Mechanism of Action of *S*-Adenosyl-L-homocysteine Hydrolase. Measurement of Kinetic Isotope Effects Using Adenosine-*3'-d* and *S*-Adenosyl-L-homocysteine-*3'-d* as Substrates[1]

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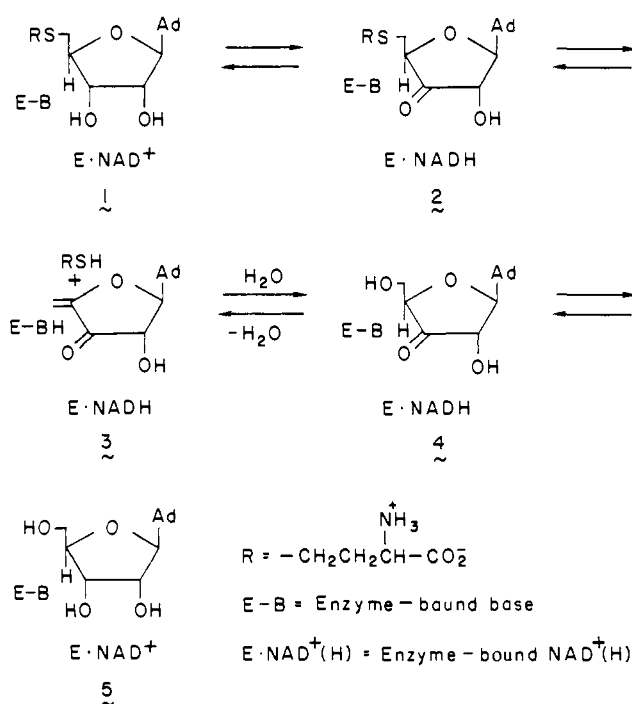
Abstract: *S*-Adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) catalyzes the reaction AdoHcy + $H_2O \rightleftharpoons$ adenosine (Ado) + L-homocysteine (Hcy). To determine if any of the proposed oxidation/reduction steps involving the 3'-position of AdoHcy or Ado and mediated by NAD^+/NADH are responsible for limiting the rate of catalysis, V_{max} isotope effects were measured by using AdoHcy-*3'-d* (**16**) and Ado-*3'-d* (**14**) as substrates. Ado-*3'-d* was synthesized from 1,2:5,6-di-*O*-isopropylidene-α-D-ribohexofuranose-*3-d* (**8**) which in turn was prepared by the virtually stereospecific reduction of 1,2:5,6-di-*O*-isopropylidene-α-D-ribohexofuranos-3-ulose (**7**) with $NaBD_4$. AdoHcy-*3'-d* was synthesized from **14** via 5'-deoxy-5'-chloro-Ado-*3'-d* (**15**). AdoHcy hydrolase was purified from bovine liver and the reactions in both the hydrolytic and synthetic directions were followed by HPLC. The isotope effects (k_H/k_D) at V_{max} were found to be 2.58 for the hydrolysis of AdoHcy and 1.33 for the synthesis of AdoHcy. The results of this study suggest that the oxidation/reduction steps are not the sole rate-limiting steps and that the release of products may be responsible for limiting the rate of catalysis.

S-Adenosyl-L-homocysteine hydrolase (AdoHcy hydrolase, EC 3.3.1.1) catalyzes[2] both the hydrolysis of AdoHcy (**1**) to adenosine (Ado, **2**) and homocysteine (Hcy) and the synthesis of AdoHcy from Ado and Hcy (Scheme I). The nucleoside AdoHcy is a product inhibitor of *S*-adenosyl-L-methionine-dependent (AdoMet) methylation reactions.[2] As AdoHcy hydrolase is the only known enzyme in eukaryotes that catabolizes AdoHcy, it plays an important role in regulating AdoMet-dependent methylation reactions.[3] Because of this role of AdoHcy hydrolase, it has become an important target for designing chemotherapeutic agents of wide applications.[4] In this regard, the detailed understanding of the mechanism of action of AdoHcy hydrolase is of considerable significance. Elegant mechanistic work of Palmer and Abeles[5] has established that the enzyme avoids direct displacement of the homocysteinyl moiety of AdoHcy and the 5'-OH of Ado during catalysis by utilizing a tightly bound NAD^+ to activate the substrates. The nature of this activation has been suggested to be the oxidation of the 3'-position of AdoHcy or Ado to produce the 3'-keto nucleosides **2** and **4**, respectively. These oxidations facilitate the abstraction of the C-4' protons (of **2** and **4**) which in turn facilitate the elimination of the C-5' substituents (homocysteinyl moiety and hydroxyl group, respectively).[6] Michael-type addition of water or homocysteine to **3** is also facilitated by the presence of the 3'-keto function. Compelling evidence has been provided[5] in support of this mechanism. However, none of the proposed intermediates has yet been isolated (because of their instability). More recently,[7] it has been shown that the elimination–addition steps at C-5' of both AdoHcy and Ado proceed with syn geometry at each step with overall retention of the configuration at C-5'.

Scheme I



Although a good deal is known about the catalytic mechanism of AdoHcy hydrolase, one important question that remains unanswered is the nature of the rate-determining step. The primary objective of this study was to determine if any of the proposed oxidation/reduction steps involving NAD^+/NADH are responsible for limiting the rate of catalysis. For this purpose V_{max} isotope effects were measured by using AdoHcy-*3'-d* and Ado-*3'-d* as substrates. We now report these results.

(1) Presented in part at the 75th Annual Meeting of American Society of Biological Chemists, St. Louis, MO, June 3–7, 1984; Abstract No. 1713 (*Fed. Proc.* **1984**, *43*, 1710).

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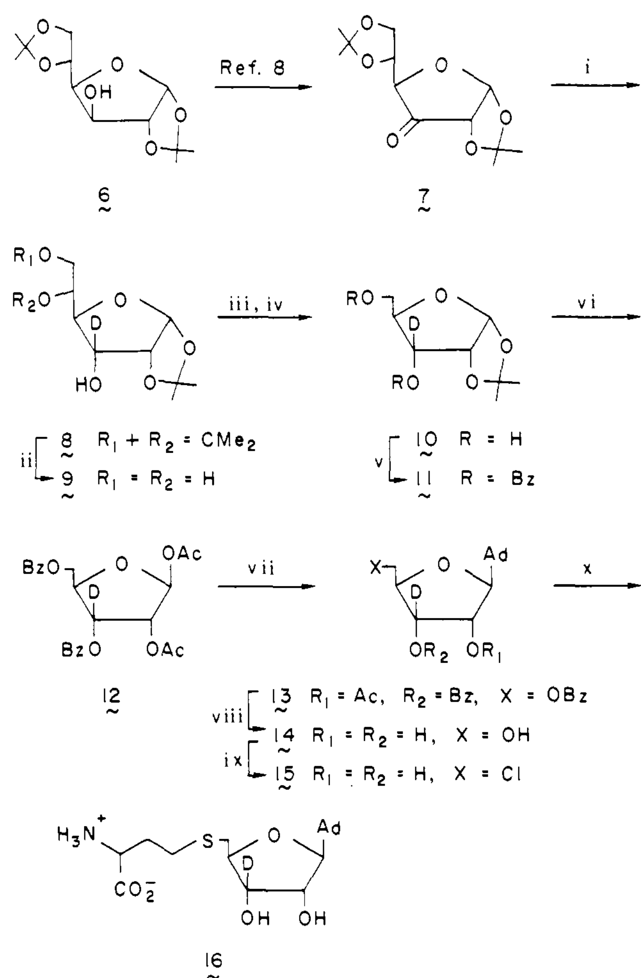
Scheme II[a]



[a] Reagents: i, $NaBD_4$, EtOH; ii, Dowex 50W-X4 (H^+); iii, $NaIO_4$, pH 6.5; iv, $NaBH_4$, EtOH; v, PhCOCl, pyridine; vi, Ac_2O, AcOH, H_2SO_4; vii, adenine, $SnCl_4$, CH_3CN; viii, NH_3, MeOH; ix, $SOCl_2$, $(Me_2N)_3PO$; x, L-homocystine, Na, liquid NH_3.

Results

Synthesis of Ado-*3′-d* and AdoHcy-*3′-d*. The observation[8] that the keto sugar **7** is reduced with $NaBH_4$ to give the alloepimer of **6** with extremely high stereoselectivity (≫99.5%) suggested a possible synthesis of Ado-*3′-d* (and hence of AdoHcy-*3′-d*) starting from **7** (Scheme II). The keto sugar **7** was readily prepared[8] by the (RuO_2/KIO_4) oxidation of the diacetonide **6** of D-(+)-glucose. Reduction of **7** with $NaBD_4$ (98 atom % D) in EtOH gave the allose-*3-d* derivative **8** in 83% yield. The mass spectrum of **8** revealed that 97 atom % deuterium had been incorporated. In the ^{13}C NMR spectrum of **8**, the carbon containing the deuterium appeared as a triplet of highly reduced intensity with $J_{C_3\text{-}D} = 22.4$ Hz. The primary isotope effect in the chemical shift[9] ($\Delta\delta$) of C-3 was +0.36 ppm. Interestingly, these values of J and $\Delta\delta$ remained essentially constant in all the subsequent synthetic intermediates for which these values were determined.

Selective acid hydrolysis of the 5,6-*O*-isopropylidene group of **8** gave the triol **9**. The C_5–C_6 bond of **9** was then cleaved with periodate, and the resulting aldehyde was reduced with $NaBH_4$ to give the ribose derivative **10**. The free hydroxyl groups of **10** were benzoylated to give **11** which upon acetolysis furnished the fully esterified sugar **12**. Coupling of **12**, with adenine was carried out in CH_3CN in the presence of $SnCl_4$. This procedure[10] was found to be superior to that involving coupling of chloromercuri-6-benzamidopurine in the presence of $TiCl_4$.[11] Ammonolysis of **13** in MeOH then gave Ado-*3′-d* (**14**). For the synthesis of AdoHcy-*3′-d*, Ado-*3′-d* was first converted[12] to the 5′-deoxy-5′-chloro intermediate **15** using $SOCl_2$ in $(Me_2N)_3PO$. Condensation[12] of **15** with the disodium salt of Hcy, generated in situ from L-homocystine in liquid NH_3, gave AdoHcy-*3′-d*.

The structures of the intermediates involved in these syntheses, as well as the target deuterium-labeled Ado and AdoHcy, were established by using spectroscopic techniques (^1H NMR, ^{13}C NMR, and mass spectrometry) and comparison with the corrresponding authentic, unlabeled homologues. It was found that the synthetic sequence did not lead to any detectable loss of the deuterium label, and the deuterium contents of Ado-*3′-d* and AdoHcy-*3′-d* were very nearly equal to that of **8**.

Unlabeled Ado and AdoHcy were also synthesized by using the reaction sequence described in Scheme II for establishing their identity with the corresponding authentic samples and also for their use as the unlabeled references in the enzymatic studies.

Enzymatic Studies. Radiolabeled substrates are normally used to measure the catalytic activity of AdoHcy hydrolase.[4b] For this study, a new HPLC assay for both the synthesis and hydrolysis of AdoHcy was developed to avoid the use of radioisotopes. A reversed-phase C-8 column gave good separation of the substrates and products in less than 15 min after injection. The retention times for inosine, Ado, and AdoHcy were 5.2, 11.4, and 13.5 min, respectively.

All experiments were done with saturating substrate concentrations and less than 10% conversion to product (V_{max} conditions). Figure 1 shows the reaction rates for the synthesis of AdoHcy and AdoHcy-*3′-d*. The slope of each line gave the V_{max} of the reaction, 186 ± 10 and 144 ± 10 pmol/min for Ado and Ado-*3′-d* as substrate, respectively. The V_{max} isotope effect (k_H/k_D) is, therefore, 1.33 ± 0.08 (after correcting for ~97 atom % deuterium content of Ado-*3′-d*).

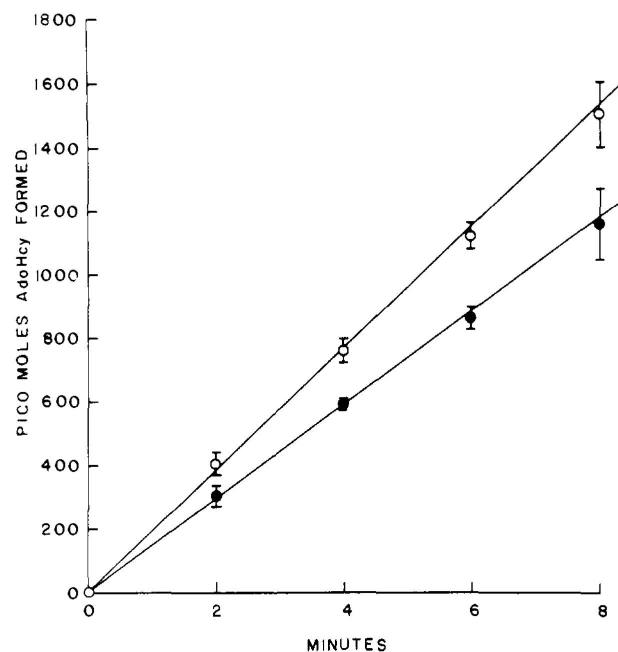


Figure 1. V_{max} reaction rates for the synthesis of AdoHcy (○) and AdoHcy-*3′-d* (●). Bovine liver AdoHcy hydrolase synthetic activity was measured as described in the Experimental Section using Ado (○) and Ado-*3′-d* (●) as substrates. Bars denote mean ± standard deviation of at least three independent determinations in duplicate.

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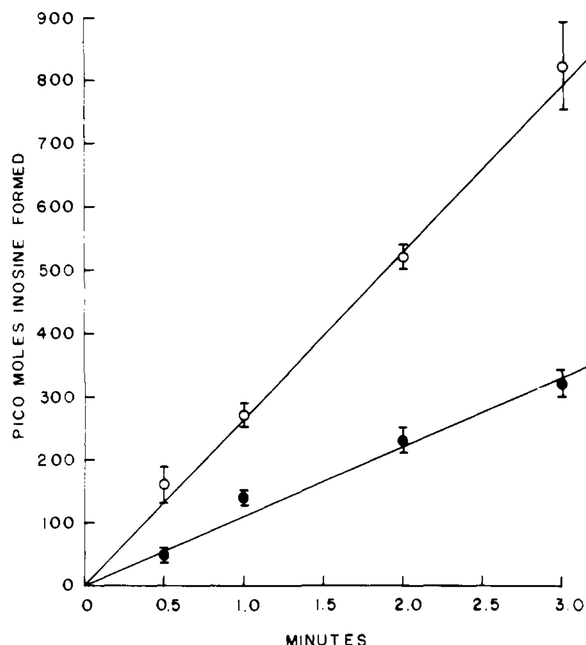

Figure 2. V_{max} reaction rates for the hydrolysis of AdoHcy (○) and AdoHcy-*3′-d* (●). Bovine liver AdoHcy hydrolytic activity was measured as described in the Experimental Section. Bars denote mean ± standard deviation of at least three independent determinations in duplicate.

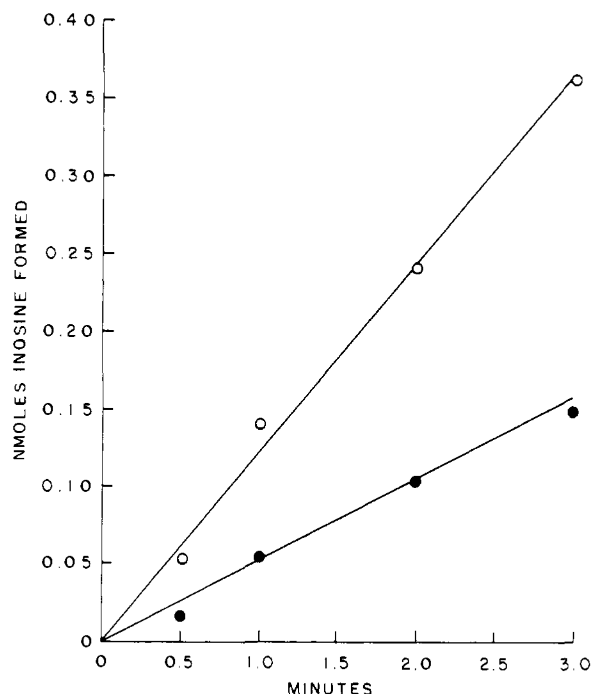

Figure 3. Effect of 200 μM DL-Hcy on the (V_{max}) rate of hydrolysis of AdoHcy (○) and AdoHcy-*3′-d* (●). For details see Experimental Section.

Unlike the synthetic reaction which was linear up to 30 min (data not shown), the hydrolysis of AdoHcy was only linear during the first 4 min. The V_{max} reaction rates for AdoHcy and AdoHcy-*3′-d* as substrates, shown in Figure 2, were 267 ± 23 and 107 ± 6 pmol/min, respectively, giving a k_H/k_D of 2.58 ± 0.22 (after correcting for ~97 atom % deuterium content of AdoHcy-*3′-d*). To determine if externally added Hcy had any effect on the isotope effect for the hydrolysis of AdoHcy, the same experiment was done in the presence of 20 and 200 μM DL-Hcy. No effect on either the reaction rate or the isotope effect was seen with 20 μM DL-Hcy (data not shown). With 200 μM externally added Hcy, the isotope effect remained unchanged ($k_H/k_D \sim 2.6$, Figure 3), but the rates for the hydrolysis of both deuterated and undeuterated AdoHcy were reduced by 60%.

Enzymatic Synthesis of AdoHcy-*3′-d* from Ado-*3′-d*. It was of interest to determine if the 3′-H abstracted by the enzyme-bound NAD^+ during oxidation of Ado was the same one that was returned during the reduction of intermediate **2** (Scheme I). Accordingly, a 20 mM solution of Ado-*3′-d* was incubated with 15–20 units of partially purified AdoHcy hydrolase in the presence of DL-Hcy at 37 °C for 3 h. Spectroscopic (^1H NMR and mass) analysis of the product showed that it is identical with synthetic AdoHcy-*3′-d* and that its deuterium content was essentially the same as in the substrate Ado-*3′-d*. These results show that the hydrogen transfer to and from the 3′-position during catalysis takes place without exchange of hydrogen from the medium.[13]

Discussion

The observed k_H/k_D values for most NAD(H)-dependent alcohol dehydrogenases[14] are larger than those obtained for AdoHcy hydrolase-catalyzed hydrolysis of AdoHcy to Ado and Hcy (k_H/k_D = 2.58 ± 0.22) and AdoHcy hydrolase-catalyzed conversion of Ado and Hcy to AdoHcy (k_H/k_D = 1.33 ± 0.08). It is very likely that the isotope effects observed with AdoHcy hydrolase are indeed less than the corresponding intrinsic, primary kinetic isotope effects and that oxidation/reduction steps at the 3′-position are not the sole rate-limiting steps. Both effects are, on the other hand, too large to be secondary or equilibrium effects so that the redox steps must participate in limiting the rate. These results further provide the first direct evidence that the cleavage of the C_3′-H bond of AdoHcy or Ado is a step on the reaction pathway.

According to the mechanism of action of AdoHcy hydrolase shown in Scheme I, a C–D bond will be cleaved at two different points during the hydrolysis of AdoHcy-*3′-d* (**1** → **2** and **4** → **5**) and during the synthesis of AdoHcy-*3′-d* from Ado-*3′-d* (**5** → **4** and **2** → **1**). It is possible that all these C–D bond cleavages proceed at different rates. Thus, in each direction of the reaction, the isotope sensitive step could be the transfer of deuterium either from the 3′-position of the substrate or to the 3′-position of the corresponding 3′-keto nucleoside.

There are several possibilities for the rate-determining steps which not only dilute the intrinsic isotope effects but also lead to the 2-fold difference in the observed isotope effects for the synthesis and hydrolysis of AdoHcy. Among the possibilities are the binding of substrates, the chemical steps not involving C–D bond cleavage, and the release of products. As the reactions were carried out under maximum velocity conditions, the differences in the rate of binding of the substrates cannot account for the values obtained.

Among the chemical steps, only the step involving the abstraction of the 4′-proton of AdoHcy has so far been kinetically defined. Palmer and Abeles[5] found that the hydrolysis of AdoHcy-*4′-d* has a V_{max} deuterium isotope effect of 1.44. This observation suggests that although the abstraction of the C-4′ proton of AdoHcy, by the enzyme-bound base, is on the reaction pathway, it is not the sole rate-limiting step. The kinetic nature of the other chemical steps, namely, abstraction of the C-4′ proton of Ado and the addition/elimination of the 5′-hydroxyl or the 5′-thioether linkage, remains undefined. Consideration of the principle of microscopic reversibility suggests that the chemical steps, once the substrates are bound, may be reversible, at least to some extent. This is also indicated by the observation that 4′,5′-dehydro-Ado, an analogue of the proposed intermediate **3**, is oxidized by the enzyme and then rapidly converted to either Ado or AdoHcy (in the presence of Hcy). The reversible nature of the chemical steps will obliterate any differences that might otherwise have existed between the chemical steps in the synthetic

(13) These observations parallel those observed with TDP-glucose oxidoreductase.[6] It was shown that this enzyme also converts TDP-[4-^{3}H]glucose quantitatively to TDP-[6-^{3}H]-6-deoxy-D-*xylo*-4-hexosulose without loss of any tritium to the medium.[6b]

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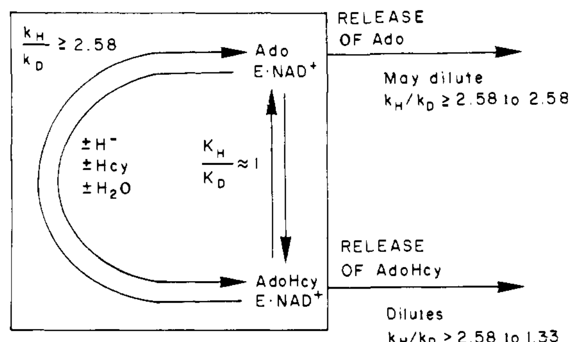

Figure 4. Possible contribution of Ado and AdoHcy release toward the rate-limiting steps of AdoHcy hydrolase.

and the hydrolytic directions. In the event that the chemical steps are reversible, the remaining difference between the synthetic and the hydrolytic reactions, under the assay conditions employed, is the release of products from the enzyme.

The possible contribution of product release to the rate-determining step is shown in Figure 4. The box represents all the chemical reactions that occur after the binding of substrates. There should be little or no equilibrium isotope effect (i.e., $K_H/K_D \approx 1$), as the chemical environment of the 3′-deuterium is similar in Ado-*3′-d* and AdoHcy-*3′-d*. On the basis of the data for the hydrolysis of AdoHcy-*3′-d*, the steps involving a C–D bond cleavage have a combined intrinsic deuterium isotope effect of at least 2.58. If the combined intrinsic isotope effect is greater than 2.58, then the release of Ado-*3′-d* dilutes this value to 2.58. The fact that increased Hcy concentration did not reduce the isotope effect (although the rate was somewhat reduced at high concentrations) is suggestive that events after a reversible Hcy release are not major contributors to limiting the rate in the hydrolytic direction, although further work will be necessary to establish the situation precisely. Release of AdoHcy, on the other hand, appears to be a significant contributor to the rate-determining step which dilutes the observed isotope effect to 1.33.

Experimental Section

General Methods. ^1H and ^{13}C NMR spectra were recorded on a Varian FT-80A spectrometer with chemical shifts reported in parts per million (ppm) downfield from Me_4Si. The isotope shifts (in ppm) for ^{13}C nuclei are defined[9] as $\Delta\delta = \delta_c$ (undeuterated) − δ_c (deuterated). Mass spectra were recorded on a Varian MAT CH-5 and Nermag R-10-10 Quadrupole mass spectrometers. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Calf intestinal Adenosine deaminase, dithiothreitol (DTT), heptanesulfonic acid, and $NaBD_4$ (98 at % D) were purchased from Sigma Chemical Co. (St. Louis, MO) and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) from Burroughs Wellcome Co., Research Triangle Park, NC.

1,2:5,6-Di-*O*-isopropylidene-α-D-ribohexofuranose-*3-d* (8). To a stirred solution of **7** monohydrate[8] (27.6 g, 100 mmol) in 200 mL of 95% EtOH at 5–10 °C was added $NaBD_4$ (2.1 g, 50 mmol) in portions. After stirring at 25 °C for 2 h, excess $NaBD_4$ was decomposed by adding HOAc with cooling. The mixture, after concentrating in vacuo, was diluted with 5% NaCl solution. The resulting mixture was extracted with CH_2Cl_2 (4 × 75 mL), and the combined CH_2Cl_2 extracts were washed with brine, dried ($MgSO_4$), and then evaporated in vacuo to dryness. Recrystallization from cyclohexane gave 21.8 g (83%) of **8**: mp 76–78 °C [lit.[15] mp 76–78 °C]; ^1H NMR ($CDCl_3$) δ 1.37 (s, 6, CH_3), 1.46 (s, 3, CH_3), 1.57 (s, 3, CH_3), 2.50 (br, s, 1, OH), 3.97–4.20 (m, 4, H-4, H-5, H-6), 4.59 (d, $J_{1,2}$ = 3.8 Hz, 1, H-2), 5.80 (d, $J_{1,2}$ = 3.8 Hz, 1, H-1); ^{13}C NMR (Me_2SO-d_6) δ 25.4, 26.2, 26.5, 26.7, 64.0, 71.1 (t, $J_{C_3\text{-}D}$ = 22.4 Hz, Δδ = 0.36, C-3), 75.1, 78.3, 79.4, 103.4, 108.5, 111.5; mass spectrum (EI), *m/z* (rel intensity) 246 (18.8), 245 (0.6). Anal. ($C_{12}H_{19}{}^2HO_6$) C, H + ^2H.

1,2-*O*-Isopropylidene-α-D-ribohexofuranose-*3-d* (9). A mixture of **8** (26.1 g, 100 mmol) and Dowex 50W-X4-200 resin (H^+ form, 28 g) in 300 mL of H_2O was stirred at 15–20 °C for 20 min. The mixture was filtered, and the filtrate was evaporated to give a solid. Recrystallization from $CHCl_3$ gave **9** (17.2 g, 78%): mp 127–129 °C; ^1H NMR (Me_2CO-d_6) δ 1.30 (s, 3, Me), 1.47 (s, 3, Me), 2.24 (s, 3, OH), 3.61–3.85 (m, 4, H-4, H-5, H-6), 4.52 (d, $J_{1,2}$ = 3.8 Hz, 1, H-2), 5.69 (d, $J_{1,2}$ = 3.8 Hz, 1, H-1); ^{13}C NMR (D_2O) 26.4, 26.6, 63.0, 70.6 (t, $J_{C_3\text{-}D}$ = 22.4 Hz, Δδ = 0.35, C-3), 71.7, 80.2, 80.4, 104.5, 114.2. Anal. ($C_9H_{15}{}^2HO_6$) C, H + ^2H.

1,2-*O*-Isopropylidene-α-D-ribofuranose-*3-d* (10). The title compound was prepared from **9** (100 mmol) in 86% yield following the procedure described by Murray and Prokop[16] for an analogous transformation: mp 86–87 °C [lit.[17] mp (of protio homologue of **10**) 85.5–86 °C]; 1H NMR (Me_2CO-d_6) δ 1.30 (s, 3, Me), 1.46 (s, 3, Me), 2.73 (s, 2, OH), 3.42–3.83 (m, 3, H-4, H-5), 4.50 (d, $J_{1,2}$ = 3.8 Hz, 1, H-2), 5.70 (d, $J_{1,2}$ = 3.8 Hz, 1, H-1); ^{13}C NMR (D_2O) δ 26.3, 26.5, 60.7, 70.5 (t, $J_{C_3\text{-}D}$ = 22.4, Hz, Δδ = 0.36, C-3), 79.9, 80.3, 104, 5, 114.1; mass spectrum (EI), *m/z* (rel intensity) 176 (19.7), 175 (0.6). Anal. ($C_8H_{13}{}^2HO_5$) C, H + ^2H.

3,5-Di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-ribofuranose-*3-d* (11). Benzoylation of **10** (50 mmol) was carried out by adapting the procedure of Jenkins and Walton.[18] The crude product was recrystallized from 2-propanol–cyclohexane to give 18.7 g (94%) of **11**: mp 101–102 °C [lit.[17] mp (of protio homologue of **11**) 100.5–101.5 °C]; ^1H NMR (Me_2CO-d_6) δ 1.31 (s, 3, Me), 1.52 (s, 3, Me), 4.35–4.76 (m, 4, H-4, H-5), 5.01 (d, $J_{1,2}$ = 3.8 Hz, 1, H-2), 5.96 (d, $J_{1,2}$ = 3.8 Hz, 1, H-1), 7.29–7.64 (m, 4, Ph), 7.92–8.09 (m, 6, Ph); ^{13}C NMR (Me_2SO-d_6) δ 26.7, 63.4, 72.5 (t, $J_{C_3\text{-}D}$ = 22.4 Hz), 73.5, 77.2, 104.4, 112.4, 128.6, 128.7, 129.1, 129.3, 129.4, 129.5, 133.4, 133.6, 165.0, 165.5. Anal. ($C_{22}H_{21}{}^2HO_7$) C, H + ^2H.

1,2-Di-*O*-acetyl-3,5-di-*O*-benzoyl-β-D-ribose-*3-d* (12). Acetolysis of **11** (46 mmol) following the procedure described by Murray and Prokop[16] for a similar compound gave 14.8 g (74%) of **12** as needles after recrystallization from 2-propanol–cyclohexane: mp 127–128 °C [lit.[17] mp (of protio homologue of **12**) 126–127 °C]; ^1H NMR (Me_2CO-d_6) δ 1.95 (s, 3, Me), 2.09 (s, 3, Me), 4.36–4.81 (m, 3, H-4, H-5), 5.50 (s, 1, H-2), 6.17 (s, 1, H-1), 7.32–7.65 (m, 4, Ph), 7.93–8.11 (m, 6, Ph); ^{13}C NMR (Me_2SO-d_6) δ 20.2, 20.5, 63.4, 70.5 (t, C-3), 74.1, 79.4, 98.1, 128.7, 128.8, 128.9, 129.2, 129.3, 129.4, 133.5, 133.8, 164.8, 165.3, 168.8, 169.2. Anal. ($C_{23}H_{21}{}^2HO_9$) C, H + ^2H.

Adenosine-*3′-d* (14). The coupling of **12** (3.54 g, 8 mmol) with adenine (1.08 g, 8 mmol) was carried out in dry CH_3CN (250 mL) in the presence of 3 equiv of $SnCl_4$ (6.25 g, 24 mmol) following the procedure of Saneyoshi and Satoh[10] who used 2 equiv of $SnCl_4$. Crude **13** was chromatographed on silica gel (50 g) by using 9:1 CH_2Cl_2–EtOH to give a glassy material which was used in the next step without further purification: ^1H NMR (Me_2CO-d_6) δ 2.01 (s, 3, Me), 4.60–4.93 (m, 4, H-2′, H-4′, H-5′), 6.38 (s, 1, H-1′), 7.40–7.65 (m, 6, Ph, NH_2), 7.98–8.15 (m, 8, Ph, H-2, H-8).

A solution of **13** (3 g) in 300 mL of CH_3OH saturated with anhydrous NH_3 was stirred at 25 °C in a stoppered flask for 20 h. Evaporation of solvent gave a white solid to which were added 120 mL of H_2O and 50 mL of $CHCl_3$. The mixture was stirred for 30 min and then the aqueous layer was collected. The aqueous layer was washed with $CHCl_3$ (3 × 30 mL) and then concentrated in vacuo to a small volume. Refrigeration gave crystalline Ado-*3′-d* (**14** 1.16 g, 75%): mp 234–236 °C; mp of a standard sample (from Aldrich) 234–236 °C; ^1H NMR (D_2O) δ 3.59–3.68 (m, 2, H-5′), 3.95 (t, *J* = 3.6 Hz, 1, H-4′), 4.59 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-2′), 5.89 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-1′), 8.15 (s, 1, H-8), 8.34 (s, 1, H-2); ^1H NMR (Me_2SO-d_6) δ 3.52–3.66 (m, 2, H-5′), 3.96 (t, *J* = 3.6 Hz, 1, H-4′), 4.59 (t, 1, H-2′), 5.08 (s, 1, 3′-OH), 5.33 (t, *J* = 6.3 Hz, 2, 2′-OH, 5′-OH), 5.88 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-1′), 7.25 (s, 2, NH_2), 8.13 (s, 1, H-8), 8.32 (s, 1, H-2); ^{13}C NMR (Me_2SO-d_6) δ 61.8, 70.5 (t, $J_{C_3'\text{-}D}$ = 22.4 Hz, Δδ = 0.36, C-3′), 73.7, 86.0, 88.3, 119.5, 140.1, 149.3, 152.6, 156.3; mass spectrum (EI), *m/z* (rel intensity) 238 (100), 237 (17.2); protio homologue 237 (100), 236 (13.9). Anal. ($C_{10}H_{12}{}^2HN_5O_4$) C, H + ^2H, N.

5′-Chloro-5′-deoxyadenosine-*3′-d* (15). The title compound was prepared in 60% yield from **14** (2-mmol scale) following the procedure of Borchardt et al.:[12] mp 188–190 °C [lit.[12] mp (of protio homologue of **15**) 188–190 °C]; ^1H NMR (Me_2SO-d_6) δ 3.83–4.15 (m, 3, 4′-H, 5′-H), 4.70 (t, 1, H-2′), 5.32 (s, 1, 3′-OH), 5.97 (d, *J* = 6.3 Hz, 1, 2′-OH), 5.90 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-1′), 7.19 (s, 2, NH_2), 8.13 (s, 1, H-8), 8.29 (s, 1, H-2). Anal. ($C_{10}H_{11}{}^2HClN_5O_3$) C, H + ^2H, N.

***S*-Adenosyl-L-homocysteine-*3′-d* (16).** The title compound was prepared from **15** (0.34 mmol) following the procedure of Borchardt et al.[12] with the following modification. The step involving ion-exchange chro-

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matography of the crude product was omitted. Instead, the crude product was directly chromatographed on preparative-layer Avicel-F plates (1 mm thick) with 3:1 ethanol/H_2O as the eluent. Recrystallization from H_2O gave **16** (71 mg, 52%) as its monohydrate: mp 216-219 °C [lit.[12] mp (of protio homologue of **16**) 217-220 °C]; ^{1}H NMR (D_2O) δ 1.85-2.10 (m, 2, CH_2-CH_2-CH), 2.54 (t, J = 7.5 Hz, 2, SCH_2), 2.85-2.95 (m, 2, H-5′), 3.40 (t, J = 6.5 Hz, 1, CH), 4.05 (t, J = 6 Hz, 1, H-4′), 4.72 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-2′), 5.3 (d, $J_{1',2'}$ = 6.1 Hz, 1, H-1′), 8.19 (s, 1, H-8), 8.34 (s, 1, H-2); mass spectrum (DCI; NH_3), m/z (rel intensity) 285 (23.1), 284 (1.5); protio homologue 284 (24.5), 283 (0.8). Anal. ($C_{14}H_{19}{}^2HN_6O_5S\cdot H_2O$) C, H + ^{2}H, N.

Purification of AdoHcy Hydrolase. Bovine liver was obtained from a local slaughter house and stored at -70 °C. The enzyme was partially purified according to the procedure of Palmer and Abeles.[5] Briefly, 500 g of tissue was homogenized in 10 mM K_2HPO_4, pH 7.2, in a Waring blender at 4 °C. The homogenate was subjected to acid and heat precipitation, ammonium sulfate fractionation, and DEAE-cellulose chromatography. The specific activity of this preparation was 12 μmol of inosine formed/min/mg of protein. One unit of AdoHcy hydrolase activity is defined as 1 μmol of inosine formed/min.

***S*-Adenosyl-L-homocysteine Assays.** The synthetic activity of AdoHcy hydrolase was measured as follows. In a total volume of 1 mL, the reaction mixture contained 250 μM Ado (or Ado-*3′-d*), 1 mM DL-Hcy, 0.1 mM DTT, 1 mM EDTA, 100 μM EHNA, and 150 mM K_2HPO_4, pH 7.6. The reaction was started by the addition of 0.4 units of AdoHcy hydrolase.

To measure the hydrolysis of AdoHcy, the coupled assay[19] utilizing Ado deaminase (to convert Ado formed to inosine) was used. In a total volume of 1 mL, the reaction mixture contained 250 μM AdoHcy (or AdoHcy-*3′-d*), 0.1 mM DTT, 1 mM EDTA, 8 units/mL of Ado deaminase, and 150 mM K_2HPO_4, pH 7.6. The reaction was started by the addition of 1.9 units of AdoHcy hydrolase.

At various times after incubation at 37 °C, 150-μL aliquots were transferred to 0.5-mL tubes containing 5 μL of 4 N $HClO_4$. All samples were stored at -20 °C until HPLC analysis.

HPLC Analysis. The samples were brought to room temperature just before injection and centrifuged in a Eppendorf microcentrifuge (8000 *g*, 1 min) to remove the precipitated material. A 50 μL aliquot of the supernatant was injected into a Perkin-Elmer Series 3 HPLC equipped with a Zorbax C-8 reversed-phase column (25 cm × 4.6 mm) and a Sigma 10B data station. A two-step gradient with acetonitrile and 50 mM NaH_2PO_4, 10 mM heptanesulfonic acid, pH 3.2, was used to elute the samples (0-5 min, 5-15% acetonitrile; 5-15 min, 15-20% acetonitrile). Absorbance at 254 nm was monitored. The retention times for inosine, Ado, and AdoHcy were 5.2, 11.4, and 13.5 min, respectively. The peak areas for AdoHcy (synthetic reaction) and iosine (hydrolytic reaction) were used to calculate the enzyme activity.

Enzymatic Synthesis of AdoHcy-*3′-d* from Ado-*3′-d*. In a total volume of 2 mL, the reaction mixture contained 150 mM K_2HPO_4, 0.1 mM DTT, 1 mM EDTA, 100 μM EHNA, 50 mM DL-Hcy, 20 mM Ado-*3′-d*, and 15-20 units of partially purified bovine liver AdoHcy hydrolase. After 3 h at 37 °C, the reaction was stopped by immersing the mixture in boiling water. (Less than 2% unreacted Ado-*3′-d* could be detected by HPLC, after the 3-h incubation period.) The precipitate was removed by centrifugation and filtration through Whatman No. 3 filters. AdoHcy-*3′-d*, present in the filtrate, was purified and characterized as described above for the chemical synthesis of AdoHcy-*3′-d*.

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EPR Evidence for Hydrogen Bond Donation to the Terminal Oxygen of Co-O_2 Model Compounds and Cobalt Oxymyoglobin

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Abstract: EPR investigations of mono-ortho and para acetamide derivatives of cobalt(II) tetraphenylporphyrins, (*o*-$NHCOCH_3$)$_1$TPPCo and (*p*-$NHCOCH_3$)$_1$TPPCo, in the presence of *N*-methylimidazole and molecular oxygen, provide information concerning the dynamics of bound dioxygen: When hydrogen bond donation is possible (from the N-H of the ortho acetamide), the EPR spectra of the dioxygen adduct are not motionally averaged, as they are for the para acetamide and all previously studied monomeric Co-O_2 complexes. These results indicate that the motion responsible for the averaging of the EPR signals in fluid solution is the rotation of the dioxygen moiety about the Co-O bond. Investigation of the room-temperature EPR spectrum of cobalt oxymyoglobin indicates that this case as well as internal rotation of bound dioxygen is prevented, presumably by hydrogen bonding of the distal histidine to the terminal oxygen atom.

Synthetic analogues of the dioxygen-carrying proteins myoglobin and hemoglobin have often utilized amide substituents to create a protected "pocket".[1-7] Recent evidence has suggested an important role for hydrogen bond donation from the amide N-H in stabilization of O_2 binding to iron(II) and cobalt(II) model hemes:[3-8] The "bis-pocket" porphyrin,[3,4] which has no amide substituents, has an oxygen affinity much lower than the "picket fence" porphyrin,[1,2] and "basket handle" and "hanging base basket handle" porphyrins having either linkages have much lower oxygen affinities and more rapid oxygen dissociation rates than those having amide linkages.[5-7] Calculations by Jameson and Drago[8]

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