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Short note

The preparation of [^{35}S]homocysteine thiolactone free of [^{35}S]methionine

Peter H. Stern, James O. Mecham and Robert M. Hoffman

Department of Pediatrics, M-009, University of California – San Diego, La Jolla, CA 92093, U.S.A.

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Introduction

The unambiguous study of homocysteine metabolism requires a source of [^{35}S]homocysteine completely free from contaminating [^{35}S]methionine. Until now, such a reagent has not been readily available.

Homocysteine is an important metabolite involved in methionine, cysteine and methylation metabolism. Homocysteine can be methylated to form methionine with either 5-methyltetrahydrofolate [1] or betaine [2] serving as the methyl donor, it can be condensed with serine to form cystathionine [3] which can be converted to cysteine, or it can be condensed with adenosine [4] to form *S*-adenosylhomocysteine, a strong inhibitor of all known cellular methylation reactions [5,6]. Additionally, there is evidence that in methionine-dependent tumor lines, methionine synthesized endogenously from homocysteine is used differently by the cell than exogenously supplied methionine [7]. In this report we describe a novel, simple and fast method of recovering methionine-free [^{35}S]homocysteine thiolactone following its synthesis.

Materials and Methods

Materials

[^{35}S]Methionine (1400 Ci/mmol) was obtained from Amersham. Hydriodic acid, hypophosphorous acid and chromatographic-grade alumina (80-325 Mesh) were obtained from Matheson, Coleman and Bell. Isopentyl alcohol, dioxane and pyridine were obtained from Mallinkrodt. The cellulose thin-layer chromatograph sheets were obtained from Eastman. Scintillation counting was performed on a Beckman LS 100 instrument.

Synthesis of [^{35}S]homocysteine thiolactone

The synthesis procedure used was a modification of the method of Baernstein [8]. [^{35}S]Methionine (2 mCi) was evaporated to dryness at 60°C under a stream of nitrogen. 20 μl hypophosphorous acid and 1 ml hydriodic acid were added and the resulting mixture was refluxed for 22 h. The solution was cooled and was evaporated at 60°C under a stream of nitrogen. Prior to evaporation, an aliquot was removed and subjected to analysis.

Isolation of methionine-free [^{35}S]homocysteine thiolactone

To isolate the product from methionine, the mixture was taken up in 200 μl of absolute methanol, and the resulting solution was placed on a dry alumina column containing 1 ml of M.C.B. Chromatographic Grade alumina 80-325 Mesh in a Pasteur pipette. The reaction vessel was washed repeatedly with 100-μl portions of methanol, each washing being placed on the column. A total of 2 ml eluent was collected in a glass vial containing 10 μl of 0.1 N HCl. The solvent was then evaporated under a stream of nitrogen, and the residue was dissolved in 1 ml of 10 mM HCl. Aliquots were removed for amino acid analysis and scintillation counting.

Determination of the purity and activity of the [^{35}S]homocysteine thiolactone

The purity of the product was first determined under standard conditions on a Beckman amino acid analyzer equipped with stream division. A standard of homocysteine thiolactone eluted between 82 and 84 min, while standards of homocysteine and methionine coeluted between 44 and 47 min. Fractions of the experimental sample were collected at 1 min intervals throughout the range of the elution. To determine the amount of methionine remaining in the reaction mixture, it was necessary to alter the above conditions of the analysis to effect a separation of the methionine and homocysteine. The separation was performed in the amino acid analyzer by elution with a diluted buffer system containing 0.05 M citrate pH 4.0 under which conditions homocysteine eluted at 38 min and methionine at 40 min. Fractions were collected at 18-s intervals between 30 and 45 min under the latter condition. All fractions were subjected to scintillation counting.

As a second method of analysis, an aliquot of the [^{35}S]homocysteine thiolactone was placed on a cellulose thin-layer chromatographic strip alongside standards of methionine and homocysteine thiolactone. The chromatogram was developed using a 1 : 1 : 1 : 1 mixture of isopentyl alcohol, dioxane, pyridine and water (S. Jacobsen and R.M. Hoffman, unpublished data). The reference methionine and homocysteine thiolactone were detected by ninhydrin. The chromatogram was cut into 1 cm strips, and the strips were subjected to scintillation counting.

Results and Discussion

Figs. 1A and B show the methionine/homocysteine region only of the standard amino acid analysis. Although not included in these figures, the presence of homocysteine thiolactone was confirmed by the analysis and is shown in Fig. 3. The

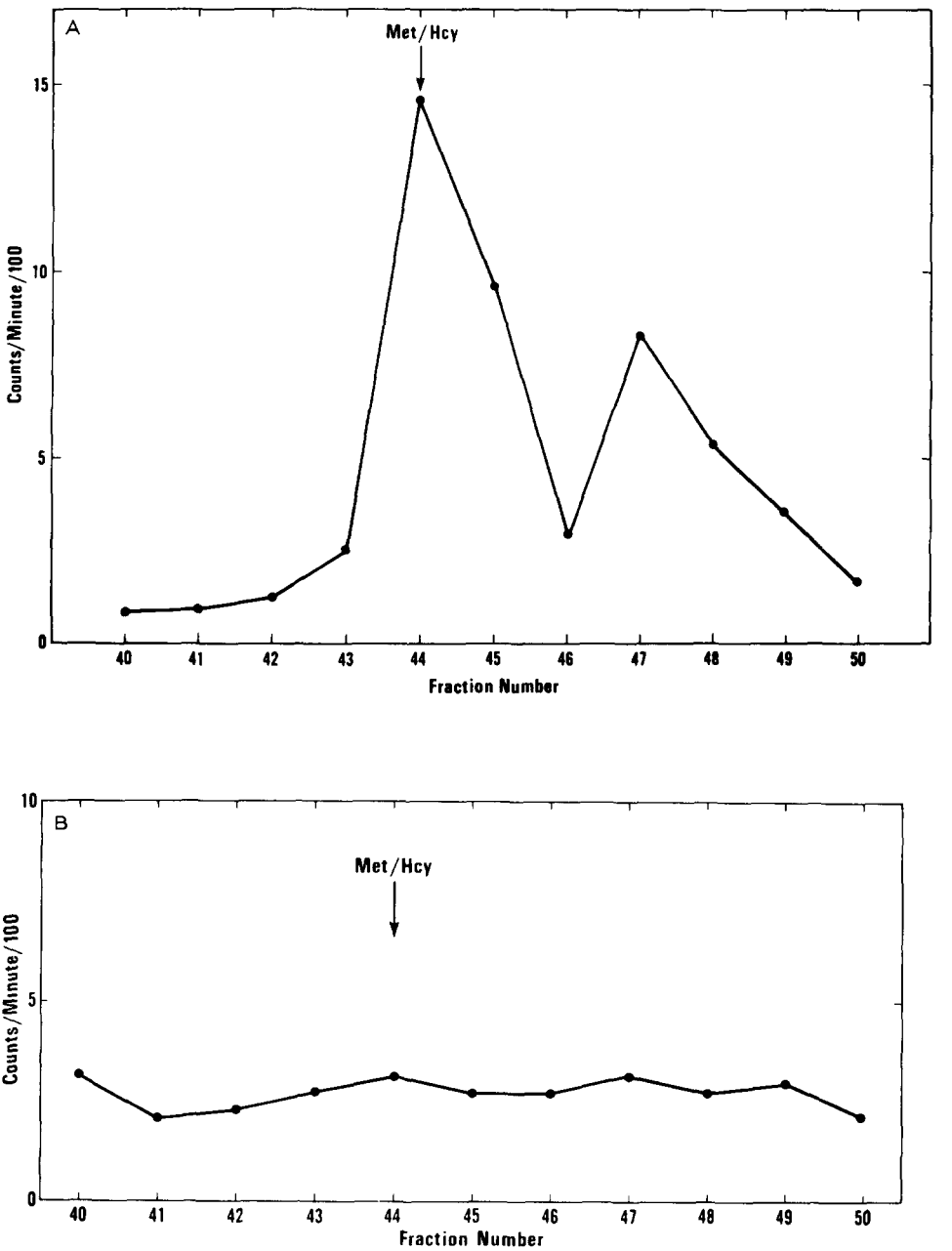

Fig. 1. A. Standard amino acid analysis of prepurified product. Fractions eluting between 40 and 50 min of a standard amino acid analysis were collected at 1 min intervals and radioactivity measured by scintillation counting. Arrow indicates the position of elution of methionine/homocysteine. See Materials and Methods for details. B. Standard amino acid analysis of alumina-column purified product. See Fig. 1A for details.

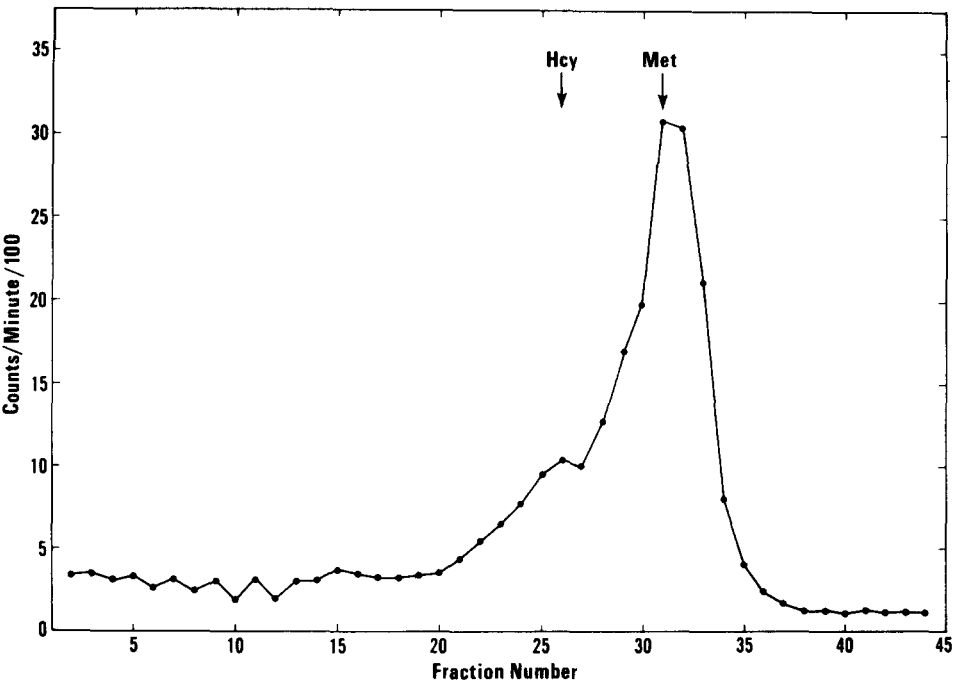

Fig. 2. Modified amino acid analysis of pre-purified product. Fractions eluting between 30 and 45 min from the amino acid analyzer run with 0.05 M sodium citrate pH 4.0 were collected at 18 s intervals. Radioactivity was measured by scintillation counting. Arrows indicate the position of methionine and homocysteine. Pre-purified product was from a different batch than the one analyzed in Fig. 1.

amino acid analyzer indicated significant amounts of methionine and/or homocysteine in the unpurified product eluting between 43 and 45 min (Fig. 1A), comprising 0.5% of the total activity. Although the percentage was low, the number of counts was too great to permit untreated product to be used in cell-labeling experiments requiring no methionine. Fig. 2 shows in a representative experiment the methionine/homocysteine region after amino acid analysis using the diluted buffer system. Homocysteine thiolactone was not analyzed under the conditions shown in Fig. 2. The majority of the pre-purification activity is associated with residual methionine. After the alumina column procedure, no peak remained in the region where methionine and homocysteine elute (Fig. 1B). Thin-layer chromatography was used as a second method to determine if the synthesized homocysteine was methionine-free. The reference methionine and homocysteine thiolactone appeared on strips 6 and 2, respectively, of the thin-layer chromatogram (Fig. 3). The counts showed a sharp peak on strip 2 with none on strip 6, indicating the absence of any [^{35}S]methionine. The presence of homocysteine thiolactone was also confirmed by standard amino acid analysis as indicated above (data not shown). The purified homocysteine thiolactone should be maintained under acidic conditions to minimize oxidation.

The specific activity of the product is identical to that of the starting material (1400 Ci/mmol). The yield of the alumina-column purified [^{35}S]homocysteine thiolactone is 5–10%, but provides a method for the complete separation of

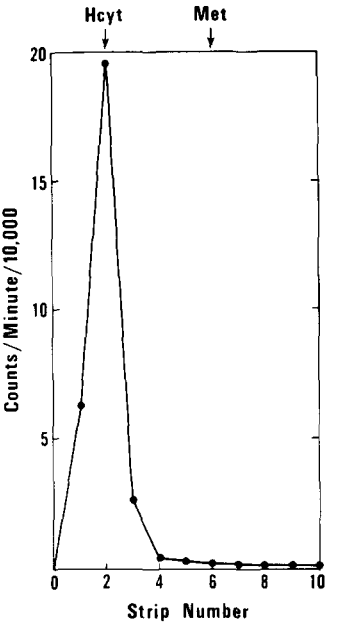

Fig. 3. Thin-layer chromatographic separation of purified product. Thin-layer chromatogram was eluted with a 1 : 1 : 1 : 1 mixture of isopentyl alcohol, dioxane, pyridine and water. Strips are numbered beginning at the solvent front. Homocysteine thiolactone and methionine are indicated by arrows. See Materials and Methods for details.

[^{35}S]homocysteine thiolactone from unreacted [^{35}S]methionine. The methionine is held strongly by the alumina through its carboxyl group, and is not eluted by methanol. Homocysteine thiolactone, lacking a carboxyl group, is eluted very rapidly. Any homocysteine is also retained by the column. The rapid and easy purification of the thiolactone, free of salts, makes a very convenient method for the preparation of methionine-free label for use in biological systems. This radioactive compound should be of important use as a tracer in studying the important reactions of homocysteine with no ambiguity due to contaminating methionine.

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