Chromatographic Analysis of the Chiral and Covalent Instability of *S*-Adenosyl-L-methionine†

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ABSTRACT: The chirality of biologically active *S*-adenosyl-L-methionine (AdoMet) is *S*,*S*, where the designations refer to the sulfur and the α-carbon, respectively. This paper describes a cation-exchange high-performance liquid chromatographic (HPLC) method for separating (*S*,*S*)-AdoMet from the biologically inactive (*R*,*S*)-AdoMet that results from racemization at the sulfur. This method was used to measure the rates of the degradation reactions of (*S*,*S*)-AdoMet as a function of pH. These reactions and the first-order rate constants, which were found at 37 °C and pH 7.5, are racemization, 1.8×10^{-6} s^{-1}; cleavage to homoserine lactone and 5′-(methylthio)adenosine, 4.6×10^{-6} s^{-1}; and hydrolysis to adenine and *S*-pentosylmethionine, 3×10^{-6} s^{-1}. Racemization showed no change in rate over the pH range from 7.5 to 1.5. The cleavage reaction persisted until the pH was lowered to 1.5, but hydrolysis ceased at pH 6. Commercial samples of nonradioactive AdoMet contained 20–30% (*R*,*S*)-AdoMet, while a sample of [*methyl*-^{3}H]AdoMet had less than 1% (*R*,*S*)-AdoMet. Preparing enzyme substrates by mixing such samples will cause an underestimate of specific activity and an overestimate of the amount of product. The (*R*,*S*)-AdoMet/(*S*,*S*)-AdoMet ratio in mouse liver was 0.03, much less than the value of 0.19 calculated from the above rate constants. An enzyme extract from mouse liver did not degrade (*R*,*S*)-AdoMet, but a more thorough search may find such an activity. In any event, the cleavage and hydrolysis reactions partially balance the racemization of (*S*,*S*)-AdoMet in vivo and prevent excessive accumulation of (*R*,*S*)-AdoMet.

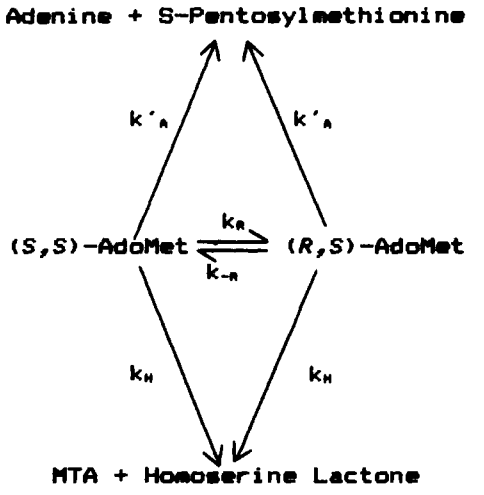
S-Adenosyl-L-methionine (AdoMet)[1] is an important metabolic intermediate that serves as a donor of methyl, α-aminobutyryl, and (after decarboxylation) aminopropyl groups to a variety of acceptor molecules. The sulfur of AdoMet is chiral, and De la Haba et al. (1959) first showed that only one of the two possible configurations is synthesized and utilized biologically.

The absolute configuration at the sulfur of naturally occurring AdoMet was determined by Cornforth et al. (1977) to be *S*. In the course of this work, these authors treated [*methyl*-^{14}C]AdoMet with base and then periodate to yield *S*-(carboxymethyl)-L-methionine. This preparation was added to a mixture of the two diastereomers of *S*-(carboxymethyl)-L-methionine and fractionated by cation-exchange chromatography on an amino acid analyzer. Two of the radioactive peaks comigrated with the partially separable diastereomers, 95% with isomer A and 5% with isomer B. This suggested that the original AdoMet contained small quantities of a sulfonium diastereomer or that racemization occurred during chemical manipulation.

Using NMR, Stolowitz and Minch (1981) found that commercial AdoMet isolated from yeast had about 20% in the biologically inactive *R* configuration at sulfur, again implying that racemization could occur.

Wu et al. (1983) reported more evidence for racemization at the sulfur of AdoMet. Starting with (*S*,*S*)-AdoMet feshly synthesized by MAT, they found that continued incubation produced material which cochromatographed with AdoMet on reversed-phase HPLC but was inactive as a substrate for catechol *O*-methyltransferase. They concluded that this material was (*R*,*S*)-AdoMet and determined the constant for racemization at the sulfur to be 8×10^{-6} s^{-1} at 37 °C and pH 7.5.

Scheme I: Reactions of (*S*,*S*)-AdoMet



This paper describes a direct method for determining (*R*,*S*)- and (*S*,*S*)-AdoMet by cation-exchange HPLC. This method is then applied to an independent verification and refinement of the results of Wu et al. (1983), measurement of the pH dependence of the rate constants for the reactions of AdoMet shown in Scheme I, analysis of the relative amounts of the AdoMet diastereomers produced by chemical methylation of

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine (when preceded by indications of chirality enclosed in parentheses, the first letter refers to the sulfur and the second refers to the α-carbon); AdoEth, *S*-adenosyl-L-ethionine; AdoHcy, *S*-adenosyl-L-homocysteine; MTA, 5′-deoxy-5′-(methylthio)adenosine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ATP, adenosine 5′-triphosphate; HPLC, high-performance liquid chromatography; MAT-III, liver-specific, low molecular weight, high K_m form of ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6).

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AdoHcy, and analysis of the diastereomer distribution in tissue extracts and commercial AdoMet samples.

Materials and Methods

Chemicals. Hepes, Mes, AdoMet, AdoMet-rich yeast, L-AdoHcy, adenine, ATP, and L-methionine were obtained from Sigma Chemical Co. Additional AdoMet samples were obtained from Calbiochem and from Boehringer-Mannheim. [*methyl*-^{3}H]AdoMet was obtained from ICN Radiochemicals, Inc. Cellex P was obtained from Bio-Rad Laboratories. Acetonitrile was Fisher HPLC grade.

Animals. Rats used as a source of MAT were female Sprague-Dawley weighing about 200 g. Mice were female Swiss Albino weighing about 25 g.

HPLC Analyses. The chromatograph was a modular system from Spectra-Physics consisting of an SP-8700 solvent delivery system with a dynamic mixer, an SP-8400 variable-wavelength detector, and an SP-4100 integrator–plotter. The system was modified slightly in that the stationary mixer was converted to a solvent-saturation column by replacing the stainless steel balls with Whatman precolumn silica gel. A 4.6 × 250 mm Sepralyte SCX column was obtained from Analytichem International (catalog no. 545817). Buffer A was made by adding 0.7 mL of concentrated NH_4OH/L of 20% acetonitrile in water and adjusting the pH to 3.0 with 88% formic acid. Buffer B was made by bringing buffer A to 50 mM $(NH_4)_2SO_4$ (Schwarz/Mann UltraPure) and then adjusting the pH back to 3.0 with concentrated sulfuric acid. Buffers prepared by this procedure were found to give the least base line shift during gradient elution.

Two different elution programs were used depending on the analyses desired. In both cases the flow rate was 2 mL/min. If the purpose was to measure only (*R*,*S*)- and (*S*,*S*)-AdoMet, isocratic elution at 60% buffer B was used, which gave retention times of 20 min for (*R*,*S*)-AdoMet and 21.5 min for (*S*,*S*)-AdoMet. This causes adenine and MTA to elute early with poor resolution, and so if measurements of these two compounds were also needed, the following program was used. A gradient was run from 0 to 50% buffer B in 2.5 min and then to 60% buffer B at 22.5 min and held at 60% buffer B until 30 min. A minimum of 7 min was allowed for equilibration with buffer A before the next sample was injected. In this HPLC system, the above solvent compositions are generated in the solvent-mixing valve at the given times, and it takes 2.5 min for this composition to arrive at the flow cell of the monitor. With this program, the retention times were 5.2 min for MTA, 6 min for adenine, 26.2 min for (*R*,*S*)-AdoMet, and 27.7 min for (*S*,*S*)-AdoMet. With either elution program the effluent was monitored at 258 nm by using a range setting of 0.01 absorbance/10 mV on the detector and an attenuation of 8–32 mV full scale on the integrator–plotter depending on the sample concentration. Compounds were quantitated by integrating the monitor output by use of response factors determined with purified standards. Minimum detectable levels were 5 pmol for each of the four compounds of interest.

Biosynthesis of (S,S)-AdoMet with MAT-III. Rat liver MAT-III was prepared as previously described (Sullivan & Hoffman, 1983). AdoMet was synthesized in reaction mixtures containing 20 mM L-methionine, 20 mM ATP, 30 mM magnesium acetate, 100 mM KCl, 2 mM dithioerythritol, 25 mM Hepes (to pH 7.5 with 1 M KOH), and MAT-III at 250 μg/mL. Reaction volumes of 5 mL were incubated for 30 min at 37 °C and then mixed with an equal volume of cold 10% perchloric acid. The remainder of the purification of AdoMet was done rapidly at 0–5 °C. After being chilled for 10 min, the acidified reaction mixture was centrifuged at 20000*g* for 10 min. The supernatant was adjusted carefully to pH 7 with 1 M KOH and again chilled and centrifuged to remove potassium perchlorate. The supernatant, now totaling about 12 mL, was applied to a 2-mL bed of phosphocellulose (Cellex P). The phosphocellulose had been hydrated with 0.5 M NaCl and then packed and extensively washed with this solvent followed by distilled water before sample application. After sample application, the column was washed with 0.05 M NaCl until the effluent had an absorbance of zero at 258 nm. AdoMet was then eluted with 0.2 M NaCl and stored frozen at −20 °C. This procedure typically gave (*S*,*S*)-AdoMet with no more than 1% contamination with (*R*,*S*)-AdoMet.

MAT-III was also used to test the chirality at the α-carbon of methionine released from (*R*,*S*)-AdoMet by acid hydrolysis. A sample of 21 nmol of purified (*R*,*S*)-AdoMet was hydrolyzed for 4 h in 6 N HCl at 100 °C, which gives high yields of methionine (Schlenk & Zydek-Cwick, 1969). The hydrolysate was dried in a stream of nitrogen. Buffer, ATP, and MAT-III were added to a final volume of 100 μL, giving concentrations identical with those of the above AdoMet synthesis mixture, except the concentration of methionine was estimated to be 210 μM on the basis of the input of (*R*,*S*)-AdoMet. Samples of 25 μL were removed at 0, 5, 10, and 15 min, mixed with an equal volume of cold 10% perchloric acid, and centrifuged. The supernatants were analyzed by HPLC for AdoMet diastereomers.

Chemical Synthesis of (S,S)-AdoMet and (R,S)-AdoMet by Reaction of AdoHcy with Methyl Iodide. Reaction mixtures contained 10 mg of AdoHcy, 200 μL of glacial acetic acid, 200 μL of 88% formic acid, and 100 μl of methyl iodide and were incubated at room temperature. Samples of 5 μL were prepared for HPLC analysis by drying in a stream of nitrogen and then redissolving in 25 μL of HPLC buffer A. In order to assess the chiral stability of AdoMet under these conditions, 10 mg of AdoMet from Sigma Chemical Co. was dissolved in acetic acid, formic acid, and methyl iodide as above and periodically analyzed by HPLC.

Purification of (R,S)-AdoMet. In earlier work this compound was purified from commercial samples of AdoMet, but after finding that chemical synthesis of AdoMet from AdoHcy as above gave 60% (*R*,*S*)-AdoMet, these reaction mixtures were used. In either case, about 10 mg of AdoMet was dissolved in 100 μL of HPLC buffer A and run on the HPLC system using isocratic elution with 60% buffer B. The diastereomers of AdoMet eluted in a broad peak between 15 and 25 min. These were collected in 0.5-mL fractions from which samples of 10 μL were then analyzed in the same system. The early portions of the broad peak contained pure (*R*,*S*)-AdoMet and were subjected rapidly to rotary evaporation to remove acetonitrile and then stored frozen at −20 °C. Owing to extensive tailing of the (*R*,*S*)-AdoMet due to the high loading, it was not possible to isolate pure (*S*,*S*)-AdoMet by this procedure.

Extraction and Analysis of AdoMet from Mouse Liver and from AdoMet-Rich Yeast. Livers were removed from ether-anesthetized mice, dropped into 3 mL of ice-cold 5% perchloric acid, and immediately homogenized with a Brinkman Polytron. Homogenates were centrifuged in the cold at 20000*g* for 10 min. Supernatants were further clarified by filtration through nylon 66 membranes of 0.2-μm pore size. Immediately after this, samples of 50 μL were analyzed for AdoMet diastereomers by using the gradient-elution mode. Samples of 100 mg of AdoMet-rich yeast were homogenized in 10 mL of 5% perchloric acid, centrifuged, and filtered as above.

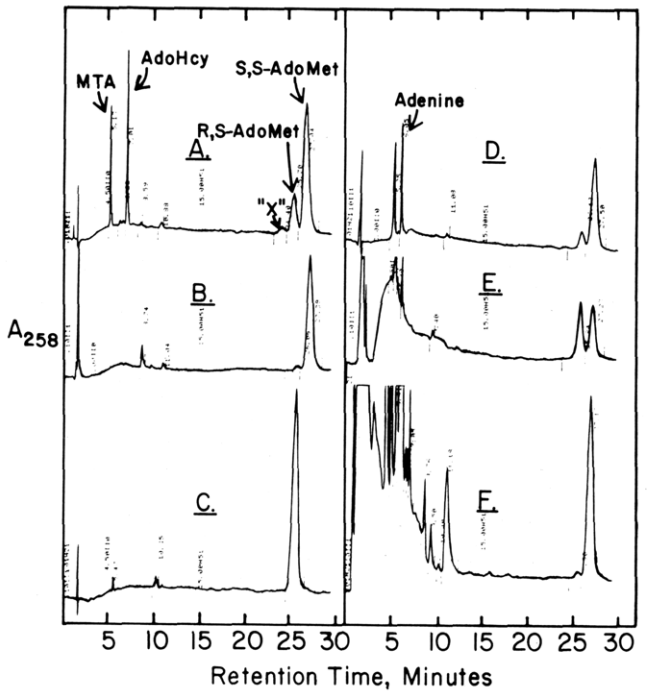

FIGURE 1: HPLC elution profiles of representative samples. Samples were prepared and chromatographed as described in the text. In all cases, fractionation was done by gradient elution. Profiles are for the following samples: (A) AdoMet from Sigma Chemical Co.; (B) MAT-synthesized, phosphocellulose-purified (*S,S*)-AdoMet; (C) (*R,S*)-AdoMet purified from methyl iodide–AdoHcy reaction mixtures; (D) (*S,S*)-AdoMet after 8 h at pH 7.5 and 37 °C (E) (*S,S*)-AdoMet after 232 h at pH 1.5 and 37 °C; (F) perchloric acid soluble extract from mouse liver. The small peaks eluting between 8 and 11 min in (A–E) are contaminants of the elution buffers since they appeared even when no sample was injected.

Samples of 10 μL were analyzed.

Stability of AdoMet. Concentrated buffers for use in adjusting AdoMet samples to various pH values were prepared as follows: 1 M Hepes to pH 7.5 with KOH, 1 M Mes to pH 6.0 with KOH, 1 M KOH to pH 4.5 with acetic acid, and 1 M KOH to pH 3.0 with formic acid. AdoMet samples were then mixed with 0.1 volume of concentrated buffer followed by measurement of pH. The most acidic mixture was prepared with 0.1 volume of 1 M HCl and gave a measured pH of 1.5. Since long incubations at 37 °C were required, chloroform was added to reaction mixtures at 50 μL/mL to prevent microbial contamination. It was found that contamination often occurred in the absence of chloroform and resulted in the consumption of adenine but had little effect on AdoMet or MTA levels. At times shown in various experiments, samples were analyzed by HPLC using the gradient-elution mode.

Biochemical stability was tested by incubating the diastereomers in a crude enzyme extract from mouse liver. This extract was prepared by homogenizing liver in 4 mL/g of 100 mM KCl, 10 mM magnesium acetate, 2 mM dithioerythritol, and 50 mM Hepes (to pH 7.5 with KOH). Homogenates were centrifuged at 20000*g* for 20 min at 0–5 °C. Purified (*S,S*)-AdoMet and (*R,S*)-AdoMet were added together to this supernatant to a concentration of 100 μM each. This mixture was incubated at 37 °C, during which samples of 50 μL were removed at 5-min intervals and mixed with an equal volume of cold 5% perchloric acid. After centrifugation at 20000*g* for 10 min at 0–5 °C, samples of 25 μL of each supernatant were analyzed by gradient-elution, cation-exchange HPLC. Control samples containing AdoMet diastereomers were incubated in the above buffer with no liver extract and analyzed similarly.

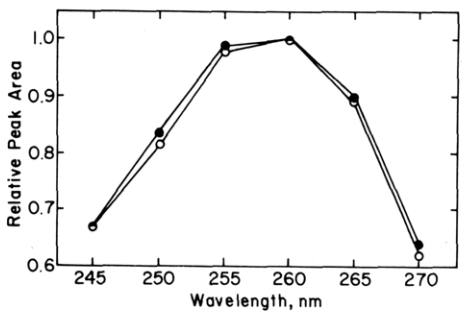

FIGURE 2: Spectral identity between (*S,S*)-AdoMet and (*R,S*)-AdoMet. Six HPLC analyses were done with AdoMet obtained from Boehringer-Mannheim. Individual runs were monitored at the indicated wavelengths, and the integrated areas of (*S,S*)-AdoMet and (*R,S*)-AdoMet peaks were each normalized with respect to their peak areas at 260 nm. Closed circles are for (*S,S*)-AdoMet, and open circles are for (*R,S*)-AdoMet.

Results and Discussion

Chromatographic Fractionation of (S,S)-AdoMet and (R,S)-AdoMet. The HPLC elution profiles of AdoMet-containing samples from various sources are shown in Figure 1. In all samples other than the purified, MAT-synthesized AdoMet, a peak of UV-absorbing material is seen to elute 1.3 min earlier than the authentic (*S,S*)-AdoMet. This peak was determined to be (*R,S*)-AdoMet on the basis of its UV spectrum, the presence of an L-methionine residue, chemical synthesis by methylation of AdoHcy, conversion to and from (*S,S*)-AdoMet, and kinetics of degradation and racemization identical with those of (*S,S*)-AdoMet, all of which are described below.

The basis for separation of the diastereomers appears to be ionic, since the inclusion of acetonitrile to prevent tailing of peaks still permitted near-base-line fractionation. The appearance of two peaks during cation-exchange HPLC of AdoMet was first reported by Van Haastert (1981), who suggested that these might be the sulfonium diastereomers. Wagner et al. (1984) reported that AdoEth from commercial samples or livers of rats treated with ethionine fractionated into two peaks during reversed-phase HPLC in the presence of the ion-pair reagent octanesulfonate.

Comparison of UV Spectra of (S,S)-AdoMet and (R,S)-AdoMet. In order to substantiate the identity of (*R,S*)-AdoMet, spectra were determined by repeated chromatographic analyses of the AdoMet obtained from Boehringer-Mannheim. The wavelength of detection was increased by 5 nm for each run until the range from 245 to 270 nm was covered. The results in Figure 2 show that the putative (*R,S*)-AdoMet has a spectrum in this range identical with that of (*S,S*)-AdoMet. This spectrum is not unique, since it would be shared by a variety of adenine-containing compounds. However, the spectral overlap combined with the strong retention similar to (*S,S*)-AdoMet on the cation-exchange column is evidence that this compound is (*R,S*)-AdoMet.

Configuration at the α-Carbon of the Methionine Residue of (R,S)-AdoMet. To ensure that the unnatural AdoMet diastereomer was not (*S,R*)-AdoMet (that is, containing L-methionine), a purified sample of (*R,S*)-AdoMet was degraded to release methionine. The configuration of this released methionine was then tested by measuring its ability to be reconverted to (*S,S*)-AdoMet by MAT-III, an enzyme specific for L-methionine. The released methionine was in the L configuration, since (*S,S*)-AdoMet synthesis occurred at a rapid rate such that 30% of the released methionine was converted to (*S,S*)-AdoMet within 15 min. This amount could not have been generated during the 4-h hydrolysis, since the

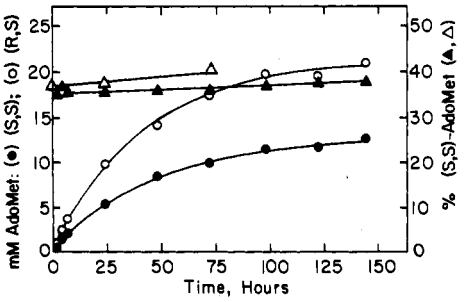

FIGURE 3: Chemical synthesis of AdoMet by methylation of AdoHcy with methyl iodide. Samples of reaction mixtures were analyzed for AdoMet distribution by HPLC at the times indicated. Filled and open circles indicate the respective concentrations of (*S*,*S*)-AdoMet and (*R*,*S*)-AdoMet. Filled triangles indicate the percentage of (*S*,*S*)-AdoMet resulting from methylation of AdoHcy, while open triangles show the chiral stability of AdoMet under the conditions of the methylation reaction.

rate of racemization of methionine at 100 °C is less than 2%/day (Boehm & Bada, 1984).

Chemical Synthesis of AdoMet. To obtain further evidence for the identity of (*R*,*S*)-AdoMet and to prepare more substantial amounts of this compound, AdoHcy was reacted with methyl iodide. Figure 3 shows the progress of this synthesis in terms of the amounts of (*R*,*S*)-AdoMet and (*S*,*S*)-AdoMet produced as a function of time. The reaction was nearly complete by 4 days and gave a total yield of the two forms of AdoMet of 32 mM, which corresponds to 64% of the input AdoHcy. Most interesting was the finding that the predominant diastereomer produced was (*R*,*S*)-AdoMet. At the earliest time point of 100 min, only 36% of the AdoMet was *S*,*S*, with a slight increase to 39% by 6 days. Previous general statements that such syntheses yielded about 50% of each diastereomer (De la Haba et al., 1959) may be due to equilibrium being reached during product purification, although Figure 1 of those authors shows that guanidinoacetate methyltransferase used only 40% of their chemically synthesized AdoMet.

Comparing the data in Figure 3 to those below, the question arises as to why the diastereomer distribution did not approach 50/50, especially after 4 days when there was little further synthesis of AdoMet. Two factors appeared to be important in this regard. First, this reaction was carried out at room temperature, which was about 16 °C less than for the later kinetic studies. Second, the solvent for the methylation reaction contained less than 5% water as compared to completely aqueous buffers used in the kinetic studies. Either or both of these conditions could decrease the rate of racemization. This was tested by measuring the chiral stability of AdoMet in such methylation mixtures. The results of this analysis are also shown in Figure 3 and verify that racemization is slower under these conditions than in aqueous solution at 37 °C. Starting with a mixture of diastereomers containing 37% (*S*,*S*)-AdoMet (similar to that synthesized initially), the composition changed at a rate of only 1%/day. Thus, the chiral composition in these reaction mixtures is determined primarily by the initial results of the more rapid methylation reaction.

Stability of AdoMet as a Function of pH. The degradation of (*S*,*S*)-Adomet at five different pH values is shown in Figure 4. At every time point, the loss of (*S*,*S*)-AdoMet was accounted for completely by the sum of MTA, adenine, and (*R*,*S*)-AdoMet. (*S*,*S*)-AdoMet is seen to be markedly unstable at the "physiological" pH of 7.5. Three different degradation processes occur: racemization to (*R*,*S*)-AdoMet, cleavage to MTA and homoserine lactone, and hydrolysis to adenine and *S*-pentosylmethionine. The covalent stabilities can be improved

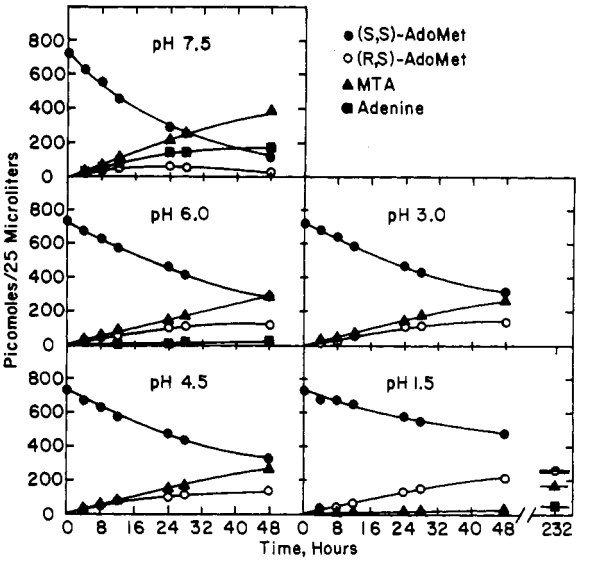

FIGURE 4: Chiral and covalent instability of (*S*,*S*)-AdoMet as a function of pH. (*S*,*S*)-AdoMet was synthesized with MAT, purified on phosphocellulose, and then incubated at 37 °C and various pH values and analyzed by HPLC at the times shown. Symbols are as shown in the key at the upper right.

by lowering the pH. Hydrolysis to adenine is strongly suppressed at pH 6 and virtually abolished at or below pH 4.5. Cleavage to MTA continues to occur at relatively high rates until the pH is reduced to 1.5. This is consistent with the intramolecular attack of the carboxylate anion on C4 of the methionine residue, leading to homoserine lactone and MTA. If the pK_a of the AdoMet carboxyl is on the order of 1.8–2.2 as for most amino acids, this reaction would not be suppressed until the pH was near or below 2. At pH 1.5, the only reaction occurring at a significant rate is racemization at the sulfonium. This permitted the analysis of the kinetics of racemization with no interference from other reactions. At this pH, one can also incubate for a sufficiently long time to verify that the rate constants for racemization in each direction (k_R and k_{-R}) are equal. This is seen in Figures 1E and 4, where the AdoMet remaining after 232 h at pH 1.5 was found to be equally divided between *S*,*S* and *R*,*S*.

On the basis of the reactions in Scheme I, the data from Figure 4 were then replotted in Figure 5 according to the rate equation (1) for reversible racemization with the rate constants equal in both directions and according to the rate equation (2) for the cleavage to MTA:

$$0.5 \ln \{(S,S)\text{-AdoMet}_0/[(S,S)\text{-AdoMet}_0 - 2 \times (R,S)\text{-AdoMet}_t]\} = k_R t \quad (1)$$

$$\ln \{(S,S)\text{-AdoMet}_0/[(S,S)\text{-AdoMet}_0 - \text{MTA}_t]\} = k_H t \quad (2)$$

The plot for racemization is linear over the entire 48 h for the data at pH 1.5, which is expected since side reactions are negligible. However, at all other pH values linearity is found only up to 12 h when total AdoMet becomes sufficiently depleted that the effects of the side reactions can no longer be ignored. On the basis of this the slope (corresponding to k_R) for each pH was calculated by linear regression analysis of the data up to 12 h and also for data over the entire 48 h at pH 1.5. The results of these calculations are shown in Table I. The rate of racemization shows little pH dependence. This is consistent with the simple inversion at a relatively unhindered sulfonium center. The plots in Figure 5 for cleavage to MTA are linear over 48 h for all five pH values, and the results of calculation of k_H are shown in Table I. These results provide

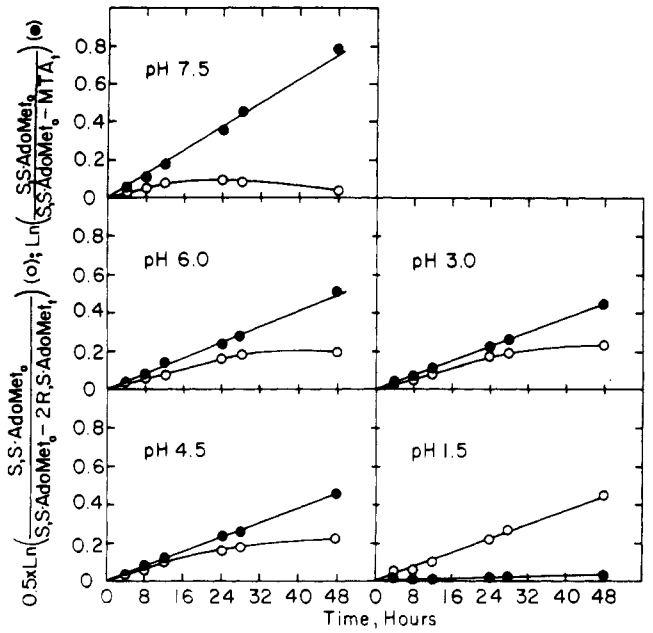

FIGURE 5: Kinetic plots for racemization and for cleavage to MTA and homoserine lactone of (*S*,*S*)-AdoMet. The data in Figure 4 were plotted according to eq 1 and 2. Filled circles are for cleavage to MTA and homoserine, and open circles are for racemization.

Table I: pH Dependence of Rate Constants for Reactions of (*S*,*S*)-AdoMet

pH	k_R ($\times 10^6$ s^{-1})[a]	k_H ($\times 10^6$ s^{-1})[b]	k'_A ($\times 10^6$ s^{-1})[c]
7.5	1.8	4.6	3.0
6.0	1.7	2.9	
4.5	2.3	2.6	
3.0	1.9	2.6	
1.5	2.1 (2.7)	0.26	

[a] k_R was calculated from data collected through 12 h, except for the value in parentheses, which included data over the entire 48 h. [b] k_H was calculated from data collected over the entire 48 h. [c] k'_A was calculated from data collected through 12 h. At pH values other than 7.5 the rate was insufficient to measure accurately.

more quantitative evidence for the pH dependence of this reaction noted above, since the largest decrease in k_H is found when the pH falls below the pK_a of the carboxyl group. The hydrolysis of AdoMet to adenine is mechanistically complex (Borchardt, 1979), but an apparent first-order rate constant (k'_A) was estimated for this reaction at pH 7.5 as shown in Table I. The rate of this reaction was insignificant at all other pH values.

The results at pH 7.5 and 37 °C in this paper differ from those of Wu et al. (1983). Their value for k_H of 6×10^{-6} s^{-1} agrees well with the value of 4.6×10^{-6} s^{-1} found in this work. However, their value of 8×10^{-6} s^{-1} for k_R is more than fourfold higher than the 1.8×10^{-6} s^{-1} found here. In their study of the stability of AdoMet, these authors used [*methyl*-^{3}H]methionine, ATP, and MAT to generate [*methyl*-^{3}H]-(*S*,*S*)-AdoMet. Degradation was assessed by reversed-phase HPLC fractionation and measurement of radioactivity in MTA and AdoMet before and after consumption of (*S*,*S*)-AdoMet with catechol *O*-methyltransferase and 3,4-dihydroxyacetophenone. The nonreacted AdoMet was assumed to be (*R*,*S*)-AdoMet. Problems with this approach that may have led to an overestimate of k_R include neglecting the hydrolysis of AdoMet to adenine, which occurs at a significant rate at pH 7.5; possible incomplete consumption of (*S*,*S*)-Adomet by catechol *O*-methyltransferase [the inhibitory effects on this enzyme of high ratios of (*R*,*S*)-AdoMet to (*S*,*S*)-AdoMet are unknown]; and calculation of results as percent

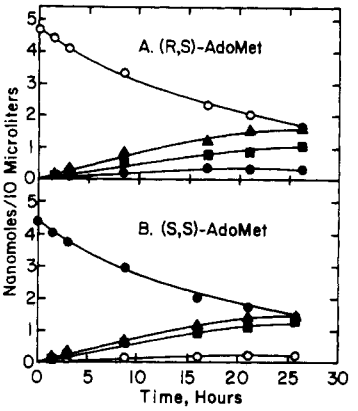

FIGURE 6: Comparison of instabilities of (*S*,*S*)-AdoMet and (*R*,*S*)-AdoMet. Samples of (*R*,*S*)-AdoMet (A) and (*S*,*S*)-AdoMet (B) were incubated at pH 7.5 and 37 °C and analyzed by HPLC. Symbols are as in Figure 4.

of recovered radioactivity at each time point, which fails to account for all products.

In a separate experiment the stability of (*S*,*S*)-AdoMet was compared to that of (*R*,*S*)-AdoMet at pH 7.5 and 37 °C, giving the results shown in Figure 6. These two compounds undergo the same three reactions of racemization, hydrolysis to adenine, and cleavage to MTA at virtually identical rates. This further verifies the identity of (*R*,*S*)-AdoMet, the equality of k_R and k_{-R}, and the assumption that k_H is the same for both diastereomers.

Biochemical and Biological Implications. When studying various AdoMet-metabolizing enzymes in vitro, a common procedure for preparing substrate solutions is to mix commercially obtained samples of highly radioactive AdoMet and unlabeled AdoMet. Since the radioactive AdoMet is prepared by using MAT, it should consist primarily of (*S*,*S*)-AdoMet. Unlabeled AdoMet is often purified from yeast cultured in high methionine, and if the purification involves having the AdoMet in solution for several days, this product could contain considerable (*R*,*S*)-AdoMet. If it were then mixed with chirally pure radioactive AdoMet, the specific activity of the biochemically active (*S*,*S*)-AdoMet would be underestimated, and calculation of the amount of product would result in an overestimate. In order to assess this possibility, commercial samples of AdoMet-rich yeast, AdoMet, and radioactive AdoMet were analyzed by cation-exchange HPLC with the results summarized in Table II.

The unlabeled AdoMet samples showed variable degrees of purity. Two of the samples were heavily contaminated with AdoHcy, which could cause problems as an inhibitor of most methyltransferases. Since there is no nonenzymic reaction known that would give this much AdoHcy from AdoMet, this compound must have copurified with AdoMet. The adenine and MTA in these same two samples may have come from degradation of AdoMet. The third sample of unlabeled AdoMet had the highest percentage of total AdoMet but the lowest proportion in the biochemically active *S*,*S* configuration. Analysis of AdoMet-rich yeast showed that most of the (*R*,*S*)-AdoMet was present in this starting material rather than generated during purification. Even when these samples were purified on conventional cation-exchange media to give pure AdoMet, such as described in this paper by using phosphocellulose, the chiral purity was not improved.

When the chiral composition of the radioactive AdoMet is considered along with that of the unlabeled AdoMet, it can be seen that, as predicted above, the specific activity of (*S*,*S*)-AdoMet in mixtures of these would be underestimated by

Table II: Chemical and Chiral Purity of AdoMet from Various Sources

source	% of 258-nm absorbing material[a]						
	MTA	adenine	AdoHcy	X[b]	AdoMet *R,S*	AdoMet *S,S*	% AdoMet as *S,S*
Calbiochem	17.9	0.7	15.7	6.1	13.4	46.2	77.5
Sigma Chemical Co.	8.1	1.0	13.2	2.2	15.6	59.9	79.3
Boehringer-Mannheim[c]	0	0	0	0	28.2	61.7	68.6
AdoMet-rich yeast	*d*	*d*	*d*	0	18.2	81.8	81.8
[*methyl*-^{3}H]AdoMet[e]	2.1	0	0	0	0.7	97.2	99.3
mouse liver[f]	*d*	*d*	*d*	0	2.9	97.1	97.1

[a] Single lots of samples from each commercial source were analyzed, and the results are assumed to be typical for that supplier. [b] Compound X is the material eluting at 24.4 min as in Figure 1A. [c] 10.1% of the 258-nm absorbing material eluted at the breakthrough volume and was not identified. [d] Present, but amounts not used in calculation of percent composition. [e] Calculated as percent of radioactivity. [f] Values given are averages from analyses of three mice. Individual percentages of (*R,S*)-AdoMet were 2.6, 3.0, and 3.1.

20–30%. To avoid this, the techniques described in this paper should be used either to synthesize and purify unlabeled AdoMet or to analyze radioactive substrate mixtures for the true specific radioactivity of (*S,S*)-AdoMet.

The final question to be addressed is whether a measurable amount of (*R,S*)-AdoMet can accumulate in cells. It has been suggested that the turnover rate of (*S,S*)-AdoMet in most tissues is so rapid that there is not time for (*R,S*)-AdoMet to accumulate (Wu et al., 1983). However, there is a steady-state level of (*S,S*)-AdoMet maintained, and so there should be a constant rate of production of (*R,S*)-AdoMet contributing to a steady-state level of this diastereomer as well. The rate constants determined in this paper can be used to predict the steady-state level of (*R,S*)-AdoMet that should occur on a strictly chemical basis. From Scheme I [with R and S as abbreviations for the concentrations of (*R,S*)-AdoMet and (*S,S*)-AdoMet, respectively, assuming S to be held constant biologically, and with k_R equal to k_{-R}], the rate of change of R with respect to time is given by eq 3 and is set equal to 0 corresponding to the steady state:

$$dR/dt = k_R S - k_R R - k'_A R - k_H R = 0 \qquad (3)$$

Rearranging this equation to solve for R/S and substituting for the rate constants, one obtains

$$R/S = k_R/(k_R + k'_A + k_H) = 0.19$$

The analyses of the mouse liver extracts shown in Figure 1F and Table II indicate that 2.9% of the AdoMet was in the *R,S* configuration, giving an R/S ratio of 2.9/97.1 or only about 0.03. This small amount might have been produced during the extraction and analysis. However, only 1 h elapsed from the time each mouse was anesthetized until the (*R,S*)-AdoMet arrived at the flow cell of the HPLC detector. The above k_R can be used to calculate that no more than 0.65% racemization could have occurred in that time at 37 °C and even less could have occurred, considering that all the procedures except for HPLC were done at 0–5 °C. This indicates that significant, but less than predicted, amounts of (*R,S*)-AdoMet exist in tissues.

One possible reason for this smaller amount of (*R,S*)-AdoMet is the existence of enzymes that degrade or utilize this diastereomer. A preliminary test for the presence of such an enzyme was conducted by incubating a mixture of AdoMet diastereomers in a crude extract from mouse liver. While 100 μM (*S,S*)-AdoMet was completely consumed by this extract in 15 min, no loss of (*R,S*)-AdoMet was found other than that attributable to nonenzymic degradation (data not shown). This was by no means an exhaustive survey, and the possibility remains that, in the presence of additional substrates or cofactors, an enzyme will be found that removes (*R,S*)-AdoMet in vivo.

An additional possibility is that a large fraction of intracellular (*S,S*)-AdoMet is stabilized in that configuration by binding to various enzymes involved in its synthesis and utilization. However, it appears that the inherent covalent instability of AdoMet, although wasteful of energy, is advantageous in partially balancing the chiral instability by diverting biochemically unusable (*R,S*)-AdoMet into adenine and MTA, which can be further metabolized.

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REFERENCES

Boehm, M., & Bada, J. L. (1984) *Biochem. Int. 8*, 603–608.
Borchardt, R. T. (1979) *J. Am. Chem. Soc. 101*, 458–463.
Cornforth, J. W., Reichard, S. A., Talalay, P., Carrell, H. L., & Glusher, J. P. (1977) *J. Am. Chem. Soc. 99*, 7292–7300.
De la Haba, G., Jamieson, G. A., Mudd, S. H., & Richards, H. H. (1959) *J. Am. Chem. Soc. 81*, 3975–3980.
Schlenk, F., & Zydek-Cwick, C. R. (1969) *Arch. Biochem. Biophys. 134*, 414–422.
Stolowitz, M. L., & Minch, J. J. (1981) *J. Am. Chem. Soc. 103*, 6015–6019.
Sullivan, D. M., & Hoffman, J. L. (1983) *Biochemistry 22*, 1636–1641.
Van Haastert, P. J. M. (1981) *J. Chromatogr. 210*, 241–254.
Wagner, J., Claverie, N., & Danzin, C. (1984) *Anal. Biochem. 140*, 108–116.
Wu, S.-E., Huskey, W., Borchardt, R. T., & Schowen, R. L. (1983) *Biochemistry 22*, 2828–2832.