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The Chemical Synthesis of High Specific-Activity [^{35}S]Adenosylhomocysteine

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The study of the family of transmethylases, critical to normal cellular function and often altered in cancer, can be facilitated by the availability of a high specific-activity *S*-adenosylhomocysteine. We report the two-step preparation of [^{35}S]adenosylhomocysteine from [^{35}S]methionine at a specific activity of 1420 Ci/mmol in an overall yield of 24% by a procedure involving demethylation of the [^{35}S]methionine to [^{35}S]homocysteine followed by condensation with 5′-chloro-5′-deoxyadenosine. The ease of the reactions, ready availability and low cost of the reagents and high specific-activity and stability of the product make the procedure an attractive one with many uses, and superior to current methodology.

KEY WORDS: Met; ^{35}S; Hcy; 5′-chloro-5′-deoxyadenosine; dimethylformamide; transmethylase.

Methylations, catalyzed by the family of transmethylases, are critical to normal cellular function and often seem to be altered in cancer (1–4). In cellular transmethylations the methyl groups are donated by *S*-adenosylmethionine (AdoMet),[1] with *S*-adenosylhomocysteine (AdoHcy) as a resulting product. AdoHcy is a competitive inhibitor for all AdoMet-mediated transmethylations (5). The inhibition constant of AdoHcy is lower than the K_m for AdoMet for most known transmethylases, indicating a key regulatory role for AdoHcy (5,6). In order to further studies on the regulatory properties of AdoHcy and on the methyltransferases themselves it is essential to have a readily obtainable source of high specific-activity radiolabeled AdoHcy.

A number of preparations of radiolabeled AdoHcy have been reported, all containing tritium or carbon-14 (7–15). With few exceptions (12,14), they have been prepared enzymatically by reaction of labeled adenosine with homocysteine in the presence of *S*-adenosylhomocysteine hydrolase. Other methods used have been a condensation reaction between radiolabeled 5′-chloro-5′-deoxyadenosine and homocysteine (14) or a demethylation of radiolabeled AdoMet, which had been prepared enzymatically (12). All the syntheses have suffered from the lack of a high specific-activity product, the highest being reported at 23 Ci/mmol.

High specific-activity AdoHcy radiolabeled in the sulfur atom would be a very important experimental tool. For example, it could have use as a photoaffinity probe that theoretically could bind to all members of the family of transmethylases. The label in the sulfur atom would also possibly offer greater stability for photolabeled products under basic conditions such as two-dimensional electrophoresis, compared to the [CH_3-^{3}H]AdoMet currently in use as a photoaffinity probe for methyltransferases (16,17). We demonstrate here that [^{35}S]AdoHcy can readily be prepared from [^{35}S]methionine at activities well in excess of 1000 Ci/mmol.

[1] Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; CM-cellulose, *O*-(carboxymethyl)-cellulose; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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MATERIALS AND METHODS

Chemicals and biochemicals. [^{35}S]Methionine (1420 Ci/mmol) was from Amersham. Adenosine, AdoHcy, and hexamethylphosphoramide were from Sigma. Dithiothreitol was from Calbiochem-Boehring. Dimethylformamide, thionyl chloride, and hydriodic acid were from Mallinkrodt. Hypophosphorous acid was from Matheson, Coleman and Bell. CM-cellulose sheets were from Baker.

Synthesis of [^{35}S]homocysteine thiolactone. Synthesis of [^{35}S]homocysteine thiolactone was performed by a modification of our previously reported procedure (18). [^{35}S]Methionine (5 mCi, specific activity 1420 Ci/mmol) was placed in a 10-ml flask and the solvent was evaporated under a stream of nitrogen. Hypophosphorous acid (20 μl), hydriodic acid (1 ml, 47% ACS reagent) and a stirring bar were added and the resulting solution was refluxed overnight. The solution was then evaporated under a stream of nitrogen, the residue taken up in 500 μl absolute methanol and placed on a 1-cm^3 alumina column (Matheson, Coleman and Bell, 80–325 mesh, chromatographic grade, packed dry). The reaction flask was washed with 500 μl portions of methanol, each portion being placed on the column. A positive pressure was applied to the column to elute the product as rapidly as possible. The basic alumina column strongly binds all acidic components but not the basic homocysteine thiolactone, making possible a simple separation of product from unreacted starting material. However, any thiolactone that is hydrolyzed on the column is also absorbed, reducing the yield. A total of 3 ml eluate over a 15-min period was collected, an aliquot was counted, and the methanol was evaporated under a stream of nitrogen. Yields of this step are as high as 80%.

Synthesis of 5′-chloro-5′-deoxyadenosine. The procedure used was that of Borchardt *et al.* (19). Hexamethylphosphoramide (10 ml) was added to a flask equipped with a drying tube. Thionyl chloride (1.5 ml) and then 1.0 g adenosine were added and the mixture was stirred overnight. Water (40 ml) was added and the volume was immediately reduced to 20 ml, *in vacuo.* Seed crystals were added and the solution was cooled overnight. It was necessary to add additional water to complete the precipitation of the product. The precipitated material was filtered *in vacuo,* washed several times with water, and dried *in vacuo.* The yield was 0.9956 g of 5′-chloro-5′-deoxyadenosine or 94%. 5′-Chloro-5′-deoxyadenosine can be purchased from Sigma but was synthesized for reasons of economy.

Synthesis of [^{35}S]AdoHcy. Twenty microliters of an argon-saturated solution of dithiothreitol (DTT) (10 mg/ml) was added to a tube containing the [^{35}S]homocysteine thiolactone. The mixture was placed under argon and was vortexed vigorously to make certain all the radiochemical had been redissolved. Sodium hydroxide (5 μl, 10 N) was added, the solution was placed again under argon and allowed to stand at room temperature for 30 min to allow the thiolactone to hydrolyze. An argon-saturated solution of 5′-chloro-5′-deoxyadenosine (10 mg) in 25 μl dimethylformamide was added, the mixture was placed under argon, vortexed, and allowed to stand overnight at room temperature. The solution was then placed on a 20 × 20 cm CM-cellulose sheet together with one spot of authentic nonradioactive AdoHcy. The chromatogram was developed with 0.08 N sodium acetate, pH 4. The position of the radioactive product corresponding to authentic AdoHcy on a strip of the chromatogram was determined by scintillation counting on a Beckman LS100 apparatus using a toluene-based fluid. The band corresponding to the product was cut from the remainder of the sheet. The cellulose was then scraped off and placed in a small disposable column. The product was eluted with 0.2 N sodium acetate, pH 4, containing 10 mg/ml DTT, and the product collected.

High voltage electrophoresis. The procedure used has been described before (20) and is a modification of the procedure of Jonas and Schneider (21). Ten microliters of the sample collected after chromatography on CM-cel-

lulose was placed on a 20 × 40 cm sheet of Schleicher & Schuell 0.22-mm paper that had been wet with a 6% solution of formic acid. A standard of authentic nonradioactive AdoHcy was added and the sample was run at 2500 V for 30 min on a Camag high voltage electrophoresis apparatus with 6% formic acid as the buffer. The paper was dried in an oven for 15 min, sprayed with 0.5% ninhydrin in methanol, and heated a further 5 min to visualize the standard. One-centimeter strips were then cut from the sheet and counted on a Beckman LS100 counter using a toluene-based scintillation fluid.

HPLC. The procedure used has been described previously (22). A volume of 40 μl of the sample collected after CM-cellulose-chromatography and 10 μl of a 10 mM sample of nonradioactive AdoHcy were injected into a Vydac cation exchange column (Separations Group, Hesperia, Calif.). The column was eluted on an Altex HPLC system using a gradient generated from two ammonium formate buffers at a flow of 2.5 ml/min. Buffer B consisted of 0.8 M ammonium formate adjusted to pH 4.0 with concentrated hydrochloric acid. Buffer A was obtained by diluting Buffer B to 0.01 M. The elution started with 100% Buffer A. Buffer B was then increased to 15% of the total over the first 10 min, to 20% over the next 10 min, to 100% over the next 5 min, and then kept at 100% for the remainder of the run. The entire run was a total of 35 min. The standard was detected at 254 nm and data processed on a Shimadzu recorder-integrator. One-minute fractions were collected on an LK B fraction collector and counted on a Beckman LS100 counter using a toluene-based scintillation fluid.

RESULTS

The chromatograph of the reaction mixture is shown in Fig. 1. The CM-cellulose sheet was cut into 20 strips and each strip was counted by liquid scintillation. The profile of the reaction mixture indicated two main products. The major product, which ran 15 to 20 cm from the origin was probably [^{35}S]homocysteic acid derived by oxidation of the [^{35}S]homocysteine. A standard of nonradioactive homocysteic acid migrated between 17 and 20 cm (data not shown). The other product migrated together with authentic AdoHcy, appeared between 11 and 13 cm from the origin, and constituted 31% of the total reaction mixture. Unreacted 5′-chloro-5′-deoxyadenosine migrated between 6 and 10 cm (data not shown). A standard of homocysteine thiolactone, a potentially troublesome impurity, migrated between 8 and 9 cm (data not shown) where there was no radioactive peak present, therefore indicating its absence, as seen in Fig. 1.

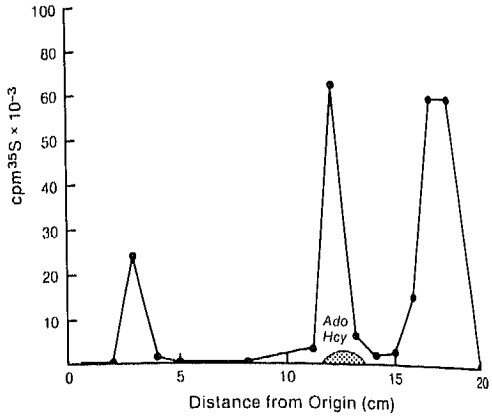

FIG. 1. Thin-layer chromatograph of [^{35}S]AdoHcy reaction mixture. Sample was streaked across a 20 × 20 cm sheet of CM-cellulose together with one spot of authentic AdoHcy, and eluted with 0.08 N sodium acetate, pH 4. A narrow strip containing the standard was removed, developed with ninhydrin, cut into 1-cm strips and subjected to liquid scintillation counting. Two main peaks were found with [^{35}S]AdoHcy comprising 31% of the total mixture. The location of the AdoHcy standard is indicated by a darkened area.

After extraction of the AdoHcy from the preparative chromatogram, CM-cellulose chromatography was repeated. [^{35}S]AdoHcy was the predominant peak and occurred between 3 and 7 cm with a very small amount of homocysteic acid at 8 cm (data not shown).

High voltage electrophoresis showed that [^{35}S]AdoHcy, which migrated together with an

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1.2 mCi or 31.3% l
[^{35}S]Hcy, which comp
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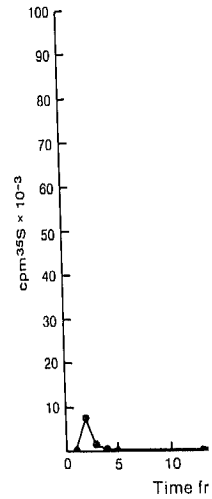

FIG. 2. HLPC of purif
sample with 10 μl of a stan
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authentic sample of AdoHcy was the only peak present and appeared between 11 and 13 cm. Any [^{35}S]homocysteic acid in the sample would have migrated off the sheet under the conditions used (data not shown).

The HPLC elution profile is shown in Fig. 2. [^{35}S]AdoHcy coeluted with an authentic sample of AdoHcy between 18 and 22 min. Minor impurities eluted between 1 and 3 min and between 24 and 27 min. The early impurity, which was 5% of the total, was likely the homocysteic acid. The later impurity, comprising 4% of the total, could not be determined. The radiochemical purity of the [^{35}S]AdoHcy was determined to be 91%.

The yield of [^{35}S]Hcy was 3.84 mCi or 77% based on the 5 mCi of [^{35}S]Met which was used. The yield of [^{35}S]AdoHcy recovered was 1.2 mCi or 31.3% based on the starting [^{35}S]Hcy, which compares favorably with the 30.9% as determined by the counting of the CM-cellulose chromatogram (Fig. 1). The overall yield from [^{35}S]Met was 24% and the product had a specific activity equal to that of the starting [^{35}S]Met which was 1420 Ci/mmol.

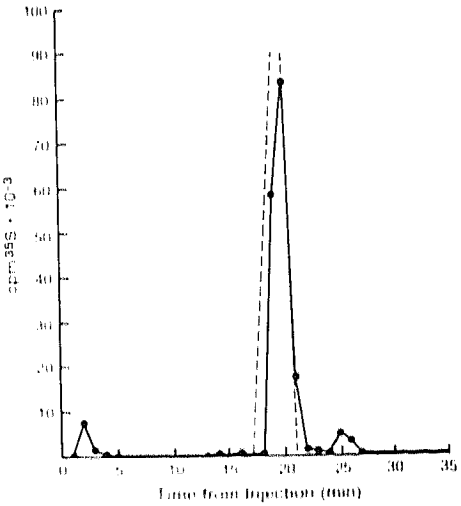

FIG. 2. HLPC of purified [^{35}S]AdoHcy. 40 μl of the sample with 10 μl of a standard were injected into a Vydac cation exchange column. The column was eluted on an Altex system using an ammonium formate gradient (pH 4) at a flow rate of 2.5 ml/min: Buffer A was 0.01 M ammonium formate, Buffer B was 0.8 M ammonium formate. The elution was initiated with 100% A. Buffer B was increased to 15% over 10 min, 20% over the next 10 min, 100% over 5 min, and kept at 100% for the remainder of the run, a total of 35 min. The location of the AdoHcy standard is given by the dashed line.

DISCUSSION

The central importance of AdoHcy as a regulatory molecule in the numerous types of cellular transmethylating reactions makes imperative a readily available source of radioactive AdoHcy for further understanding the mechanism of regulation and for study of the transmethylases themselves. The methodology outlined here starting with commercially available [^{35}S]methionine, and using our previously published facile synthetic procedure for highly pure [^{35}S]Hcy (18), allows a rapid and inexpensive synthesis of very high specific-activity [^{35}S]AdoHcy (>1000 Ci/mmol). The relative simplicity and speed of the reactions involved, the ready availability and relatively low cost of the reagents, the elimination of preparing enzymes to carry out the reaction, and the high specific-activity and purity of the product all make the procedure an attractive one with wide use, and superior to current methodology (7–15). In particular, the high specific-activity of the [^{35}S]AdoHcy should be of use as a specific photoaffinity probe for the labeling of most if not all of the members of the methyltransferase family.

The chemical synthesis described here could also allow the synthesis of high specific-activity ^{35}S analogs of AdoHcy using various adenosine derivatives, which may be of use in metabolic studies or as photoaffinity probes (23) with possible very high affinities to particular methyltransferases (24–26). These probes could lead to the identification and eventual cloning of the genes of the large family of transmethylases, which are critical to normal cellular function and seem, at least in part, to be altered in cancer (1–4,27).

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