An Enzymatic Derivative Double Isotope Assay for L-Methionine*

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SUMMARY

A highly sensitive and specific enzymatic derivative method for the measurement of L-methionine is described. The procedure depends upon the addition of tracer quantities of L-[methyl-3H]methionine and its conversion to [H,14C]-S-adenosyl-L-methionine in the presence of [8-14C]ATP and of purified Escherichia coli ATP: L-methionine S-adenosyltransferase (EC 2.4.2.13). The S-adenosyl-L-methionine is separated from the radioactive reactants and its "C:HI ratio is a linear function of the quantity of L-methionine originally present. The method has been applied to the measurement of L-methionine in various tissues.

In the course of recent studies on the control of tissue levels and turnover of S-adenosyl-L-methionine (1) it became desirable to carry out measurements of the concentrations of free L-methionine in tissues. It has been previously reported by Schlenk (2) and Baldessarini (3) that the levels of L-methionine in animal tissues appear to exert an important controlling influence on the concentrations of S-adenosyl-L-methionine. It has also been shown that excess dietary L-methionine increases the activity of a number of enzymes, such as arginase, tryptophan pyrrolase, glutamate-oxalacetate aminotransferase, and ornithine &transaminase has been specifically studied in rat liver and kidney (5) and in Chang's liver cells (4).

It is also of interest that the feeding of DL-methionine to animals at concentrations above 2% causes numerous, profound effects, most of which are pathological, including depression of growth, atrophy of liver, decreased nitrogen retention, and pancreatic damage (reviewed in Reference 4).

Among the 12 amino acids required for the growth of mouse L strain fibroblasts, only L-methionine and L-tryptophan inhibited the multiplication of the L cells when added in excess of concentrations required for maximal growth (6). Excess L-methionine is also toxic to Chang's liver cells in suspension culture (4). Virtually all of the above observations have been made without direct measurement of L-methionine levels in the tissues in which the pathological or biochemical changes occur. Moreover, there appear to be no systematic studies on L-methionine levels in various animal tissues.

A comprehensive discussion of analytical procedures for the determination of methionine has been given by Greenstein and Winitz (7). A number of titrimetric and colorimetric methods are now largely of historical interest. More modern methods for measuring L-methionine are the following: (a) the nitroprusside colorimetric technique of McCarthy and Paille (8), which has been modified by Vasantha, Moorjani, and Sreenivasan (9); (b) microbiological assays which utilize bacterial production of lactic acid which may be titrated (10); (c) paper and thin layer chromatographic separation, or high voltage electrophoretic separation, followed by estimation with ninhydrin (11, 12); (d) gas chromatographic separation (13); or (e) automatic amino acid analyzer techniques which utilize ion exchange chromatography (14). Whereas the last four techniques possess sufficient sensitivity for tissue analyses, all are time-consuming, tedious, and unsuitable for large numbers of samples. Moreover, some of the manipulations may result in the oxidation of L-methionine to its sulfoxide and sulfoxide which may not be measured by certain of the analytical techniques.

A rapid, sensitive, and highly specific method for measuring L-methionine forms the subject of this paper. The principle of the method depends upon the addition of a tracer quantity of L-[methyl-3H]methionine (of high specific radioactivity) to the reaction mixture containing the sample to be analyzed, followed by enzymatic conversion of the L-methionine to S-adenosyl-L-methionine in the presence of [8-14C]ATP and a purified preparation of ATP: L-methionine S-adenosyltransferase (EC 2.4.2.13) which may be conveniently obtained from Escherichia coli. The reaction promoted by this enzyme, as first described by Cantoni and Durell (15, 16), is the following:

\[
L-[\text{methyl-}^3\text{H}]\text{Methionine} + [8-\text{14C}]\text{ATP} \xrightarrow{\text{Mg}^{2+}} [\text{H,14C}]\text{S-adenosyl-L-methionine} + \text{PP} + \text{P}
\]

The cationic product (S-adenosyl-L-methionine) which carries a positive charge by virtue of its sulfonium function may be easily separated from the radioactive reactants by retardation and elution from ion exchange resins, and its radioactivity may be determined. The ratio of the two isotopes remains constant unless...
there has been dilution with unlabeled L-methionine (or ATP). In order to measure L-methionine, therefore, special precautions are required to eliminate interference from ATP which is commonly present in tissue extracts at considerably higher levels than L-methionine. Under specified conditions, the ratio of the two isotopes in the S-adenosly-L-methionine is a direct and linear measure of the L-methionine concentration and may be obtained from appropriate standard curves in which known quantities of L-methionine have been carried through the reaction system. The theoretical justification for these derivations has been considered by Baldessarini and Kopin (17) in an analogous system for the measurement of S-adenosly-L-methionine. Apart from its simplicity and sensitivity, the method has the advantages that it does not depend upon quantitative recoveries or conversions, and once the endogenous L-methionine has been diluted with tracer L-[methyl-3H]methionine, partial oxidations or losses of the L-methionine are without influence on the final result. The specificity of the method depends upon the high degree of substrate specificity of the adenoslytransferase which has recently been systematically defined (18).

**EXPERIMENTAL PROCEDURE**

**Materials**—All solutions were prepared from reagent grade chemicals in deionized, glass-distilled water. L-Methionine, ammonium sulfate (special enzyme grade), and DL-norleucine were supplied by Mann. Standard solutions of L-methionine and DL-norleucine were prepared in 0.01 N HCl. Reduced glutathione was purchased from P-L Biochemicals. The amino acid analyzer was calibrated with a standard mixture of 20 amino acids obtained from Beckman-Spinco. 2-Mercaptoethanol was supplied by Eastman and distilled under reduced pressure (b.p. 58-60° at 23 mm Hg). Tris(hydroxymethyl)aminomethane base was obtained from Sigma. The primary and secondary scintillators, 2,5-diphenyloxazole and p-bis[2-(5-phenyloxazolyl)]benzene, used in making Bray's scintillation fluid (19) were purchased from Matheson, Coleman, and Bell. Tetraphosphothioate [8-3H]adenosine 5'-triphosphate (33.4 µCi per µmole in 50% ethanol), L-[methyl-14C]methionine (53.6 µCi per µmole), S-adenosyl-L-[methyl-14C]methionine (52.3 µCi per µmole), and L-[methyl-H]methionine (2.60 mCi per µmole) were purchased from Schwarz BioResearch and Amersham-Searle Corporation, Arlington Heights, Illinois. The commercial [8-14C]ATP was evaporated in a stream of N₂, redissolved in a small volume of water, and passed over a small Dowex 50W-X2 column in order to reduce the radioactivity of the controls (see below). The cation (Dowex 50) and anion (Dowex 1) exchange resins designated AG 50W-X2 (100 to 200 mesh) and AG 1-X2 (100 to 200 mesh), respectively, were supplied by Bio-Rad, Richmond, California. Sprague-Dawley male rats (120 to 160 g) were purchased from Spengis-Dawley, Madison, Wisconsin. E. coli strain B (midlogarithmic) cells were obtained packed from Grain Processing, Muscatine, Iowa. Glass beads (microspheres; 210 to 297 µm) were purchased from LaPine Scientific Company, Chicago, Illinois.

**Determination of Radioactivity**—All measurements of radioactivity were carried out in glass scintillation vials containing 15 ml of Bray’s solution and 2.0 ml of concentrated ammonium hydroxide. Discriminator settings were selected in such a way that the counting efficiency of 14C was 42% and that of 3H was 14%. The spillover of 14C into the 3H channel under these circumstances was 27 to 28%, whereas that of 3H into the 14C channel was 1 to 2%.

All measurements of radioactivity reported in this paper were obtained under these counting conditions. Sufficient counts were accumulated to reduce the error below ±5%.

**Calculations**—The spillover of the 14C counts into the tritium channel was determined by adding S-adenosyl-L-[methyl-14C]-methionine (20,000 cpm) in 10 ml of water to the Dowex 50W-X2 columns (following the same procedure as is used in the L-methionine assay described below), washing with water, eluting with 2 ml of concentrated ammonium hydroxide, and counting with 15 ml of Bray’s solution. The percentage of spillover after passage through the column (27%) is slightly lower than that found by the direct addition of 2 ml of ammonium hydroxide to a scintillation vial containing S-adenosyl-L-[methyl-14C]methionine (spill = 30%) owing to the extra water contained in the columns.

Corrections for both the spillover of the 14C into the tritium channel and for the 3H spillover into the 14C channel were made by solving the following simultaneous equations on a Wang 300 series calculator, model 360K.

\[
{\text{3H counts per min corrected}} = \text{3H counts per min} - \frac{\% \text{14C spill}}{100} \times \left(\frac{14C \text{ counts per min} - \% \text{3H spill}}{100} \times \text{3H counts per min corrected}\right) \\
\times \frac{\% \text{3H spill}}{100} \times \text{14C counts per min corrected}
\]
most of the enzymatic activity was present in the fraction precipitating between 30 and 50% of saturation. The ammonium sulfate precipitate (30 to 50% saturation) was dissolved in 50 mM Tris-HCl, pH 7.0, containing 5 mM 2-mercaptoethanol. The enzyme solution was then heated for 6 min at 80°C, and the denatured protein precipitate was removed by centrifugation and discarded. The supernatant fluid was again fractionated with ammonium sulfate (20 to 50% saturation), dialyzed overnight against 50 mM Tris-HCl buffer containing 5 mM 2-mercaptoethanol and 20% glycerol, and stored in small aliquots at -15°C. The purification was approximately 40-fold with a recovery of 30% of the initial activity. The enzyme could be stored at -15°C for at least 3 months without loss in activity. The final specific activity obtained was 3.5 units per mg of protein when calculated in the first column were added directly to the assay system. None of the measurements of radioactivity are corrected for back-

**Table I**

| L-Methionine | Carbon channel | Tritium channel | Ratio 14C:3H |  
|--------------|----------------|-----------------|-------------|
| nmoles       | 14C            | Control^a       | 14C corrected for 3H spill (21.2%) | 14C corrected for 3H spill (21.2%) |
|--------------|----------------|-----------------|-------------|
| 0.10         | 6,053          | 980             | 5,073       | 4,500 |
| 0.25         | 11,377         | 980             | 10,397      | 9,777 |
| 0.50         | 19,070         | 980             | 18,000      | 17,446 |
| 1.00         | 38,537         | 980             | 37,557      | 36,878 |
| 2.50         | 77,596         | 980             | 76,616      | 76,008 |
| 5.00         | 136,952        | 980             | 135,952     | 133,363 |
| 10.00        | 238,192        | 980             | 237,212     | 236,727 |

^a No added (labeled or unlabeled) L-methionine.

**Preparation of Tissues for Assay of L-Methionine**—The animals were killed by cervical fracture and the tissues were immediately excised and washed in cold 0.9% NaCl to remove extraneous blood. The tissues were then frozen in liquid nitrogen, and thereafter all operations were carried out in the cold. The tissues were weighed in the frozen state and homogenized with 2 volumes of cold 2% perchloric acid in an ice bath driven Teflon-glass homogenizers. The homogenates were centrifuged for 15 min at 10,000 × g, and the supernatant fluids were neutralized with solid potassium bicarbonate and recentrifuged as above. Small anion exchange columns (Dowex AG 1-2X2, 2.5 × 30 mm, 100 to 200 mesh; in the chloride form) were utilized to remove endogenous ATP from the tissue supernatants in order to avoid dilution of the specific activity of the exogenous [8-14C]ATP added to the incubation mixture. After preparation of the columns with Dowex resin suspended in water, they were partially dried with a stream of nitrogen to remove trapped water. The above described neutralized supernatant (0.3 ml) was applied to the columns and eluted with water (0.3 ml), and again the trapped solu-

**Assay for L-Methionine**—The conversion of L-methionine to S-adenosyl-L-methionine was carried out in systems originally designed for measurement of adenosyltransferase activity by Mudd et al. (21) and modified by Lombardini et al. (18). The reaction mixture contained the following components (in micromoles) in a final volume of 0.35 ml: glutathione, 2; KCl, 50; Tris-HCl, pH 7.6, 80; MgCl₂, 75; [8-14C]ATP, 0.012 (containing 600,000 cpm); L-[methyl-3H]methionine, about 0.1 n mole (containing 80,000 cpm); neutralized tissue aliquot or solution to be analyzed (normally in 0.1 ml); and purified E. coli adenosyltransferase preparation, 0.4 unit. Incubations were carried out at 37°C with agitation for 2 hours. The reaction was terminated by diluting with 10 ml of cold water and applying the total mixture to Dowex AG 50X8-X2 columns (6 × 22 mm, 100 to 200 mesh) in the ammonium form. A minimum of 200 ml of water was used to wash unreacted L-[methyl-3H]methionine and [8-14C]ATP through the columns. The S-adenosyl-L-methionine (containing 3H and 14C) was eluted from the columns with 2 ml of concentrated ammonium hydroxide, and counted for 3H and 14C in a liquid scintillation spectrometer with 15 ml of Bray's scintillation fluid. Two controls in which either the radioactive L-methionine or the ATP was omitted were also included. A series of standard L-methionine concentrations was also run with each set of determinations to provide a standard curve.

**Amino Acid Analyzer**—The long column of the Beckman 120 C amino acid analyzer was used with the standard buffers to
Estimation of L-Methionine

Vol. 246, No. 14

Fig. 1. Standard curve for the assay of pure L-methionine. Aliquots (0.1 to 10.0 nmole) of unlabeled L-methionine were added to the assay system which contained a constant quantity of L-[methyl-3H]methionine and [8-14C]ATP. The change in the 14C: H ratio of the S-adenosyl-L-methionine formed is proportional to the dilution of the radioactive L-methionine with unlabeled L-methionine. Details of the assay are discussed under "Experimental Procedure." Typical measurements of radioactivity are recorded in Table I. The determinations for low levels of L-methionine are expanded in the inset. The graph does not pass through the origin because no correction for the quantity of radioactive tracer was made.

determine the L-methionine content of the tissue homogenates. A calibrated amino acid mixture and L-norleucine were used to calibrate the instrument.

RESULTS AND DISCUSSION

Standard Curve—A linear relationship between the 14C: H ratio of the S-adenosyl-L-methionine eluted from the columns and the L-methionine concentration is consistently obtained, as shown in Fig. 1, which covers the range of 0.1 to 10 nmole of pure L-methionine. Some of the actual measurements of radioactivity are given in Table I in order to illustrate the accuracy of the method, to give closer insight into the degree of enzymatic conversion obtained, and to show the magnitudes of the experimental values. It should be noted that under these conditions about 75 to 80% of the total amount of L-methionine present in the system is converted to S-adenosyl-L-methionine at low concentrations (0.1 to 1.0 nmole) and that this percentage decreases somewhat at higher concentrations of L-methionine. In order to maintain the highest sensitivity it is desirable that about 75 to 80% of the total amount of L-methionine present in the system is converted to S-adenosyl-L-methionine at low concentrations (0.1 to 1.0 nmole) and that this percentage decreases somewhat at higher concentrations of L-methionine. In order to maintain the highest sensitivity it is desirable that the fraction of L-methionine converted to S-adenosyl-L-methionine be relatively high, although the degree of conversion does not influence the accuracy of the method, other than by virtue of its influence on the accuracy of counting.

With each set of determinations, two controls are incubated containing all reactants except that L-methionine (labeled and unlabeled) is omitted from one and [8-14C]ATP from the other.

Table II

| L-Methionine added | Corrected radioactivity | Amount of L-methionine present | Recovery of added L-methionine |
|---------------------|-------------------------|-------------------------------|-------------------------------|
|                     | 14C | 3H | 14C:3H | nmoles | %       |
| None . . . . . . . . |    | 3H | 1.14   | 1.14   | 102     |
| 1.64 nmoles . . . . | 31,300  | 31,100  | 1.11 | 1.14 | 102 |

Table III

Quantitative removal of ATP and recovery of L-methionine from rat tissues after chromatography on Dowex 1 chloride

A liver homogenate was prepared in perchloric acid, neutralized, and centrifuged as described under "Experimental Procedure." To an aliquot of the processed tissue supernatant (0.3 ml) either [8-14C]ATP (21,700 cpm) or L-[methyl-14C]methionine (58,500 cpm) was added and then the solution was passed through Dowex AG 1-X2 (100 to 200 mesh) columns (2.5 x 30 mm) in the chloride form. The Dowex anion exchange columns were then washed with 0.3 ml of water to elute the L-[methyl-14C]methionine quantitatively or to check the retention of the [8-14C] ATP on the Dowex resin. Aliquots of the wash were then counted for radioactivity.

Table:<br>Radioactive component<br>Before chromatography | After chromatography | Recovery<br>[8-14C]ATP | 21,700 | 18 | 0.3<br>L-[methyl-14C]Methionine | 58,500 | 57,800 | 99<br>* Corrected for background.
be used longer than about 2 months since it undergoes extensive radiochemical decomposition.

The range of quantities of L-methionine that can be measured in the specified system can be extended up to at least 100 nmol without significant deviation from linearity (results not shown). The sensitivity of the method permits measurement of 0.1 nmol of L-methionine or even less.

Since the standard curve shown in Fig. 1 is based only on the amount of exogenous L-[methyl-14C]methionine which is added to the reaction system and not the L-[methyl-3H]methionine (about 0.1 nmol) which is also present, the graphical representation of the data as expected does not pass precisely through the origin.

Recovery of L-Methionine Added to Tissue—In order to evaluate the reliability of recovery of L-methionine added to a tissue extract, a series of experiments was conducted, of which Table II gives a representative example. Recoveries were studied when known quantities of L-methionine were added to a centrifuged, neutralized brain homogenate and assayed for L-methionine. When 1.64 nmol of L-methionine were added to an aliquot of a brain tissue homogenate containing 1.14 nmol of L-methionine, the total recovery of L-methionine was 102% of theory (Table II). Recoveries of added L-methionine have been carried out with each set of tissue analyses, and have ranged from 90 to 105%.

Removal of Endogenous ATP—Although the measurement of L-methionine in many solutions presents no problem, the enzymatic isotopic procedure requires that the endogenous ATP present in tissues be quantitatively removed so as not to lower the specific activity of the added radioactive ATP. This removal is accomplished by passing the neutralized, centrifuged tissue samples through a Dowex 1 column before subjecting them to the enzymatic conversion of L-methionine to S-adenosyl-L-methionine. Table III shows the results of the passage of an aliquot of liver homogenate (prepared as described under “Experiments” in Table II) through Dowex 1 column before subjecting them to the enzymatic conversion of L-methionine to S-adenosyl-L-methionine. The sensitivity of the method permits measurement of 0.1 nmole; without significant deviation from linearity (results not shown).

Advantages of Method—There are two advantages of the new enzymatic-isotope method of analysis of L-methionine. First, the method is extremely sensitive, estimating with ease 0.1 nmol, and second, the simplicity permits analysis of a large number (approximately 40) of tissue samples by one person in a single day. If necessary, the sensitivity can be easily extended to 10 nmol of L-methionine, or perhaps even lower. All the reagents are commercially available except for the E. coli transferase which can be purified in about 2 working days.

It should be considered that, if analyses are carried out on tissue extracts both before and after passage through Dowex 1 column for removal of ATP, it should be possible to measure both ATP and L-methionine levels in the same samples.

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