SYNTHESIS OF CARBOXY-LABELLED ℓ-CARNITINE

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SUMMARY

A method for the production of carboxy-labelled ℓ-carnitine is described. The first step is the chemical synthesis of 4-N-trimethylammoniobutanoate (butyrobetaine) from the precursors 4-aminobutanoate and iodomethane. The second step involves the hydroxylation of butyrobetaine to form ℓ-carnitine using butyrobetaine hydroxylase partially purified from bovine calf liver. The method also can be used to synthesize Me-labelled and uniformly-chain-labelled ℓ-carnitine.

Key words: ℓ-carnitine, 4-N-trimethylammoniobutanoate, butyrobetaine hydroxylase

INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylammoniobutanoate) radioactively labelled in various positions has been used in the study of fatty acid oxidation as well as in the study of the biosynthesis and metabolism of carnitine. Commercial sources supply radioactive carnitine as a racemic mixture only; we have found that certain lots of d,ℓ-[carboxy-^{14}C]carnitine contain a radioactive contaminant possessing approximately 5% of the total radioactivity. Furthermore, d-carnitine is not entirely inert and interferes with certain experiments. Consequently, a method for the production of ℓ-[carboxy-^{14}C]carnitine was sought.

Techniques for the resolution of d,ℓ-carnitine (1,2) or its unmethylated precursor, d,ℓ-3-hydroxy-4-aminobutanoate (3,4) are available. However, the low recoveries of starting materials when using these methods prohibit their use in radiochemical synthesis.

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Butyrobetaine (4-N-trimethylammoniobutanoate) has been shown to be converted into ℓ-carnitine in vivo, and radioactive analogs have been used to obtain optically pure radioactive ℓ-carnitine (5,6). Lindstedt studied the in vitro conversion of butyrobetaine into ℓ-carnitine using a partially purified rat liver enzyme, butyrobetaine hydroxylase (7). The enzyme has a low specific activity, and the small total activity extractable from each rat liver would necessitate the use of large numbers of animals to obtain enough enzyme to produce labelled ℓ-carnitine in useful quantities. Therefore, a more economical source of the enzyme was calf liver.

This paper describes a general method for the production of labelled ℓ-carnitine. Using chemically synthesized [carboxy-^{14}C] butyrobetaine, we have studied the conversion into ℓ-carnitine by butyrobetaine hydroxylase as a method to obtain ℓ-[carboxy-^{14}C] carnitine. Although developed to prepare ℓ-[carboxy-^{14}C] carnitine, it has been used effectively to produce ℓ-[Me-^{14}C] carnitine ℓ-[Me-^{3}H] carnitine and ℓ- uniformly-chain-labelled-^{14}C carnitine (ℓ-3-hydroxy-4-N-trimethylammonio-[U-^{14}C]butanoate).

MATERIALS

4-Aminobutanoate was obtained from Eastman Organic Chemicals Company, Rochester, New York, U.S.A.; 4-amino-[carboxy-^{14}C] butanoate (21.5 mCi/mmol) from Schwartz/Mann, Orangeburg, New York, U.S.A.; 4-amino- [U-^{14}C] butanoate (232 mCi/mmol) from the Radiochemical Centre, Amersham, England; iodomethane and scintillation grade toluene from Fisher Scientific Company, Cleveland, Ohio, U.S.A.; ^{14}C iodomethane (50 mCi/mmol) and ^{3}H iodomethane (1.9 Ci/mmol) from ICN, Cleveland, Ohio, U.S.A.; Triton X-100, 2,5-diphenyloxazole (scintillation grade), and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (scintillation grade) from Packard Instrument Company, Inc., Downers Grove, Illinois, U.S.A.; type IV porcine heart isocitric dehydrogenase from Sigma Chemical Company, St. Louis, Missouri, U.S.A.; lyopholized beef liver catalase from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.; and NADPH from P-L Biochemicals, Milwaukee, Wisconsin, U.S.A. The resins AG1X8, 200-400 mesh (Cl^- form); AG 50X8, 200-400 mesh (H^+ form) and AG 11A8, 50-100 mesh were obtained from Bio-Rad Laboratories, Inc., Richmond, California, U.S.A. The AG1X8 (Cl^- form) was converted to its OH^- form as suggested by the manufacturer. All other chemicals used were of reagent grade.

METHODS

The detection of quaternary amines was accomplished by either forming the Reineckate salt using a 2% aqueous solution of ammonium Reineckate or by spraying a spotted filter paper with iodoplatinate solution (8,9). A 0.5% solution of ninhydrin in butanol was used to detect the presence of primary amines. Specific radioactivities of butyrobetaine were determined by the periodide assay (10); butyrobetaine was standardized by converting to carnitine and carnitine was enzymatically determined by the method of Marquis and Fritz (11). Radioactivity measurements were done in a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Company, Inc., LaGrange, Illinois). Aliquots of 0.05 ml were diluted to 1 ml with H_2O and then suspended in 10 ml of the following mixture: 31.35 g 2,5-diphenyloxazole; 0.95 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene; 3.8 liters toluene; and 1.9 liters Triton. The efficiencies for counting ^{14}C and ^{3}H were generally 92% and 43% respectively.

Butyrobetaine Hydroxylase: Calf liver was obtained from a local slaughter-house shortly after the death of the animal, sliced and placed in ice. The remaining steps were performed at 4°C. The liver was diced, minced and taken to 33% wet weight per volume with 220 mM mannitol, 70 mM sucrose and 5 mM 4-morpholinopropane sulfonate, pH 7.4 (MSM). After homogenization at low speed for ten seconds in a Waring blender the dense particles were removed by a 20 min centrifugation at 400xg. Mitochondria were sedimented at 25,000xg for 20 min. The supernatant was centrifuged at 100,000xg for 1 hr sedimenting the microsomes. The microsomes were resuspended in MSM and stored at -80°C. The 100,000xg 60 min supernatant represented the crude enzyme extract. Further purification involved an ammonium sulfate fractionation similar to that of Lindstedt (7) in which the 40-70% saturation fraction contained 68% of the enzymatic activity (Table 1). Further purification of the enzyme was not essential. As shown in Table 1 the specific activity of butyrobetaine hydroxylase in beef liver cytosol was much lower than either calf or rat liver and fractionation with ammonium sulfate does not produce a purification. Calf and rat liver activities were about the same in specific activity and degree of purification. Calf liver then served as a more economical source of butyrobetaine hydroxylase. The activity of the enzyme was assayed by incubation with butyrobetaine and the necessary co-factors according to the procedure of Lindstedt (7).

Table 1

PURIFICATION OF BUTYROBETAINE HYDROXYLASE

	100,000xg Supernatant	Ammonium Sulfate Fractions 0-40%	40-70%	70%
RAT LIVER[1]				
Protein[2]	6,100		2,400(39%)	
Specific Activity[3]	0.12		0.28	
Total Activity[4]	732		672(92%)	
BEEF LIVER				
Protein[2]	17,700	1,433	12,550(71%)	5,348
Specific Activity[3]	0.008	0.002	0.0077	0.0079
Total Activity[4]	141.6	2.9(1%)	96.6(68%)	42.1(30%)
CALF LIVER				
Protein[2]	40,800	1,700	15,923(39%)	20,792
Specific Activity[3]	0.143	0.0035	0.272	0.080
Total Activity[4]	5,734	5.95	4,331(68%)	1,663(30%)

[1] From Lindstedt (7) based on 25 rat livers.
[2] mg biuret protein
[3] mU/mg protein (nmol/min/mg)
[4] mU (nmol/min)

Synthetic: Unlabelled 4-butyrobetaine was prepared according to the method of Lindstedt and Lindstedt (12) with modification (8). The resulting white needle-like crystals had a melting point of 211-213°C with decomposition (Ref. 12; m.p. 209-211°C dec.). The tetrachloroaurate melted at 183-185°C with decomposition (Ref. 12; m.p. 183-184°C dec.). and the hexachloroplatinate melted with decomposition at 213°C.

4-[carboxy-^{14}C]butyrobetaine was prepared as follows: 1 mCi 4-amino[carboxy-^{14}C] butanoate, 4-aminobutanoate (200 μmoles), $Ba(OH)_2$ (900 μmoles) in 80% methanol (v/v) and a tenfold excess of iodomethane were stirred in a tightly stoppered reaction flask at room temperature for 20 hours. The mixture was neutralized and chromatographed on a 1.5 cm x 20 cm column of AG1X8, 200-400 mesh (OH^- form) and eluted with water. Radioactive fractions were pooled, neutralized and evaporated in vacuo. The residue was suspended in H_2O and chromatographed on a 1 cm x 50 cm column of AG 50X8, 200-400 mesh (H^+ form) with 1.5 N HCl. The radioactive fractions were pooled and evaporated in vacuo. The residue was dissolved in water and specific radioactivity was 6.2 μCi/μmol. The yield was 90% based on recovery of 4-amino[carboxy-^{14}C]butanoate.

Uniformly-chain-labelled butyrobetaine (4-N-trimethylammonio-[U-^{14}C] butanoate; specific radioactivity = 232.2 μCi/mol) was synthesized from 4-amino-[U-^{14}C]butanoate without adding unlabelled 4-aminobutanoate.

4-[Me-^{14}C]butyrobetaine and 4-[Me-^{3}H]butyrobetaine were prepared by using 1mCi [^{14}C] iodomethane or 25 mCi [^{3}H] iodomethane during the first 20 hours. At the end of this period a tenfold excess of unlabelled iodomethane was added and the reaction continued for an additional 20 hours. The yields varied from 50-70% based on recovery from the radioactive iodomethane.

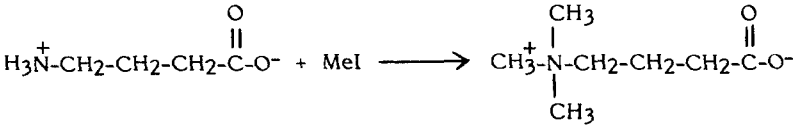
The radiochemical purity of the labelled compounds was assessed by thin-layer chromatography using three systems: A. Cellulose plate, (Eastman 6064) with methanol/NH_4OH (28%)/H_2O (93/2/5, by vol.), R_f = 0.50; B. Cellulose plate, (Eastman 6064), with isopropanol/methyl-ethyl ketone/0.1 N HCl (5/3/2 by vol.), R_f = 0.35; C. Silica plate, (Eastman 6061), with methanol/ acetone/ concentrated HCl (5/45/2 by vol.), R_f = 0.20. Each of the labelled compounds (20-35,000 dpm) was chromatographed; the only detectable area of radioactivity migrated with the appropriate carrier. Recoveries were quantitative. Therefore, the radioactive compounds contain less than 0.05% radioactive impurities.

RESULTS AND DISCUSSION

The general scheme used to prepare radioactive ℓ-carnitine involves two steps:

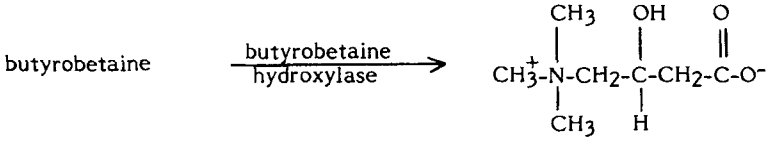
Scheme 1

Step 1.



4-aminobutanoate iodomethane 4-N-trimethylammoniobutanoate (butyrobetaine)

Step 2.



ℓ-carnitine

The first step is the chemical synthesis of butyrobetaine from 4-aminobutanoate. The method used results in high yields of butyrobetaine. The next step takes advantage of the product stereospecificity of the enzyme butyrobetaine hydroxylase to produce ℓ-carnitine. The assay medium for butyrobetaine hydroxylase produces less than a μmole of ℓ-carnitine. To effect large scale production of carnitine the following system was utilized: The reaction mixture contained in a volume of 50 ml: 1000 μmoles Tris-HCl, pH 7.8, 1000 μmoles KCl, 2300 μmoles nicotinamide, 65 μmoles $MgCl_2$, 200 μmoles sodium (+)-isocitrate, 1 mg isocitrate dehydrogenase, 40 mg catalase, 2 μmoles NADPH, 500 μmoles sodium ascorbate, 10 μmoles ferrous sulfate, 50-60 μmoles labelled butyrobetaine, and 1.2-1.5 g partially purified butyrobetaine hydroxylase. These amounts represent optimal concentrations for maximal conversion; microsomes were found not to be necessary and thus were not added to the incubation. The incubations were performed in an unstoppered 250 ml Erlenmeyer flask with shaking in a water bath at 37°C. The reaction was terminated after three h with the addition of 5 ml 60% perchloric acid. After one h on ice, this mixture was centrifuged, the supernatant decanted and neutralized with KOH. Following one h at 0-4°C the mixture was centrifuged to sediment $KClO_4$. The supernatant was then processed by ion exclusion chromatography on a 2 cm x 20 cm column of Ag 1X8, 200-400 mesh (OH^- form) and eluted with water. The fractions containing radioactivity were pooled, neutralized and evaporated in vacuo and this residue dissolved in water and passed through a 1 cm x 60 cm column of the ion retardation resin AG 11A8, 50-100 mesh to desalt the carnitine and butyrobetaine. The radioactive fractions were pooled and evaporated in vacuo. The residue was dissolved in water and chromatographed on a 1 cm x 50 cm column of AG 50X8, 200-400 mesh (H^+ form) and eluted with 1.5 N HCl. Five ml fractions were collected. The tubes containing carnitine were pooled, evaporated in vacuo and dissolved in water. Between 60-70% of the radioactive butyrobetaine was converted into carnitine in this system. The tubes containing butyrobetaine were combined and could be recycled. Therefore, with this recycling it was possible to achieve almost 100% conversion.

The synthesized carnitine was radiochemically pure (as defined above) in the three thin layer systems by co-chromatography with commercial carnitine. The specific activity of the carnitine was determined by using carnitine acetyltransferase (11) (ℓ-[carboxy-^{14}C] carnitine, 5.9 μCi/ μmol; ℓ-[uniformly-chain-labelled-^{14}C]carnitine {ℓ-3-hydroxy-4-N-trimethylammonio-[U-^{14}C]butanoate}, 222.3 μCi/μmol).

The synthesized ℓ-carnitine was compared to d,ℓ-[Me-^{14}C]carnitine as a substrate for carnitine palmitoyltransferase. The carnitine palmitoyltransferase assay 1 system was used (13). There were twice as many counts incorporated into palmitoylcarnitine with the synthesized carnitine as compared to the d,ℓ-carnitine (Table 2). This would be expected assuming that only one-half of the d,ℓ-carnitine is bioactive (14) and is consistent with the data of Stokke and Bremer (15) for methyl-labelled ℓ-carnitine.

Table 2

Incorporation of ℓ-[Me-^{14}C] carnitine and ℓ-[carboxy-^{14}C]carnitine into palmitoyl-ℓ-carnitine by mitochondria

Palmitoyl-ℓ-carnitine formation was measured in a 0.5 ml incubation containing unlabelled ℓ-carnitine (1 μmole), approximately 0.075 μCi ^{14}C carnitine either commercial d,ℓ-[Me-^{14}C]carnitine, synthetic ℓ-[Me-^{14}C]carnitine, or ℓ-[carboxy-^{14}C]carnitine, 100 nmoles palmitoyl-CoA, 0.1 mg sonicated rat liver mitochondria in 80 mM KCl, 50 mM morpholinopropane sulfonate (pH 7.0), 4 mM dithiothreitol, 0.1 mM EDTA and 0.5 mg defatted bovine serum albumin. The incubations were conducted for 5 min at 37°C. The reaction was stopped with 1.5 ml 1 N HCl, palmitoyl-ℓ-carnitine extracted with n-butanol (16), and the n-butanol layer counted for radioactivity. The average of 2-4 incubations are given.

label added	μCi per incubation	dpm in butanol	nmol palmitoyl-ℓ-carnitine formed/min · mg
d,ℓ-[Me-^{14}C]carnitine	0.084	1170	25.2+
ℓ-[carboxy-^{14}C]carnitine	0.063	1913	27.3
ℓ-[Me-^{14}C]carnitine	0.095	2779	26.4

+Assuming that ½ of the ^{14}C is present as the levarotatory isomer and therefore, biologically active.

We have described a method that leads to almost quantitative conversion of carboxy-labelled 4-aminobutanoate into carboxy-labelled ℓ-carnitine. The radioactively labelled ℓ-carnitine is chromatographically pure by both cation-exchange and thin layer chromatography. The procedure also desalts the labelled carnitine. Although designed to produce

carboxy-labelled carnitine we have used the method to produce either [^{14}C] or [^{3}H] methyl-labelled carnitine with good results and purity.

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