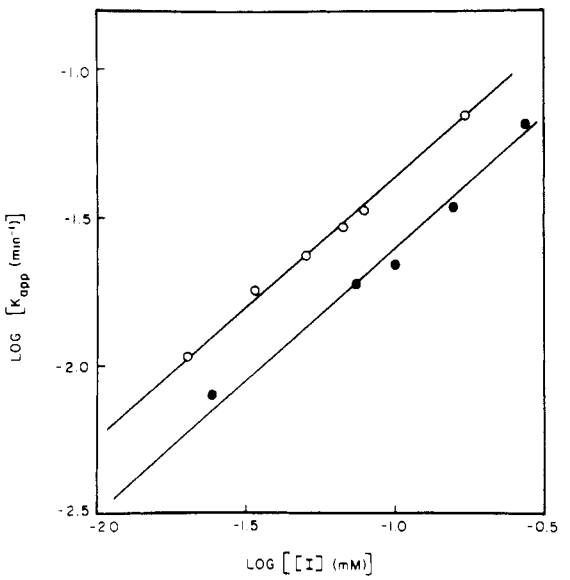


FIGURE 4: A plot of logarithm of the pseudo-first-order rate constants of inactivation, k_{app}, vs. the logarithm of the concentration of the inhibitors: (○) AdoMet dialdehyde; (●) L-AdoHcy dialdehyde.

TABLE IV: Substrate Protection of HMT from Inactivation by D-AdoHcy Dialdehyde.

reaction mixture	additions[a] (mM) AdoMet	histamine	D-AdoHcy	% residual act. after 60 min at 37 °C[b]
1				13
2	1			99
3		1		43
4			0.04	54
5		1	0.04	96

[a] The standard preincubation mixture consisted of D-AdoHcy dialdehyde (50 μM), phosphate buffer, pH 7.6 (40 mM), and purified HMT (65 μg) in a total volume of 0.25 mL. The preincubation was carried out for 60 min at 37 °C, after which the samples were assayed as described in the Materials and Methods section. [b] Residual activity was calculated with respect to the activity of samples incubated for zero time.

Incorporation Studies. To provide further evidence that these dialdehydes are indeed inactivating HMT by affinity labeling the active site, L-[2,8-^{3}H]AdoHcy dialdehyde was synthesized and its incorporation into the enzyme was studied. In Figure 5 is shown a comparison of the rate of HMT inactivation produced by L-[2,8-^{3}H]AdoHcy dialdehyde vs. the amount of radioactivity incorporated into protein. The incorporation of radioactivity appears to parallel quite closely the loss in enzymatic activity.

To confirm that L-[2,8-^{3}H]AdoHcy dialdehyde was incorporated into HMT, a sample of the ^{3}H-labeled protein was chromatographed on Sephadex G-100. The elution pattern of the ^{3}H-labeled protein was compared with the elution pattern of HMT activity and the results are shown in Figure 6. It appears that the majority of the ^{3}H-labeled protein cochromatographed with HMT activity.

Discussion

One approach to the design of an affinity labeling reagent is to modify a substrate or inhibitor which has a known affinity for the active site of the enzyme in question. This modified ligand often binds to the protein with lower affinity than that of the parent molecule resulting in poor specificity as an affinity labeling reagent (Wold, 1977). Therefore, in the design of an affinity labeling reagent it is important to consider the nature of the chemical modification and its possible effects on the ability of the reagent to form a dissociable complex with the enzyme.

In the case of AdoMet-dependent methyltransferases, one approach to the design of affinity labeling reagents would be to attach a chemically reactive group to AdoHcy, the product inhibitor of these reactions. Possible sites of attachment on AdoHcy would include the functional groups on the homocysteine portion (e.g., the terminal carboxyl group, the terminal amino group, or the sulfur atom) or those on the base portion (e.g., 6-amino group). However, in earlier studies (Borchardt, 1977; Borchardt & Wu, 1974, 1975, 1976a,b; Borchardt et al., 1974, 1976a,b), we showed that, in general these functional groups are crucial in the binding of AdoHcy to AdoMet-dependent methyltransferases. Therefore, it would be unlikely that these functional groups could be modified in the synthesis of affinity labeling reagents and still retain affinity and specificity for methyltransferases.

In affinity labeling reagents described in this paper, we have maintained these crucial functional groups on the amino acid and base portion of AdoHcy, but chemically activated the molecule by periodate oxidation of the 2′,3′-diol functionality. In preliminary studies (Borchardt et al., 1977) the resulting L-AdoHcy dialdehyde was shown to produce rapid and irreversible inactivation of HMT. In contrast, L-AdoHcy dialdehyde did not inhibit other AdoMet-dependent enzymes (e.g., catechol O-methyltransferase, phenylethanolamine *N*-methyltransferase, and hydroxyindole *O*-methyltransferase) suggesting that it was not a general affinity labeling reagent for all AdoMet-dependent methyltransferases.

To examine the specificity of this protein–ligand interaction, we synthesized the dialdehyde derivatives of D-AdoHcy, a known reversible inhibitor of HMT (Borchardt & Wu, 1974), and AdoMet, the methyl donor for the HMT catalyzed reaction. Both D-AdoHcy dialdehyde and AdoMet dialdehyde, like L-AdoHcy dialdehyde, produce rapid and irreversible inactivation of HMT. The crucial role of the aldehydic functionalities of these molecules in this inactivation is evident from the fact that the diols obtained by $NaBH_4$ reduction of L-AdoHcy dialdehyde were inactive as inhibitors of HMT (Borchardt et al., 1978).

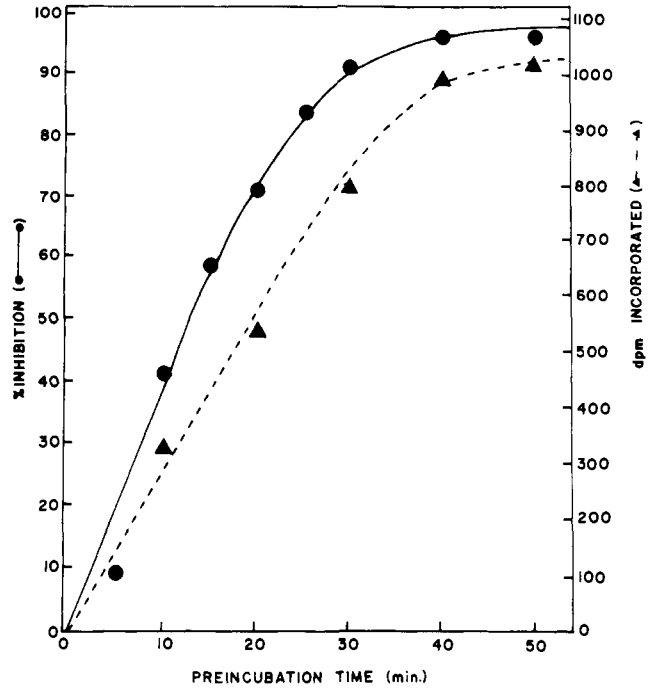


FIGURE 5: Extent of HMT inactivation produced by L-[2,8-^{3}H]AdoHcy dialdehyde as compared with dpm of radioactivity incorporated. Preincubation with HMT was carried out using L-[2,8-^{3}H]AdoHcy dialdehyde (160 μM; specific activity, 0.47 mCi/mmol). HMT was purified through Sephadex G-100 chromatography. The HMT activity remaining and the radioactivity incorporated were determined as described in Materials and Methods.

To further examine the specificity of this interaction, we oxidized L-AdoCy, L-AdoHcy sulfoxide, and adenosine, which have been shown to exhibit little or no affinity for HMT (Borchardt & Wu, 1974; Borchardt et al., 1976a) to the corresponding dialdehydes. As would be predicted, L-AdoCy dialdehyde, L-AdoHcy sulfoxide dialdehyde, and adenosine dialdehyde were poor irreversible inhibitors of HMT. For example, adenosine dialdehyde exhibited a steady-state constant of inactivation (K_I = 3768 ± 46 μM) 17 times greater than L-AdoHcy dialdehyde (K_I, 216 ± 1.6 μM). These results suggest a rather specific initial binding interaction between L-AdoHcy dialdehyde (or D-Ado-Hcy dialdehyde, L-AdoMet dialdehyde) and HMT. This interaction appears to be dependent upon structural features of the ligand known to enhance formation of a dissociable enzyme–inhibitor complex.

The generally accepted experimental criteria for an affinity labeling reagent include: (1) a rate saturation effect on the rate of inactivation of the enzyme by the affinity labeling reagent; (2) protection against inactivation by substrate or competitive inhibitor; and (3) stoichiometric incorporation of one reagent molecule per binding site (Meloche, 1967; Wold, 1977). In the present study we have shown that L-AdoHcy dialdehyde (or D-AdoHyc dialdehyde, L-AdoMet dialdehyde) inactivates HMT through the formation of a dissociable enzyme–inhibitor complex rather than by a nonspecific bimolecular reaction. The kinetic order of the reaction was determined to be unity suggesting that one molecule of the inhibitor is bound to one molecule of HMT when inactivation occurs. Therefore, these results satisfy the first criteria for an affinity labeling reagent (Meloche, 1967).

The second criteria was satisfied from substrate protection studies which showed that AdoMet, the methyl donor, would protect HMT from inactivation by L-AdoHcy dialdehyde (or D-AdoHcy dialdehyde, L-AdoMet dialdehyde). Histamine, the methyl acceptor for the reaction, provided only partial

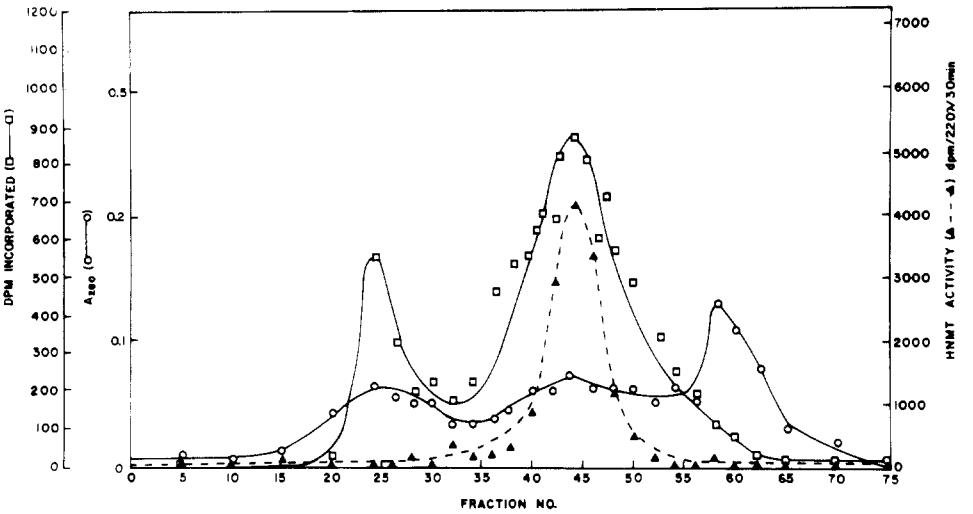


FIGURE 6: Chromatographic properties of HMT activity as compared with ^{3}H-labeled protein obtained by incubating partially purified HMT with L-[2,8-^{3}H]AdoHcy dialdehyde. HMT was incubated with L-[2,8-^{3}H]AdoHcy dialdehyde as described in Materials and Methods. After extensive dialysis to remove excess inhibitor, the ^{3}H-labeled protein along with an aliquot of active HMT was applied to a Sephadex G-100 column (1 × 100 cm), which had been previously equilibrated with 10 mM phosphate buffer, pH 7.4. Aliquots from each fraction were assayed for HMT activity (▲---▲), protein concentration (○—○), and ^{3}H-label (□—□).

protection of the enzyme from inactivation. These results are consistent with the initial, reversible binding of the affinity labeling reagent to the AdoMet binding site on HMT.

Incorporation studies using L-[2,8-^{3}H]AdoHcy dialdehyde satisfied in part the third criteria for an affinity labeling reagent. It was shown that the incorporation of L-[2,8-^{3}H]-AdoHcy dialdehyde into protein paralleled the loss of HMT activity. In addition, the majority of radioactivity from a preparation of HMT inactivated with L-[2,8-^{3}H]AdoHcy dialdehyde cochromatographed on Sephadex G-100 with enzyme activity from an untreated HMT preparation. These results are suggestive that specific incorporation of L-AdoHcy dialdehyde into HMT had occurred.

The results of these studies suggest that L-AdoHcy dialdehyde, D-AdoHcy dialdehyde, and L-AdoMet dialdehyde are affinity labeling reagents for HMT. The reagents initially bind through dissociable complexes to the AdoMet binding site on HMT, followed by reaction of the aldehydic functionalities of the reagents with an amino acid residue in the proper juxtaposition. The exact nature of this amino acid residue is unknown. The most likely possibility is a lysine residue, because of its ability to form a Schiff base adduct with aldehydes. The interaction of other ribonucleoside dialdehydes with proteins apparently occurs by Schiff base–adduct formation with lysine residues. For example, the periodate oxidation product of 6-methylmercaptopurine ribonucleoside inhibits *E. coli* RNA polymerase (Wu & Goldthwait, 1969; Krakow & Frank, 1969; Wu & Wu, 1974; Wu et al., 1974) and *E. coli* DNA-dependent DNA polymerase I (Salvo et al., 1976; Kimball, 1977) by covalently binding to an ϵ-amino group of a lysine residue at the enzymes' initiation sites.

However, in the case of HMT, the totally irreversible nature of interaction with L-AdoHcy dialdehyde is not consistent with a simple Schiff base formation, since one would expect such an imine bond to be reversible upon dialysis. Considering the multitude of hydrated species observed for the simple dialdehydes (Rowen et al., 1951; Hurd et al., 1953), an interaction more complex and more stable than a simple Schiff base adduct might be possible with amino acid residues present at the active site of HMT. Adducts similar to III, where one or more of the hydroxyl groups are substituted by protein ligands (e.g., amino or sulfhydryl residues), might account for the stability of this protein–ligand interaction. The identification of the specific amino acid residues being modified on HMT will have to await degradation studies of the inactivated protein. Such studies are presently under investigation in our laboratory.

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Conformational Changes and Local Events at the AMP Site of Glycogen Phosphorylase *b*: A Fluorescence Temperature-Jump Relaxation Study†

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ABSTRACT: The relationship between nucleotide binding kinetics and the activation process of glycogen phosphorylase *b* has been studied with two fluorescent AMP analogues, 1,N_6-etheno-AMP (ϵAMP), an activator of phosphorylase *b*, and 1,N_6-etheno-2′-deoxy-AMP (ϵdAMP). This latter probe binds to the same nucleotide site but does not activate the enzyme. A major relaxation process consistent with a bimolecular association is observed after perturbation of the phosphorylase *b*-ϵAMP or ϵdAMP complex in 50 mM glycylglycine buffer. Residence times of these two nucleotides are of the same order of magnitude ($\simeq$500 μs at 11 °C). After addition of the anionic substrates, orthophosphate or glucose 1-phosphate, to the enzyme-nucleotide complex, a rapid chase ($\leq$3 ms) of ϵAMP and ϵdAMP is observed, which presumably reflects competition for the nucleotide binding site. This rapid chase is followed by a slow uptake of ϵAMP on the enzyme. This process is not observed when ϵAMP is replaced by ϵdAMP and reflects an isomerization of the enzyme in the 10-min range at 11 °C. Temperature-jump relaxation studies have shown that this isomerization is accompanied by a concomitant change of the binding kinetics of ϵAMP, a new binding process in the 20-ms time range becoming predominant. The residence time of ϵAMP on the corresponding enzyme conformation is 28 ms at 11 °C: this is very close to the value observed with phosphorylase *a* without any added substrate (11 ms at 24 °C). In addition to phosphate and glucose 1-phosphate, a variety of anionic effectors (sulfate, citrate) or cationic effectors (magnesium, spermine), as well as 5′-AMP, were found to produce the transition from the "fast-binding" to the "slow-binding" enzyme forms. In the light of these findings, we finally discuss the role of the nucleotide and of these various effectors in the activation of phosphorylase *b*, in the context of current models for allosteric transitions.

Glycogen phosphorylase is at the center of all regulation mechanisms controlling glycogen degradation. The molecular basis of regulatory effects on phosphorylase activity has been interpreted in terms of conformational changes, the more active states being stabilized either by covalent phosphorylation or through AMP binding.

On the basis of binding and activation studies, several groups (Madsen and Shechosky, 1967; Buc, 1967) suggested that the allosteric regulation of phosphorylase *b* conforms to the two-state concerted model (Monod et al., 1965). However, additional conformational states have been postulated to explain the activity of phosphorylase *b* at low AMP concentrations (Kastenschmidt et al., 1968) or the steady-state enzymatic response to AMP analogues (Black and Wang, 1968; Morange et al., 1976). Other conformational states have been proposed in order to account for the changes of protein structure upon the addition of ligand, detected by paramagnetic and spectroscopic reporter groups (Birkett et al., 1971; Madsen et al., 1976). However, the precise interpretation of most static measurements in terms of conformational changes is obscured by the fact that only conformation-averaged properties are recorded. Moreover, it is often difficult in spectroscopic work to decide whether the observed effects reflect gross changes in the tertiary or quaternary structure of the protein or minor structural modifications in the vicinity of the spectroscopic probe.

† From the Institut Pasteur, 75015 Paris, France. *Received February 14, 1978.* This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale.

0006-2960/78/0417-4153$01.00/0