SYNTHESIS OF [2-^{3}H] CREATINE

Marie M. Daly and I. Lalezari

Departments of Biochemistry and Medicine

Albert Einstein College of Medicine, Yeshiva University

1300 Morris Park Avenue, Bronx, New York 10461

ABSTRACT

A method for preparing [2-^{3}H] creatine with high specific activity is described. [2-^{3}H] Sarcosine is prepared from [2-^{3}H] glycine and purified by ion-exchange chromatography. [2-^{3}H] Creatine is then synthesized by reaction of the labeled sarcosine with cyanamide, and isolated by ion-exchange chromatography. Radiochemical purity of the labeled creatine was verified by isotope dilution analysis and by thin-layer chromatography.

Key words: creatine, sarcosine, glycine, specific tritium labeling

Several types of cells have been shown to take up creatine from the extracellular medium by saturable processes with values for apparent Km in the range of 0.02 - 0.04 mM (1,2). In studies conducted thus far, it has not been possible to distinguish unequivocally events required for transport of creatine into the cell from those involved in its metabolism. Characterization of the process of transport requires accurate measurements of initial rates of uptake of creatine by cells or plasma membrane vesicles prepared from them. In turn, such measurements require labeled creatine of high specific activity. The radioactive creatine that is available commercially, however, is labeled with ^{14}C and has specific activities of 23 mCi/mmol or less. We report here a convenient procedure for synthesis of [2-^{3}H] creatine with specific activity 309 mCi/mmol. The method is based on published procedures (3) that have been adapted for use with a few mg of starting materials, and further modified by purification of intermediate and final products by ion-exchange chromatography.

0362-4803/83/030377-07$01.00
© 1983 by John Wiley & Sons, Ltd.

Received August 31, 1982
Revised October 22, 1982

MATERIALS AND METHODS

[2-^{3}H] creatine is synthesized by the series of reactions summarized in figure 1.

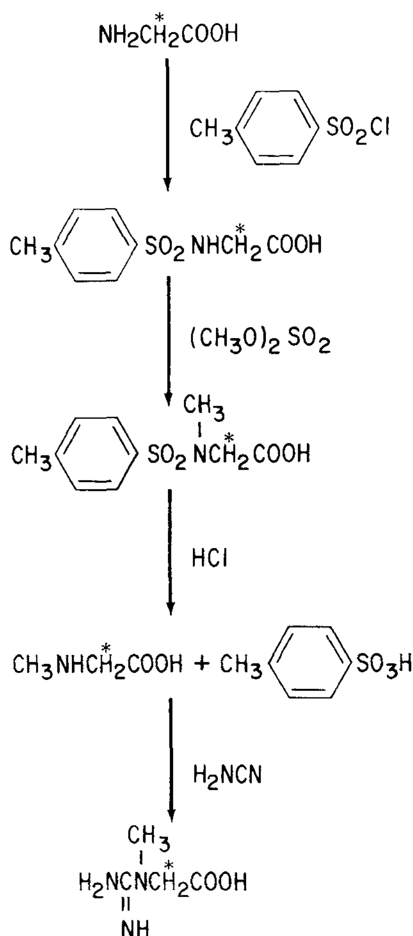

Figure 1. Sequence of reactions used for synthesis of [2-^{3}H] creatine.

* Indicates site of labeled atoms of ^{3}H.

Preparation of [2-^{3}H] sarcosine: [2-^{3}H] glycine (15 Ci/mmol) was obtained from the New England Nuclear Corp. as a solution of 25 mCi in 25 ml of 0.1 N hydrochloric acid. Unlabeled glycine (67 µmol) was added to this solution, which was then transferred, in portions, to a 5 ml ampule (Vacule Gold Band, Wheaton Industries) and evaporated to dryness in a stream of nitrogen. Thirteen mg p-toluenesulfonyl chloride, 0.6 ml 1 N NaOH, and 10 drops of p-dioxane were added to the residue, and the mixture was heated at 50°C for 2 h. After removal of dioxane by evaporation, 0.4 ml 1 N NaOH and 1 drop of dimethyl sulfate were added, and the reactants allowed to stand at room temperature for 15 min. The mixture was evaporated to dryness, and 2 ml of concentrated hydrochloric acid were added. The ampule was sealed and placed in an oven at 100° for 20 h. The ampule was then cooled in ice and opened. Supernatant fluid was separated from the residue of salt by means of a Pasteur pipet, and the residue was washed with a few drops of concentrated hydrochloric acid. Supernatant and washings were combined and evaporated to dryness. The residue was dissolved in 1 ml water, the pH adjusted to approximately 3, and the sample placed on 1 x 50 cm column of Dowex-50W-X4 (Na^{+} form, 200-400 mesh,) to separate sarcosine from unreacted glycine (4). The column was eluted with 0.1 M sodium citrate buffer, pH 3.42, at room temperature, and labeled compounds located by counting aliquots of the eluate. Fractions corresponding to sarcosine were pooled and desalted by passage through an 0.5 x 7 cm column of Dowex-50W-X4 (H^{+} form, 200-400 mesh). The column was washed with 5 ml water, and sarcosine was eluted with 5 ml 1 N ammonium hydroxide. The yield of sarcosine was 13 µmol.

Preparation of [2-^{3}H]-creatine: The solution of sarcosine was evaporated almost to dryness in a small conical tube. Two drops of concentrated ammonium hydroxide and 2 drops of cyanamide (50% solution in water, Aldrich Chemical Co.) were added. The mixture was allowed to stand at room temperature for 1 h. It was then diluted with 0.5 ml of water, and the pH adjusted to approximately 6. Creatine was isolated by chromatography on Amberlite CG-50 (100-200 mesh) (5) that had been washed with 5% (v/v) acetic acid, followed by deionized water,

before use. Fine particles were removed by decantation and discarded. The sample was applied to an 0.7 x 30 cm column of Amberlite and eluted with water. Two ml fractions were collected; aliquots of the fractions were mixed with Hydrofluor (National Diagnostics) and the radioactivity was measured, with an efficiency of 36%, in a liquid scintillation spectrometer (Searle Analytic, Inc., Mark III). Fractions were analyzed for creatine by enzymatic (6) and colorimetric (7) methods. Reagents in the Sigma Diagnostic Kit No. 520 were used for colorimetric analysis.

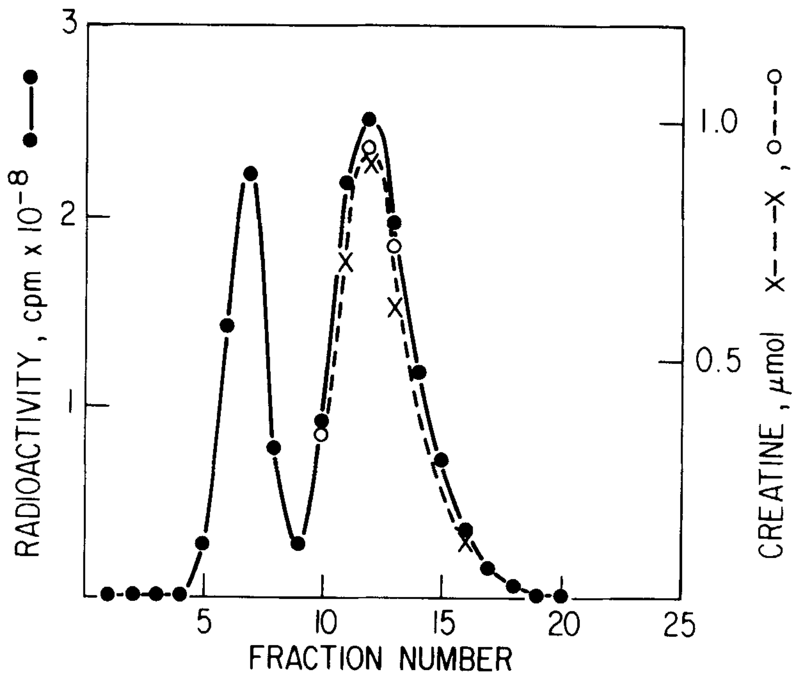

Figure 2. The pattern of elution of radioactivity (o——o) and creatine, determined enzymatically (o-----o) or colorimetrically (x-----x), is shown.

Preliminary experiments established that unreacted sarcosine, cyanamide, and its decomposition products were separated from creatine on this column.

Salt that had been present in the final reaction mixture was eluted from the column before creatine. A residue of salt was readily visible upon evaporation of aliquots of fractions preceding the creatine peak. The absence of salt in the effluent containing creatine was confirmed by analysis for Na^+ by flame photometry (IL Flame Photometer Model 143).

For isotope dilution analysis, an authentic sample of unlabeled creatine monohydrate (Aldrich Chemical Co.) was recrystallized from water made alkaline with ammonia. Fifty mg samples of this material were mixed with known quantities of radioactivity from various fractions of the peak containing creatine and dissolved in 2 ml warm water containing ammonia. Ethanol was added dropwise to induce crystallization. Samples were refrigerated overnight; crystals were collected and allowed to dry to constant weight at room temperature. The specific activity of the creatine was determined, and crystallization repeated. The specific activity of the creatine was constant after the first crystallization.

For thin-layer chromatography, 1 or 2 µl samples of fractions from the Amberlite column were applied to layers of silica gel G, 0.25 mm thick. The mobility of the radioactive material was compared with that of creatine standards in three solvent systems, n-butanol:formic acid:water (63:20:17), isopropanol:ammonium hydroxide:water (100:5:10), and n-butanol:pyridine:water (1:1:1).

RESULTS AND DISCUSSION

The pattern of elution of radioactivity during purification of creatine on Amberlite CG50 is shown in figure 2. The first radioactive peak contains unreacted sarcosine while the second contains creatine. The specific activity of creatine was constant in fractions 10-16. Colorimetric analysis of three samples taken from the creatine peak gave specific activities of $2.44 \pm .22 \times 10^8$ cpm/µmol while enzymatic analysis of six samples gave $2.49 \pm .36 \times 10^8$ cpm/µmol. From the average of all values, ($2.47 \pm 0.29 \times 10^8$ cpm/µmol), and the efficiency of our liquid scintillation counting system, we calculated that the specific activity of the creatine was 309 mCi/mmol. Isotope dilution analysis of fractions

10,12,14 and 15 (figure 2) showed that the radiochemical purity of the creatine was 104 ± 1.4%. Samples of fractions 10,12,14 and 15 were subjected to thin-layer chromatography in three different solvent systems. Radioactive scanning of the chromatograms showed that 99 ± 1% of the radioactivity coincided with creatine standards.

The procedure yields 7 µmol of labeled creatine, 10% of the theoretical yield. This yield is not very different from that obtained in syntheses conducted with unlabeled compounds on a much larger scale (3). In our procedure, labeled glycine can be recovered during the purification of labeled sarcosine by chromatography on Dowex-50W-X4 (Na^+) and used for the preparation of additional labeled creatine. In one experiment, for example, the fractions corresponding to glycine (24 µmol) were pooled, desalted by passage through an 0.7 x 15 cm column of Dowex-50W-X4 (H^+) and recycled through the entire procedure yielding an additional 2 µmol of creatine.

The radiochemical purity of the creatine is close to 100%, and its specific activity is considerably higher than that of the labeled creatine available commercially. Since there was little change in yield when the synthesis was conducted with 24 µmol of glycine instead of 67 µmol, it is possible that the procedure can be adapted for preparation of creatine of even higher specific activity by using a smaller quantity of unlabeled glycine as carrier. This preparation should be useful not only for studies of creatine transport, but also for investigations of other biological interactions with high affinity for creatine.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Research Grants AM 26601 and 2 P01 AG 00374.

We are grateful to Miss Tina Marie Chieco, who provided technical assistance, and to Mr. Demetrios Kambosos, who performed the flame photometry.

REFERENCES

1. Ku, C-P. and Passow, H. Biochim. Biophys. Acta 600: 212 (1979).

2. Daly, M.M. and Seifter, S. Arch. Biochem. Biophys. 203: 317 (1980).

3. McChesney, E.W. and Swann, W.K. Jr., J. Am. Chem. Soc. 59: 1116 (1937).
   Fischer, E. and Bergmann, M. Ann. 398: 96 (1913).
   Rosengarten, F. and Strecker, A. Ann. 157: 1 (1871).

4. Moore, S. and Stein, W.H. J. Biol. Chem. 192: 663 (1951).

5. Berlet, H.H. Z. Anal. Chem. 252: 159 (1970).

6. Graber, W., Mollerung, H. and Bergmeyer, H.V. in Methods of Enzymatic Analysis (Bergmeyer, H.V. ed.) 2nd ed. p. 1772, Academic Press, New York (1974).

7. Hughes, B.P. Clin. Chimica Acta 7: 597 (1962).