

SYNTHESIS OF [methyl-¹⁴C]CROTONOBETAINE FROM DL-[methyl-¹⁴C]CARNITINE

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Summary

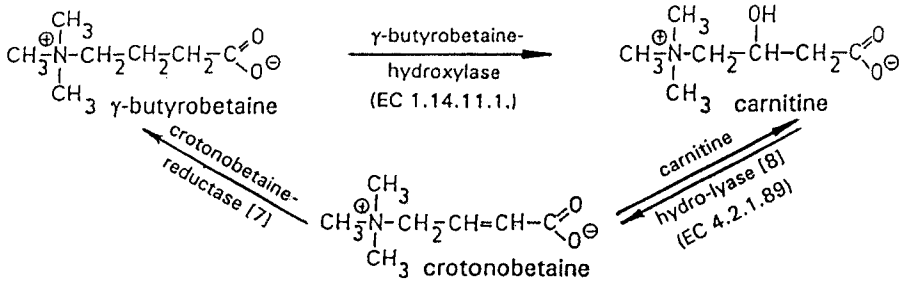
The causes of carnitine deficiency syndromes are not completely understood, but decomposition of L-carnitine *in vivo* is likely to be involved. Carnitine is metabolized to γ -butyrobetaine, and crotonobetaine is probably an intermediate in this pathway. To validate experimentally the precursor-product relationship between the three physiologically occurring γ -betaines - L-carnitine, crotonobetaine, γ -butyrobetaine - labelling with stable or radioactive isotopes became necessary. Methyl-labelled carnitine isomers (L(-), D(+)- or DL-) or γ -butyrobetaine can be easily synthesized by methylation of 4-amino-3-hydroxybutyric acid isomers or 4-aminobutyric acid, respectively. Because of problems with the 4-aminocrotonic acid, we synthesized labelled crotonobetaine from labelled carnitine. Thus, DL-[methyl-¹⁴C]carnitine was dehydrated by reaction with concentrated sulfuric acid. After removal of the latter the products were separated and purified by ion exchange chromatography on DOWEX 50 WX8 (200 - 400 mesh) and gradient elution with hydrochloric acid. In addition to the labelled main product [methyl-¹⁴C]crotonobetaine (yield about 50 %), [methyl-¹⁴C]glycine betaine and [methyl-¹⁴C]acetyltrimethylammonium (ATMA) were formed. The end products were identified by combined thin layer chromatography/autoradiography and quantified by liquid scintillation counting.

Key words: carnitine, crotonobetaine, acetyltrimethylammonium, TLC/autoradiography, liquid chromatography

INTRODUCTION

L(-)-carnitine (L(-)-4-N,N,N-trimethylamino-3-hydroxybutyrate) is essential for the β -oxidation of fatty acids in the mitochondria of mammalian cells, because the carnitine system is responsible for the transport of long-chain fatty acids through the mitochondrial inner membrane [1]. Consequently, carnitine deficiency is combined with disturbances of the lipid metabolism, for instance with lipid storage myopathies [2]. The increase in case reports of carnitine deficiency syndromes [2] forced detailed studies of carnitine metabolism, especially with respect to its catabolism [3].

After the administration of L-carnitine by injection or orally either to animals or humans, one can find γ -butyrobetaine in their blood, urine and faeces [4, 5]. γ -Butyrobetaine is also formed after the administration of crotonobetaine [6]. Therefore it can be assumed that crotonobetaine is the intermediate in the formation of γ -butyrobetaine and that a circle exists between the three physiological γ -betaines:



Enterobacteria, like *Escherichia coli* [9] or *Salmonella typhimurium* [10], are able to form γ -butyrobetaine from crotonobetaine. In mammalia this conversion is carried out only by microorganisms of the gastrointestinal tract. In germ free rats no conversion was found [11].

For a more detailed characterization of the enzymes involved and for the determination of the precursor-product relationship in vivo, labelled crotonobetaine had to be synthesized.

MATERIALS

The 4-amino-3-hydroxybutyric acid was purchased from Merck GmbH (Darmstadt), ^{14}C -methyl iodide (925 MBq [25 mCi]; 120 mg; $A_{\text{Sp}} = 1110 \text{ MBq (30 mCi/mmol)}$) from Amersham (Braunschweig) and the ion exchanger Dowex 50 WX8 from Serva (Heidelberg).

The DL-(methyl- ^{14}C)carnitine was synthesized by permethylation of 4-amino-3-hydroxybutyric acid with ^{14}C -methyl iodide according to [12] and purified with anion exchanger Dowex 1X8 (200 - 400 mesh; OH^- -form) (Serva, Heidelberg) and cation exchanger Merck IV (Merck, Darmstadt). Sulfuric acid and barium oxide are common substances.

The thin layer chromatograms were carried out on silica gel plates and the autoradiograms on X-ray films TF 13 and macro-autoradiography films AF 3 and AF 4 (Filmkombinat ORWO, Wolfen). To determine the radioactivity the samples were counted on a Packard TRI-CARB 300C liquid scintillation spectrometer.

For large scale separation of carnitine and crotonobetaine using the anion exchange resin IRA-400, a patent had been taken out [18]. After testing several ion exchange resins, a good separation of crotonobetaine from the byproducts was achieved with the cation exchanger DOWEX 50 WX8 (200-400 mesh) and gradient elution with hydrochloric acid. The crude product was dissolved in 1 ml water, placed in a column filled with 50 ml of DOWEX 50 WX8 (H^+ -form), eluted with a flow rate of 1 ml/min, and fractions of 5 ml each were collected (Fig. 1):

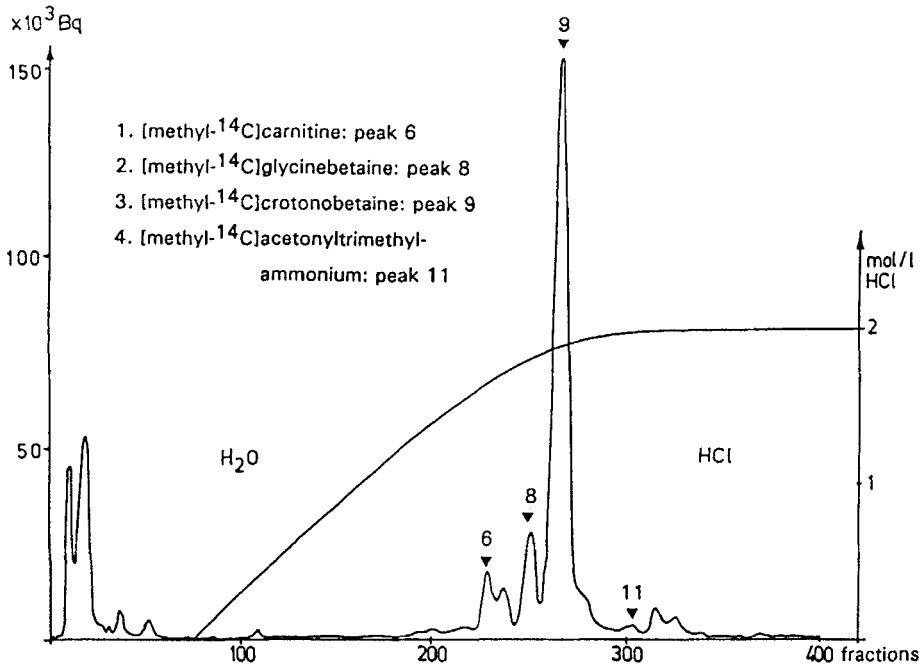


Fig. 1: Separation of [methyl- ^{14}C]crotonobetaine from its byproducts by means of ion exchange chromatography on DOWEX 50 WX8 (200 - 400 mesh) and gradient elution with hydrochloric acid (0-2 mol/l).

Up to fraction 72 the column was washed with water, removing the uncharged molecules and the anions. Then the column was eluted in a non linear, convex gradient of hydrochloric acid (0 - 2 mmol/l) to separate more efficiently the betaines from byproducts in the suitable range. From fraction 300 to 400 the column was further eluted with hydrochloric acid (2 mmol/l). The

radioactivity of all fractions was measured in 10 μ l amounts by means of liquid scintillation counting.

The elution profile of figure 1 shows two main peaks: the fractions 248 - 256 ([methyl-¹⁴C]crotonobetaine) and fractions 264 - 272 ([methyl-¹⁴C]glycinebetaine). 79,8 % of the total radioactivity was eluted in the main peaks of the profile, the remaining activity of 20 % was accounted for by the unreacted carnitine, unidentified substances, tailing and underground (Tab. 1):

Table 1: Distribution of the radioactivity on the various fractions of the ion exchange chromatography:

Peaks	Fractions	Radioactivity [Bq]	Amount on the total activity (%)
1	8 - 11	2 015.5	4.48
2	15 - 20	3 816.5	8.49
3	35 - 39	452.9	1.00
4	49 - 54	351.2	0.78
5	108 - 111	89.0	0.20
6 (carnitine)	225 - 234	1 921.7	4.27
7	237 - 242	1 027.4	2.28
8 (glycinebetaine)	247 - 256	2 994.0	6.66
9 (crotonobetaine)	259 - 276	21 707.3	48.32
10	299 - 307	464.9	1.03
11 (ATMA)	313 - 318	640.7	1.43
12	325 - 329	399.6	0.89

The main peak (> 25 kBq) and three other peaks were identified by thin layer chromatography/autoradiography with unlabelled reference substances as inner standards in at least 3 solvent systems [4].

Four substances, with a total activity of 60.3 %, could be identified as

1. [methyl-¹⁴C]crotonobetaine hydrochloride :48.3 %
2. [methyl-¹⁴C]glycinebetaine hydrochloride : 6.7 %
3. [methyl-¹⁴C]carnitine hydrochloride : 4.3 %
4. [methyl-¹⁴C]acetyltrimethylammonium chloride : 1.4 %

The marginal fractions were evaporated separately and for the main product, the [methyl- ^{14}C]crotonobetaine, only the fractions 263 - 273 were used.

The total yield of [methyl- ^{14}C]crotonobetaine can be estimated from its fraction on the radioactivity to the total activity. Because the specific radioactivity cannot be changed during the reaction, a calculation of the yield of the [methyl- ^{14}C]crotonobetaine can be made:

$$m = \frac{\text{total activity} \times \text{molecular mass}}{\text{specific activity}} = \frac{35\,203.3 \text{ Bq} \times 143.2 \text{ mg}}{2\,000.45 \text{ kBq}} = \underline{2.52 \text{ mg}}$$

Figure 2 demonstrates the purity of the [methyl- ^{14}C]crotonobetaine formed. For the thin layer chromatogram, unlabelled crotonobetaine was added as an inner standard, so that the autoradiogram can be compared with the thin layer chromatogram:

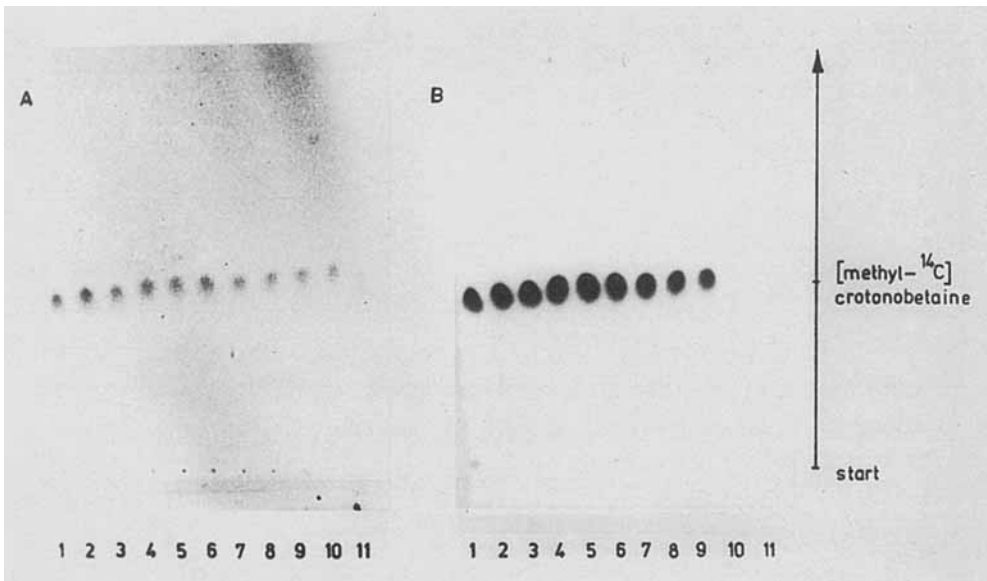


Fig. 2: Thin layer chromatogram (A) and autoradiogram (B) of [methyl- ^{14}C]crotonobetaine from the same plate:
 solvent: phenol/ n-butanol/ ammonia (25 %): 50/50/20 (v/v),
 film: AF 4; exposition: 7 d
 peaks 1-9: fractions 264-272 (2 x 1 μl),
 peaks 10, 11: unlabelled crotonobetaine 1 μl (conc. 10 $\mu\text{g}/\mu\text{l}$)

It is remarkable that a large amount of labelled glycinebetaine was formed. It could be a consequence of the strong oxidable activity of the concentrated sulfuric acid that it first forms dehydrocarnitine (ketocarnitine), which is not stable in the non esterified form, and then forms acetyltrimethylammonium (ATMA) by spontaneous decarboxylation and glycinebetaine by release of acetate, respectively [19]. In mamalia acetyltrimethylammonium (ATMA) is formed especially from the D (+)-isomer of carnitine [20].

For non labelled crotonobetaine, produced by the same method, YOKOZEKI et al. [21] proved by ¹H-NMR spectroscopy that it has the trans-configuration.

The method described has the advantage that a yield of about 50 % was achieved in respect to the precursor substance and that in addition two other metabolites formed from carnitine in vivo were obtained in the same experiment.

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