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S-Adenosylmethionine: Studies on Chemical and Enzymatic Synthesis

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Several methods for the chemical and enzymatic synthesis of (−)-*S*-adenosylmethionine (AdoMet) are described and compared. Studies on the effects of solvents, pH, methylating reagents, and KI on the coupling of sodium homocysteine thiolate and 5′-chloro-5′-deoxyadenosine led to an improved procedure for the synthesis of (±)-AdoMet. The use of trimethylsulfonium iodide as a methylating agent under acidic conditions results in a higher content of the desired (−)-epimer than does the use of CH_3I. The enzymatic synthesis of (−)-AdoMet using AdoMet synthetase from an over-producing strain of *Escherichia coli* is demonstrated and the effect of product inhibition on preparative-scale synthesis is illustrated. A new HPLC technique for separation of the epimeric mixture of AdoMet, which allows small-scale preparation of optically pure AdoMet from the enzyme product, has been developed. With this HPLC technique, evidence that (−)-AdoMet is the sole epimer formed by the enzyme has been shown.

(−)-*S*-Adenosylmethionine, (−)-AdoMet,[4] is a cofactor involved in various biochemical transformations, of which transmethylation is the most important. Since the discovery of this compound (1), a great deal of research directed toward its synthesis (2–4), elucidating the stereo- and regiospecificity in biological transmethylation reactions (5), determining the function of methylation reactions in DNA (6), RNA, or during post-translational modification of proteins (7), and determining its roles in carcinogenic cells has been carried out. Because of its analgesic and antiinflammatory properties (8), the importance of developing a convenient and practical route to AdoMet and its derivatives is obvious.

Chemical approaches toward AdoMet generally involve the methylation of *S*-adenosylhomocysteine (SAH) with CH_3I. For this reason, most strategies involve the improvement of SAH synthesis. The chemical synthesis of SAH is carried out by coupling of homocysteine (Hcys) and *S*-benzylhomocysteine (9), both generated from L-methionine (10) or homocystine (3), or a homocystine derivative (11). An

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[4] Abbreviations used: AdoMet, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; Hcys, homocysteine; SMM, *S*-methylmethionine; THF, tetrahydrofuran; SAM, *S*-adenosyl-L-methionine; HOAc, acetic acid; TMSI, trimethylsulfonium iodide.

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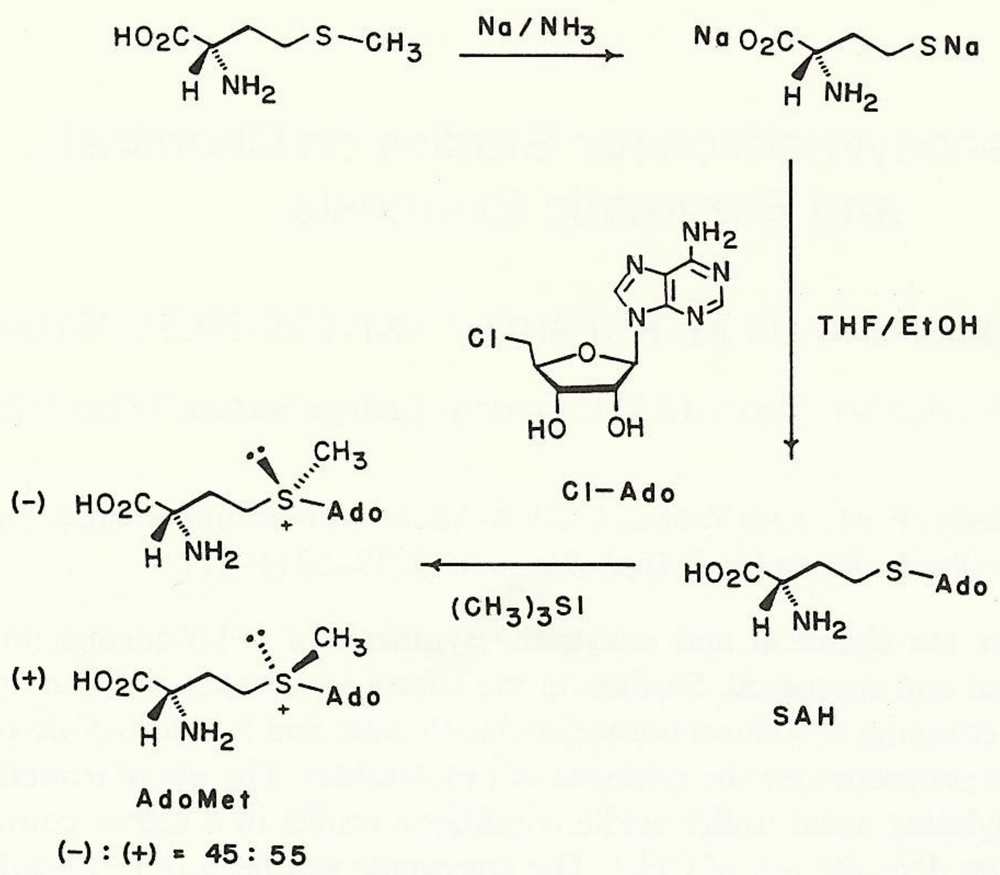

$$\text{ATP} + \text{Met} \xrightarrow[\text{E2}]{\text{E1}} (-)\text{-AdoMet} + 3\text{Pi}$$

SCHEME 1. Chemical and enzymatic synthesis of SAH and AdoMet. E1 = AdoMet synthetase; E2 = inorganic pyrophosphatase.

enzymatic method wherein L-Hcys and adenosine are coupled by SAH hydrolase from beef liver (12) and bacteria (13) results in high yields of SAH. Methylation of SAH with CH_3I results in the formation of a diastereomeric mixture of AdoMet (4). The microscale enzymatic method for the synthesis of (−)-AdoMet involves the stoichiometric coupling of ATP to L-Met in a reaction catalyzed by AdoMet synthetase from *Escherichia coli* (14, 15), yeast (16), or rat liver (17). These approaches result in the asymmetric synthesis of (−)-AdoMet (18), although it has been suggested that the (+)-epimer is also formed (19). (−)-AdoMet is currently prepared in a preparative scale by isolation from yeast grown in media supplemented with L-Met (16). This preparation gives a product of 60–80% purity containing approximately 20–30% of the undesired epimer (the (+)-form).

In connection with our efforts to develop practical methods for the synthesis of (−)-AdoMet, we describe in this paper improved procedures for the synthesis of SAH and AdoMet and identify the factors which inhibit preparative-scale synthesis of AdoMet chemically and enzymatically (Scheme 1).

EXPERIMENTAL

The following compounds were obtained from the given sources: homocystine, ATP, AdoMet tosylate, catechol-*O*-methyltransferase, protocatechuic acid, Sigma; L-Met, D-(+)-10-camphorsulfonic acid, Aldrich; AdoMet sulfate, Boehringer; AdoMet synthetase from *E. coli,* Dr. George D. Markham.

^{1}H NMR spectra were obtained on EM-390 (90 MHz) and Varian XL-200 (200 MHz) instruments. Pertinent chemical shifts for methylation byproducts (D_2O):

δ 2.97 (s, 6H, s-CH_3, SMM) (20), 2.95 (s, 3H, N^6-Ado) (21), 3.49 (s, 3H, 2′-OCH_3, Ado) (21), 3.60 (s, 3H, 3′OCH_3 Ado) (21), 2.89 (s, 9H, SCH_3, (CH_3Sl)). HPLC analyses were run on a Gilson chromatograph integrated to an Apple IIe computer and equipped with a VYDAC C_{18} reverse-phase column. The conditions were as follows: 1.0 ml/min, 1.70×10^3 psi, 5% ethanol, 243 mM acetic acid, 1 mM EDTA, 4 mM heptanesulfonic acid, pH 3.75, adjusted with NaOH, uv detector at 259 nm ($\epsilon = 15400\ M^{-1}$). Retention times (R_t) were (+)-AdoMet, 10.6 min; (−)-AdoMet, 11 min; SAH, 22–24 min; SMM, 12 min. Before the HPLC analyses were run with the heptanesulfonic acid buffer, the column was preequilibrated with 2.3 mM (1*S*)-(+)-10-camphorsulfonic acid buffer (using the same concentration of other chemicals as described) for 1 h at 1.0 ml/min. This camphorsulfonic acid-preequilibrated column can be used with the heptanesulfonic acid buffer for 2.5–3.0 h and must then be reequilibrated with the camphorsulfonic acid solution as before. Although a resolution of 0.4–0.6 min is achieved, it is not sufficient to allow absolute quantitation of the individual epimers due to peak overlap. TLC analyses are carried out on ANSPEC aluminum silica gel 60 F_{254} plates. Compounds are visualized by uv light and/or 0.2% ninhydrin (v/v) in ethanol; the solvent systems used in the separations are described individually where applicable.

L-Homocysteine

Method 1. This is a modification of a previously reported procedure. L-Methionine (5.0 g) is added to 13 to 75 ml of liquid ammonia under argon. Enough solid Na is slowly added to keep an intense blue color for at least 20 min. Evolution of methane can be observed by an oil bubbler or the reaction may be monitored by TLC with butanol:acetic acid:water (60:15:25), where $R_f = 0.38$ and R_f Hcys = 0.34. Once all the L-Met has been consumed, the color is allowed to dissipate by itself and the ammonia is evaporated under a stream of argon. The white precipitate is taken up in a minimal amount of ethanol (anhydrous). Since Na-Hcys is very hygroscopic and extremely sensitive to air oxidation, as evidenced by the yellow discoloration upon exposure to the atmosphere, the salt is precipitated out with anhydrous ether and centrifuged. The supernatant is decanted and the salt is desiccated over P_2O_5 under vacuum. Yield 87%; mp > 300°C (dec); ^{1}H NMR (D_2O) δ 2.2 (m, 2H, Hβ); 2.6 (t, 2H, Hγ; 3.5 (t, 1H, Hα); lit. mp > 300°C (dec) and same ^{1}H NMR (7). For further synthesis, the precipitate was used directly without further purification.

If L-Hcys is desired in the zwitterionic form, the initial solid obtained after evaporation of ammonia is dissolved in a minimal amount of deoxygenated water and precipitated out with cold acetone. Yield 89%; mp 270–275°C (dec); lit. mp 270–275°C (dec) (22). If the hydrochloride salt is desired, concentrated HCl is added directly to the deoxygenated water solution until precipitation is complete. Yield 97%; mp > 200°C (dec).

Method 2. In a manner similar to that above, L-homocystine is subjected to Birch conditions. Thus, to 20 ml of liquid NH_3 under argon is added 1.0 g L-homocystine and enough Na is slowly added until a deep blue color persists for approximately 20 min. The reaction should then be allowed to quench itself followed by evaporation of the ammonia under a stream of argon. The resultant white solid is taken up in a minimal amount of deoxygenated water and the zwitterionic L-Hcys precipitated out with cold acetone. Yield 87%; mp 270–275°C (dec). (lit. mp 270–275°C (dec)) (22).

S-Adenosylhomocysteine

Method 1. This method is similar to that employed by Ranalingam and Woodard (3) wherein the sodium salt of L-Hcys prepared from homocystine and Cl-Ado are coupled. We generated Na-Hcys from L-Met (as described above) and used directly after; care is taken to keep the atmosphere as well as the water deoxygenated to reduce formation of excessive byproducts under the extremely basic conditions. For optimal yield, 2.5:2.0 Na-Hcys:Cl-Ado mole ratio and 1% (w/v) KI are required. Thus, to 7.0 ml of deoxygenated water is added 0.394 g (2.19 mmol) Na-Hcys, 0.07 g KI, and 0.5 g (1.76 mmol) Cl-Ado and the solution is slowly heated to 110°C for 4 h. By this time, decomposition of SAH has become a major side reaction, so the reaction mixture is cooled immediately and made acidic to pH 3.5. The sample is then applied to a 100-ml Dowex 50-×8 (NH_4^+) column (2.5 × 8.0 cm) and the column is washed with 170 ml water until little to no Hcys is visualized by ninhydrin on TLC with butanol:acetic acid:water (60:15:25), where R_f SAH = 0.41, R_f Cl-Ado = 0.56, and R_f Hcys = 0.35. The product is then obtained by eluting with 1 N NH_4OH; it is imperative that the column be run extremely slowly, about 1 dp/17, s and that 7.0-ml fractions for optimal resolution from some impurities still remain on the column after washing. Solid SAH is obtained by concentration of the fractions containing it, usually the next 15–40, followed by lyophilization. Yield 57% (even after several attempts, the literature value of 81% has not been reproduced); mp 209–211°C (lit. 212°C) (3). ^{1}H NMR (D_2O) δ 2.15 (m, 2H, Hβ); 2.7 (t, 2H, Hγ); 3.0 (m, 2H, H5′); 3.85 (t, 1H, Hα); 4.35 (m, 1H, H4′); 4.4 (t, 1H, H3′); 4.85 (t, 1H, H2′); 6.0 (d, 1H, H1′); 8.15 (s, 1H, H2); 8.3 (s, 1H, H8); (lit. same ^{1}H NMR) (5).

Method 2. This method is designed to reduce the amount of impurities formed throughout the above reaction and to decrease the solvent involvement by decreasing its nucleophilicity; 0.50 g Cl-Ado and 0.40 g Na-Hcys are dissolved in 10.0 ml of deoxygenated anhydrous THF/EtOH (3:2 v/v). The reaction is allowed to reflux for 16 h, after which time the solvent is evaporated. The resulting precipitate is taken up in 2.0 ml of 1 N HCl and applied to the same Dowex 50-×8 column as above following the same isolation procedure. The yield is 56%.

(±)-*S-Adenosyl-L-methionine*

Method 1. The procedure used is based on that by Borchardt and Wu (4) except that the product is recovered as the tosylate salt due to the known great stability and pharmaceutically important quantities of SAM-sulfonic acid salts. The yield is determined by HPLC using an external standard of (−)-AdoMet tosylate. Thus 30 mg of SAH is dissolved in 1.0 ml 85% formic acid and 1.0 ml methyliodide; the solution is kept in the dark at room temperature for 3–5 days at which time the reaction is virtually complete. Unreacted methyliodide is quenched with 5.0 ml water and the remainder is extracted with 5.0 ml cold ether. The water layer is then lyophilized, redissolved in a minimal amount of water, and subsequently applied to a refrigerated Amberlite IRC-50 S column (2.5 × 8.0 cm) previously equilibrated with 0.01 M KH_2PO_4/K_2HPO_4, pH 7.0, buffer. Unreacted SAH is removed by elution with 100 ml of the same buffer solution and the column is washed with 50 ml of 0.25 *N* HOAc. (±)-AdoMet is eluted with 4 N HOAc and all the 5.0-ml fractions containing product are lyophilized; fractions are inspected by TLC using the same solvent as for SAH,

where R_f SAM = 0.05. Usually, the first 10–30 fractions following the void volume contain SAM. The solid is taken up in a 0.27 mM solution of *p*-toluenesulfonic acid monohydrate based on an 87% literature yield and a 4:1 tosylate:AdoMet mole ratio. This adaptation is a result of the virtually unreferenced work of Fiecchi wherein the stable sulfonic acid salts of SAM were isolated by precipitation directly from solution (8). The final sample is again lyophilized and product yield is determined by HPLC with an internal standard. Yield 69%; E_{259} AdoMet = 15,400; solvent: 5% ethanol, 243 mM acetic acid, 1 mM EDTA, 2.3^{-4} mM *n*-heptanesulfonic acid, pH 3.8, with NaOH; conditions: 1.0 ml/min, 1.38 kpsi and preequilibration with (1*S*)-(+)-10-camphorsulfonic acid as described above. The respective retention times are mentioned above. ^{1}H NMR (D_2O) δ 3.81 (t, 1H, Hα); 2.36 (q, 2H, Hβ); 3.7, 3.5 (m, 2H, Hγ); 3.9, 4.05 (m, 2H, H5′, 5″); 2.96 (5, 3H, (+)-SCH_3); 3.01 (s, 3H, (−)-SCH_3); 4.6 (m, 1H, H4′); 4.85 (m, 1H, H3′); 4.98 (m, 1H, H2′); 6.10 (d, 1H, H1′); 8.25 (s, 1H, H2); 8.28 (s, 1H, H8) (5).

Method 2. To a 1.0-ml solution of formic acid containing 0.6 mmol SAH is added 1.5 mmol $(CH_3)_3SI$ and the flask is placed in the dark for 1 day at room temperature while being monitored by HPLC for the consumption of SAH and formation of AdoMet. The solution, which is slightly discolored due to the iodine, is then concentrated and purified as before by an Amberlite IRC-50 S (H^+) column or by prep HPLC using repeated sample application and recovery of the AdoMet at 11 min after injection. Yield 30%.

Method 3. This procedure is a modification of Method 2 and was used to obtain the data in Fig. 5. Using equal concentrations of the methylating agent in parallel reactions, 2.0 ml 85% formic acid solution is made 0.05 mM in SAH and 0.1 mM in either CH_3I or $(CH_3)_3SI$ and left in the dark at 23°C. The reactions are monitored by HPLC as described previously. The solution is concentrated *in vacuo* to 1.5 ml. The yields are determined *in situ* by HPLC and are not the isolated yields. Yields: 90% with CH_3I and 30% with $(CH_3)_3SI$. It should be noted here that, in some instances, reversed mole ratios of the (+):(−) epimers were obtained using CH_3I as the methylating agent, although no obvious change in conditions aside from shaking was done.

S-Methylmethionine

The procedure used is the same as Method 3 under (±)-*S*-adenosyl-*L*-methionine, above, except that four parallel reactions are run using 1.0 and 0.15 mM of the methylating agent and 0.1 mM of L-methionine. SMM is isolated by HPLC using repeated sample application and recovery followed by lyophilization: R_t SMM = 12 min. ^{1}H NMR (D_2O) δ 3.80 (t, 1H, Hα); 2.35 (q, 2H, Hβ); 2.97 (s, 6H, S-$(CH_3)_2$); 3.6 (t, 2H, Hγ).

Enzymatic Synthesis of (−)-AdoMet

A 2.0-ml solution of 200 mM KCl, 10 mM $MgCl_2$, 100 mM Tris buffer, pH 6.8, 1 mM ATP, 1 mM L-Met, 0.3 mg pyrophosphatase, and 1 mg AdoMet synthetase from *E. coli* is allowed to react overnight at room temperature. The reaction is monitored by HPLC, as before; the reaction was stopped at 82%, probably due to product inhibition. The crude AdoMet salt may now be isolated by addition of the sulfonic acid in a 4:1 acid:AdoMet mole ratio, centrifugation (the enzyme has become dena-

TABLE I

Solvent Effect on SAH Synthesis According to Method 1 or Method 2 under Experimental

Reactants	*Solvent*	*Time (h)*	*(Reaction) and isolated yield (%)*
Na-Hcys, Cl-Ado	H_2O	4	(75) 57
	THF/EtOH (3/2 v/v)	16	(75) 56
	THF	10	(<5) 0
	DME	10	(<5) 0
	DMF	10	(10) 1
Hcys, Cl-Ado	H_2O, pH 83	10	(<5) 0
Hcys, Cl-Ado	H_2O, pH 3.3	10	(<5) 0

tured after $\frac{1}{2}$ h of acid addition), and finally, lyophilization (the centrifugation is carried out at 1500 rpm). If very pure (−)-AdoMet is desired, the buffer salts may be removed by passing the original reaction solution through an Amberlite column as before. Yield 67%: (+):(−) = 6:94 when isolated by prep HPLC and (+):(−) = 11:89 when isolated by the Amberlite column, as determined by ^{1}H NMR resonance intensities.

Assay of AdoMet Synthetase

The assay mixture (final volume 1 ml, pH 7.8) contained 100 mM Tris/HCl, 2 mM $MgCl_2$, 5 mM ATP, 5 mM L-Met, and an aliquot of soluble or immobilized enzyme solution. A blank was prepared simultaneously without Met and both were incubated at 25°C. The reaction was monitored by measuring the formation of AdoMet with HPLC as described above.

Enzyme Immobilization

The enzymes were immobilized by the polymerization condensation method (23) in polyacrylamide gels. Organic pyrophosphatase (200 U, 0.4 mg of protein) was immobilized in 1 g of PAN-800 in the presence of 5 mM pyrophosphate to afford 70 U of immobilized enzyme (16). AdoMet synthetase (22 U, 10 mg of protein) was immobilized in 1 g of PAN-800 in the presence of ATP (2 mM), L-Met (2 mM), and $MgCl_2$ (2 mM) to afford 10 U of immobilized enzyme.

RESULTS AND DISCUSSION

Synthesis of SAH

One of the most recent developments in the synthesis of SAH is the coupling of L-Hcys sodium thiolate (Na-Hcys), prepared from L-homocystine, to 5′-deoxy-5′-chloradenosine (Cl-Ado) in water (3). This approach has some disadvantages aside from our not being able to reproduce the 81% reported yield (an average 50% yield due to product and reagent decomposition has been obtained): L-homocystine is not an easily available substance ($50/g), and the extremely basic reaction conditions in water cause product and reagent decomposition. In an attempt to maximize the yield, solvent effects on the coupling reaction of Na-Hcys or Hcys and Cl-Ado were studied (Table I). In the reactions, Na-Hcys was made directly from L-Met via demethylation

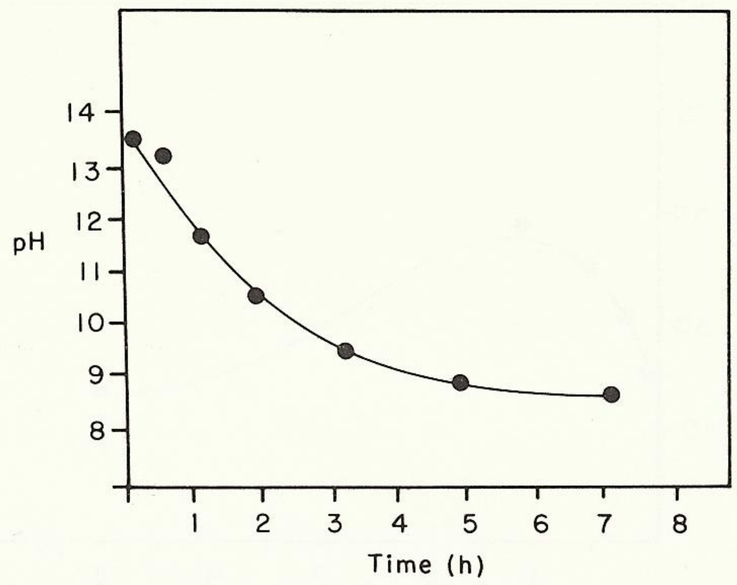

FIG. 1. Time-course study of the change of reaction pH in the synthesis of SAH from Na-Hcys and Cl-Ado in water. Conditions are those of Method 1 under SAH in the experimental section.

under Birch conditions (10) followed by precipitation of the product with ether. The product Na-Hcys precipitated out of solution was then treated with Cl-Ado (2.5:2.0 mole ratio for Na-Hcys:Cl-Ado) in THF/EtOH (3:2, v/v) containing 1% of KI. The mixture was then refluxed for varying time periods. As shown in Table I, the polarity of solvent used in the coupling is critical. Of the solvents tested, THF/EtOH is the best and it is comparable with water in terms of reaction yield. Very poor yields were obtained for the other aprotic solvents with less polarity. No detectable reaction occurred in aqueous solution at either pH 8.3 or 3.3. In order to understand the ionization status of the thiol group during the reaction in aqueous solution, we measured the reaction pH vs time in the reaction with Na-Hcys as a starting material. As shown in Fig. 1, the reaction pH, initially > 13, drops rapidly within the first two h. This pH effect correlates directly to a decrease in the reaction rate vs time as determined by product isolation at various times. The actual limiting factor of the coupling reaction in water is the decomposition of both Cl-Ado and SAH under the extremely basic conditions. The limiting factor in the THF/EtOH system, however, is the increased reaction time due to the decreased concentration of the thiolate anion, but much less product and substrate decomposition is observed under this anhydrous and deoxygenated condition.

A reported system where the reaction is run in liquid ammonia (24) also has a comparable yield, as does the most recently reported procedure using *N,N*-bis[trifluoroacetyl-L-homocystine dimethyl ester coupling to adenosine in the presence of tri-*N*-butyl or triethylphosphine in pyridine (11). These procedures, however, are not as convenient as those carried out in water or THF/EtOH and the reagents used are relatively expensive. Also of interest is the effect of KI concentration on the reaction yield. As shown in Fig. 2, the optimum ratio of Cl-Ado to KI is 4 to 1. At lower Ki concentrations, decomposition predominates due to the small extent of catalysis, whereas at high KI concentrations, the iodide seems to promote decomposition of both adenosine and SAH (as shown by testing the stability of both components in separate experiments under similar conditions).

Although the conditions described above are similar to those employed by Ramalingam and Woodard (3) and have similar yields, the advantage in using anhydrous/

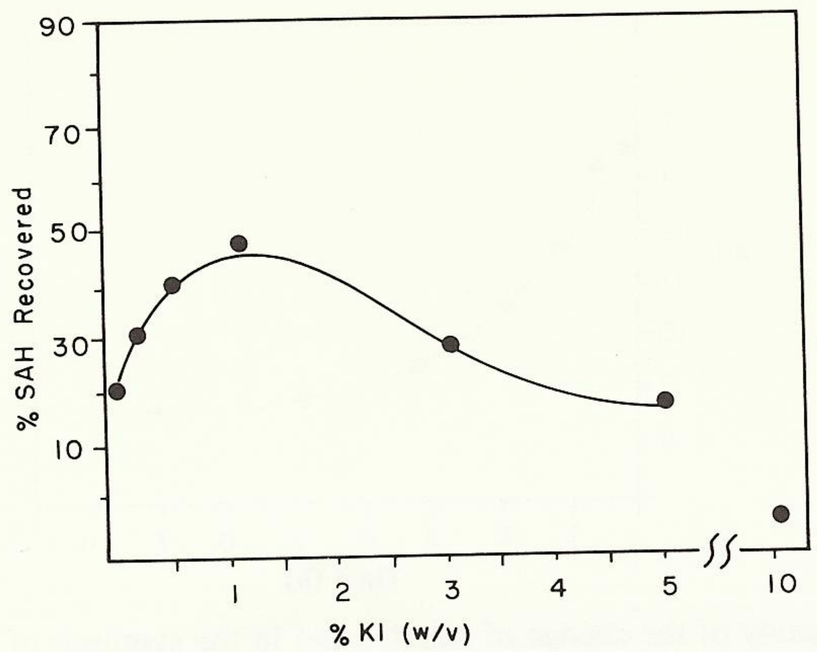

FIG. 2. Effects of KI on product yield. Reaction conditions are those of Methods 1 and 2 under Experimental.

deoxygenated THF/EtOH (3:2 v/v), however, is that much purer SAH is obtained from the Dowex 50 (NH_4^+) column due to fewer impurities being formed in the reaction and subsequently coeluting with SAH even after extended washing with H_2O.

An alternate route to SAH is through the enzymatic coupling of L-Hcys and adenosine by SAH hydrolase from beef liver (12). The literature reports an 80% yield with direct precipitation of the product from the reaction solution; the reaction has been scaled up to 18 g (1.2 g SAH/g liver). Were it not for the need to isolate the beef liver enzyme, this would be the most feasible route.

Chemical Synthesis of AdoMet

The most common method toward AdoMet is through the methylation of SAH with CH_3I (4); however, this method has the disadvantage of forming the biologically

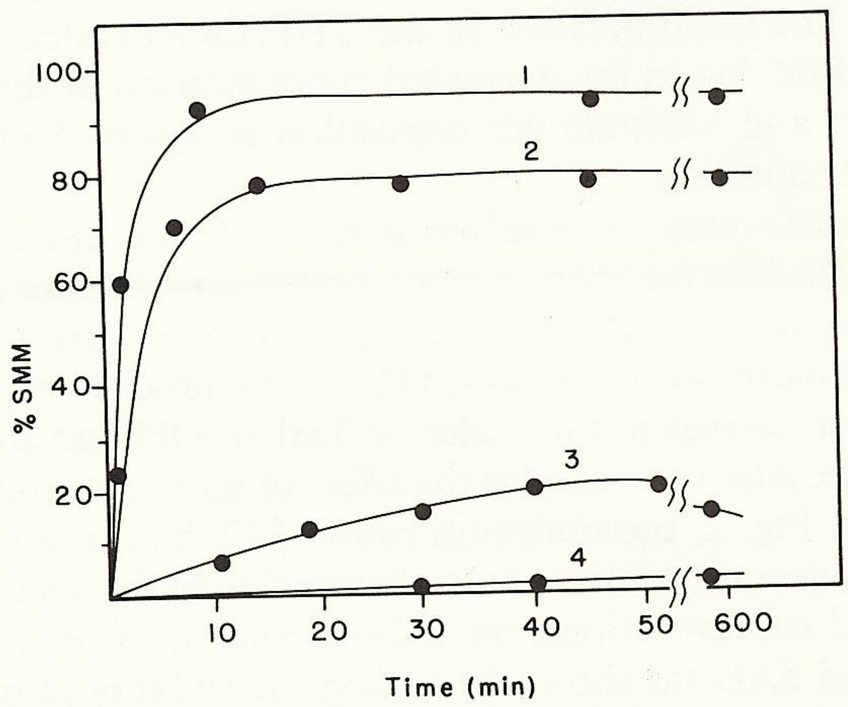

FIG. 3. Methylation of L-Met (0.1 mM) to form *S*-methyl-L-methionine (SMM). Conditions are described under Experimental. (1) 1.0 mM CH_3I; (2) 0.15 mM CH_3I; (3) 1.0 mM $(CH_3)SI$; (4) 0.15 mM $(CH_3)_3SI$. Results are based on yields determined by HPLC.

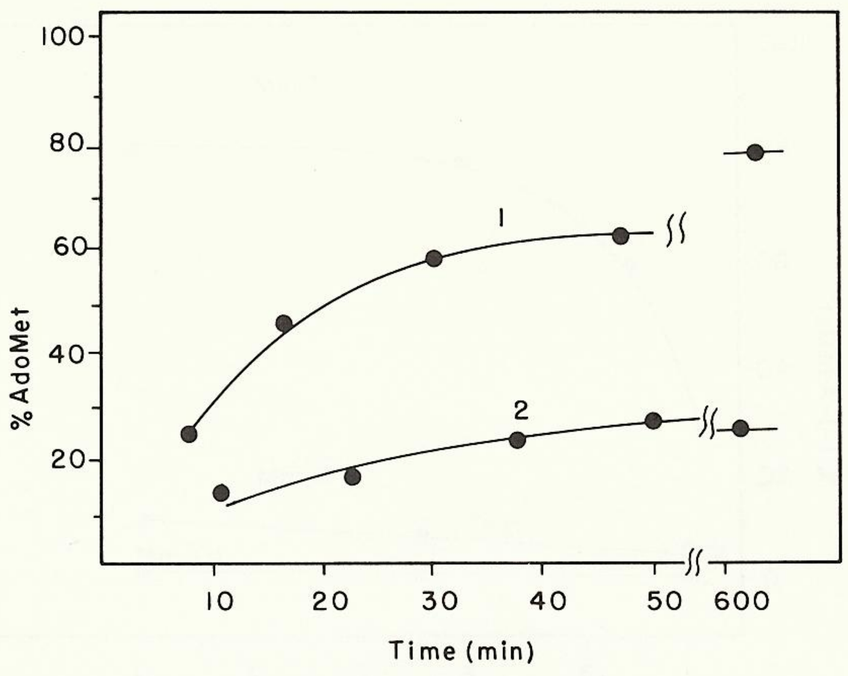

FIG. 4. Methylation of SAH (0.05) to form AdoMet. Conditions are those described for Method 3 under Experimental. (1) 0.1 mM CH_3I and (2) 0.1 mM $(CH_3)_3SI$.

inactive isomer as a major product in a 60:40 (+):(−) mole ratio (5). In an attempt to overcome this disadvantage, we have used trimethylsulfonium iodide (TMSI) as a methylating reagent and compared it to CH_3I for efficiency and stereo- and regioselectivity. This reagent was used previously for the methylation of hydroxyl, sulfhydryl, carboxyl, and amino groups under neutral conditions (20, 21, 25, 26), but no attempt was made to use this reagent to methylate thioether derivatives such as SAH. It is feasible to suggest that perhaps by using a smaller excess of the reagent the concentration of dimethylsulfide formed after methylation can be kept to a minimum and methylation might predominate at least in the early course of the reaction. This strategy was applied toward the synthesis of *S*-methylmethionine and AdoMet by treatment of Met and SAH, respectively, with the reagent. Shown in Fig. 3 and 4 are the results of several reactions using CH_3I vs Me_3SI at varying concentrations while keeping the relative concentration of substrates the same. At either concentration of reagent, CH_3I is seen to be the better reagent by producing the product in less time and allowing the product to stay in solution with only minimal decomposition. Both studies show that as the concentration of Me_2S increases, the demethylation reaction competes with the methylation reaction and finally reaches equilibrium. Me_3SI also methylates the 2′- and 3′-hydroxyls and N^6 positions in 10:10:5%. The use of other solvents such as H_2SO_4, H_2SO_4/MeOH, or CH_3OH results in excessive formation of methylation byproducts due to loss of regioselectivity. One interesting aspect, however, is that an increase of (−)-AdoMet in the epimeric mixture has been observed: (+):(−) mole ratios of 55:45 for the Me_3SI reaction and 65:35 for the CH_3I reaction. Although Me_3SI is not an efficient methylating agent for L-Met and SAH, it does show that more than one methylating agent can be used and the possibility of developing a reagent capable of asymmetrically synthesizing AdoMet may exist.

Enzymatic Synthesis of (−)-AdoMet

As described before, (−)-AdoMet has been prepared enzymatically from ATP and L-MET on milligram scales for biochemical studies. The preparative (7 mmol scale)

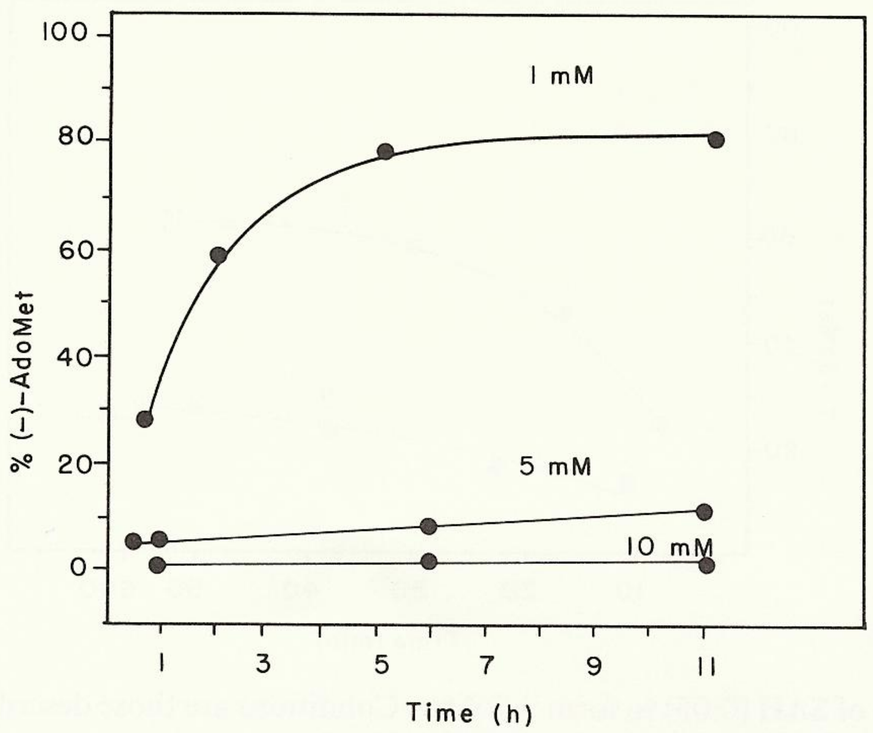

FIG. 5. Results of the enzymatic synthesis of AdoMet under different concentrations of substrates (ATP and Met) using AdoMet synthetase (soluble or immobilized) from an overproducing strain of *E. coli*. Conditions are those described under Experimental except that ATP and L-Met concentrations are varied while keeping a 1:1 mole ratio.

enzymatic method toward (−)-AdoMet using AdoMet synthetase has the drawback that the enzyme is difficult to isolate: 400 g of dried yeast gave 8 U of AdoMet synthetase with specific activity of 0.05 U/mg (1 U is 1 μmol of (−)-AdoMet formed per minute). In an attempt to improve the enzymatic procedure, we have studied the synthetic utility of AdoMet synthetase from an overproducing strain of *E. coli* (14, 15) which produces 80-fold more AdoMet synthetase than wild-type *E. coli* and permits the preparation of about 100 mg of homogenous enzyme from about 120 g of cells. The results of several small-scale experiments as shown in Fig. 5, however, indi-

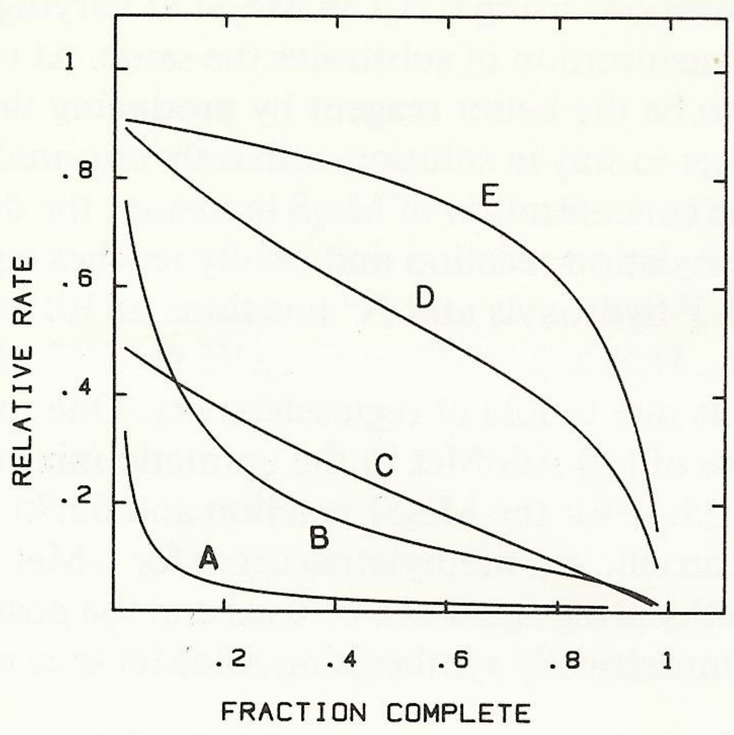

FIG. 6. Prediction of the reaction yield as a function of relative rate in AdoMet synthetase (from the *E. coli* strain) catalyzed synthesis. The results are generated from Eq. [1] with different K_i and K_m values. (A) $A_0 = K_m$, $K_i = K_m$; (B) $A_0 = 10\ K_m$, $K_i = K_m$; (C) $A_0 = 100\ K_m$, $K_i = K_m$; (D) $A_0 = 10\ K_m$, $K_i = 10\ K_m$; (E) $A_0 = 10\ K_m$, $K_i = 1000\ K_m$. A_0 is the initial concentration of Met or ATP.

TABLE II

Parameters of AdoMet Synthetase from Varying Sources[a]

Enzyme source	*Km, Km APP*[b] *L-Met*	*ATP (mM)*	*Ki (mM), L-Met*	*AdoMet vs ATP*	*Specific activity (U/mg)*	*Total activity (U/100 g cells)*	$M_r \times 10^{-3}$
Yeast[c]							
Isozyme I	0.11[a]	0.074[a]	5.4 (U)[d]	3.5 (U)[d]	0.623	10[e]	55
Isozyme II	0.14	0.047[a]	5.4 (U)[d]	3.5 (U)[d]	0.367	30[e]	55
Rat Liver							
Isozyme I	0.043[f]	0.145[g]	0.4 (U)[d,i]	0.4 (U)[d,i]	0.0012[f,h]	2.32	208
Isozyme II	0.005[f]	0.07–0.14[g]	0.4 (U)[d,i]	0.4 (U)[d,i]	0.01[f,h]	0.75	190
Isozyme III	1.3[f]	2.70[g]	—[j]	—[j]	0.021[f,h]	24	2 × 47
E. coli	0.08[k]	0.11[k]	0.01 (N)[k]	0.01 (C)[k]	2.2[l]	140	4 × 43

[a] Abbreviations: U = uncompetitive; N = noncompetitive; C = competitive.

[b] Determined by approximating linearity for substrate concentrations up to 0.5 mM in Lineweaver–Burk plots using L-Met and ATP as the variable substrates. This approximation is necessary due to the strong positive cooperativity of AdoMet synthetase.

[c] Chiang, P. K., and Cantoni, G. L. (1977) *J. Biol. Chem.* **252,** 4506.

[d] Obtained from a mixture of isozymes.

[e] Obtained from 400 g dried yeast.

[f] Hoffman, J. L., and Kunz, G. L. (1980) *Fed. Proc.* **39,** 1690.

[g] Hoffman, J. L. (1983) *in Methods in Enzymology* (Tabor, H., and Tabor, C. W., Eds.), Vol. 94, p. 223, Academic Press, Orlando, FL.

[h] Obtained from three rat livers (27 g) of 200- to 300-g female Sprague–Dawley rats.

[i] Lombardini, J. B., Chou, T. C., and Talalay, P. (1973) *Biochem. J.* **135,** 43.

[j] No product inhibition ((Ref. (27)).

[k] Ref. (14).

[l] Obtained from 50 g wet cells (Ref. (14)).

cate a problem in product inhibition. The reaction starting with 1 mM each of ATP and Met stops completely when 0.82 mM of AdoMet is produced. The 5 and 10 mM reactions do not even form 1 mM of AdoMet. The same results were observed with immobilized enzymes.

The decrease in the rate of enzymatic synthesis of AdoMet at high concentrations of ATP and methionine can be readily explained by the noncompetitive product inhibition of AdoMet vs methionine. Markham *et al.* (14) have shown with the *E. coli* enzyme that AdoMet is a strong noncompetitive inhibitor versus methionine. The K_{ii} and K_{is} values are 0.06 and 0.01 mM, respectively. As illustrated in Fig. 5 the noncompetitive inhibition by the products is more severe at high initial substrate concentrations than at low concentrations. The theoretical plots in Fig. 6 were generated by substitution of various parameters into the equation for noncompetitive inhibition

$$v = \frac{VA}{A(1 + I/K_{ii}) + K(1 + I/K_{is})} \qquad [1]$$

where v = velocity, $V = V_{max}$, A is the concentration of the substrate, I is the concentration of the product inhibitor, and K_{ii} and K_{is} are the intercept and slope inhibitor

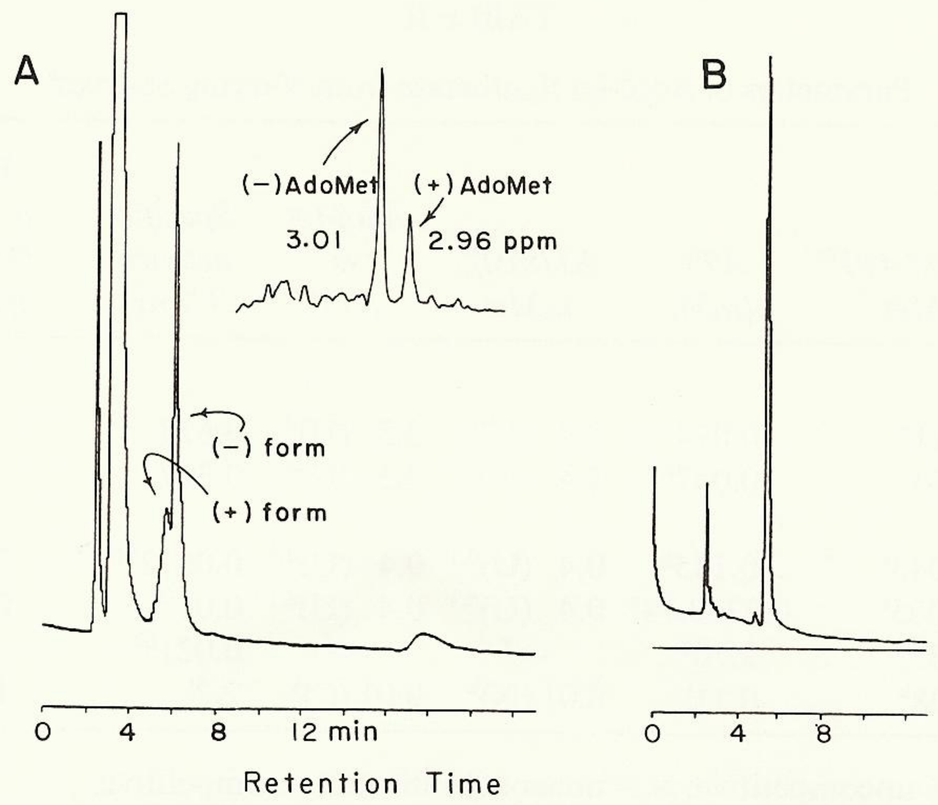

FIG. 7. (A) HPLC analysis of standard AdoMet tosylate from Sigma. The chromatography conditions are described under Experimental. Insert: ^{1}H NMR spectrum of the sulfonium methyl group. $\delta = 2.96$ ppm for the (−)-form and 3.0l ppm for the (+)-form. (B) HPLC analysis of the enzyme product from 1 mM reaction after denaturation of enzyme and centrifugation.

constants. The relative rate (v/V) was calculated with this equation as the initial concentration of A was decreasing toward zero and the concentration of the inhibitor was increasing toward the initial substrate concentration throughout the time course of the reaction. The K_{ii} and K_{is} values were assumed to be equal. Plots A–C (Fig. 6) illustrate the effect of increasing the initial substrate concentration from a value equal to the K_m level to $100 \times K_m$ when $K_i = K_m$. As the initial substrate concentration becomes larger, the reaction rate actually becomes slower after the reaction has gone to 10% completion. Plots B, D, and E illustrate how the reaction rate is directly effected by the K_i value. As the K_i value becomes larger and larger the overall rate becomes faster and faster. With the enzyme from *E. coli,* the noncompetitive inhibition by AdoMet would require the synthesis to be completed at levels of substrate of about 1 mM. We compare the affinity constant (K_m), the inhibition constant (K_i), and other parameters of AdoMet synthetase from different species (Table II) and conclude that the use of AdoMet synthetase from *E. coli* in the synthesis of AdoMet suffers the most severe problem in product inhibition. Although it has the highest specific activity, the enzyme can only be used for small-scale preparation of AdoMet or derivatives in a very dilute solution (~1 mM). The enzymes from other species such as yeast and rat liver (isozyme III) (16, 17) have less of a problem with product inhibition and could be useful for larger scale preparation, if they were prepared in larger quantities by recombinant DNA technology.

In the various enzymatic studies done on AdoMet synthetase, it has been suggested that the enzyme forms the sulfonium in an 88:12 (−):(+) mole ratio (19); however, no direct evidence aside from ^{1}H NMR analysis of isolated product has been presented. We found that depending on the method used for isolation, varying results are obtained. Where the method involving Amberlite IRC-50 S cation-exchange purification is used, an 88:12 (−):(+) mole ratio is obtained. Where the isolation of Ad-

oMet involving HPLC purification of the enzyme reaction is used, yields of 94:6 (−):(+) mole ratio are obtained. Where the isolation of (−)-AdoMet is accomplished by precipitation as described by Fiecchi (8), mole ratios of 80–90:20–10 are obtained. It should be evident from these results that contamination of (−)-AdoMet by (+)-AdoMet is not a characteristic of the enzyme catalytic reaction; rather its presence is a function of the method of isolation and temperature at which the enzyme reaction was done (Matos and Wong, unpublished). The enzyme product is only (−)-AdoMet. This is further proved by the observation of only this isomer in the HPLC analysis of the enzyme reaction mixture before isolation. In any event, a virtually epimerically pure (−)-AdoMet can be obtained in small quantities via rapid enzymatic synthesis followed by HPLC purification (Fig. 7).

CONCLUSION

With the newly developed HPLC technique, optically pure (−)-AdoMet can be obtained from a mixture containing the undesired (+)-isomer prepared chemically or enzymatically. The AdoMet synthetase from the *E. coli* strain can be useful only for small-scale synthesis of (−)-AdoMet. For larger scale synthesis, the enzyme from rat liver or yeast is a better choice because of problems due to product inhibition, but the enzyme from these sources are difficult to isolate. If cloned, they would be good catalysts for the preparative synthesis of (−)-AdoMet.

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