SELF-DECOMPOSITION OF DL-/METHYL-^{14}C/CARNITINE TO LABELLED ß-METHYLCHOLINE AND ACETONYLTRIMETHYLAMMONIUM

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Summary

The self-decomposition rate of DL-/methyl-^{14}C/carnitine (32 mCi/mmol) in the crystalline betaine form was studied. It was found to be decomposed to 39.5 % in 4.5 years. On this basis a self-decomposition coefficient G = 4.2 was calculated for the solid state. DL-/N-methyl-^{14}C/ß-methylcholine was the most important labelled product of decomposition (12 %). /N-methyl-^{14}C/acetonyltrimethylammonium was also present among the radiolytic products (2 %). It was detected as its 2,4-dinitrophenylhydrazone by means of thin-layer chromatographic separation and autoradiography.

Key words: ^{14}C-labelled DL-carnitine, radiolysis, trimethylammonium compounds, ß-methylcholine

Introduction

L(-)-carnitine (L(-)-3-hydroxy-4-N,N,N-trimethylaminobutyrate) is essential for the oxidation of long chain fatty acids in the mitochondria of mammals and humans. Because the impairment of physiological L-carnitine concentration leads to carnitine deficiency syndromes (1), the metabolism of carnitine is being intensively studied. To this end, and for the detection of six L-carnitine-specific enzymes (2), ^{14}C-labelled carnitine is

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used. Numerous chemical and biochemical methods for the synthesis of ^{14}C-labelled racemic carnitine and especially its optical isomers have been described and modified in the last few years (3-8). During our studies of the catabolic metabolism of carnitine in mammals (9), we found that the use of nonlabelled carnitine and /methyl-^{14}C/carnitine stored for some years did not give identical results. In analogy to choline, self-decomposition of the ^{14}C-labelled carnitine could be expected, therefore it was necessary to produce a pure radiochemical and to look for biochemically relevant substances among the radiolytic products of DL-/methyl-^{14}C/carnitine.

Material and Methods

DL-/methyl-^{14}C/Carnitine was purchased from Isocommerz-GmbH, Berlin. DL-ß-Methylcholine (VEB Berlin-Chemie) and 2,4-dinitrophenylhydrazine (Schönert-KG, Leipzig) were purchased, the ion exchanger Dowex 50 WX8 also (Serva, Heidelberg).

The acetonyltrimethylammonium chloride (ATMA) was synthesized by oxidation of DL-carnitine (Merck, Darmstadt) with potassium bichromate in glacial acetic acid (10) or reaction of chloroacetone with trimethylamine (11).

The thin-layer chromatography (TLC) was accomplished on silica gel plates (9) and the autoradiography (AR) on X-ray film TF 13 and macro-autoradiography-film AF 3 and AF 4 resp. (VEB Filmkombinat ORWO, Wolfen).

The radioactive samples were counted as 10 µl quantities in a solution of 10 ml dioxane-scintillator on a Packard Tricarb liquid scintillation spectrometer, model 3375, and the quench-ratio was determined by means of a standardization curve.

Results and Discussion

4.5 years ago the commercial DL-/methyl-^{14}C/carnitine hydrochloride was purified by combined ion exchange resin passages (Wofatit SBK, OH^- and Merck IV, H^+). Thus the DL-/methyl-^{14}C/carnitine was produced free of all cations and anions for biochemical use, without examination of the separated radiolytic products. The labelled carnitine was evaporated to dryness in vacuo and has been stored in the solid state as free betaine in a desiccator in darkness at 4°C for 4.5 years (1668 d). After this storage time the labelled, nonvolatile products of self-decomposition are demonstrated in Fig. 1, first sample.

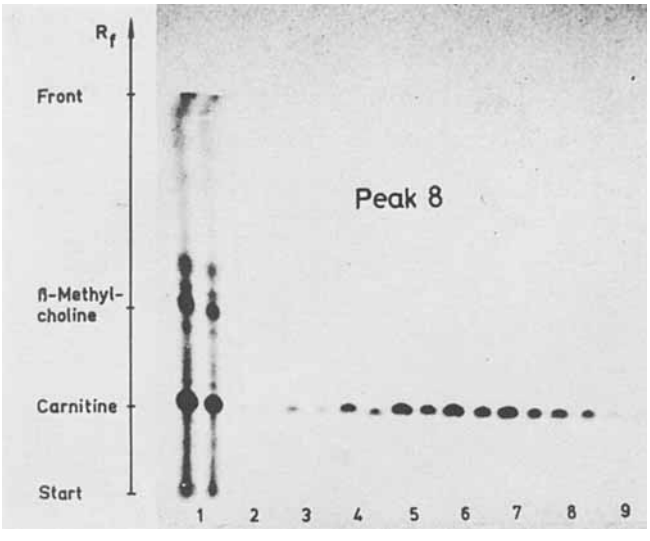


Fig. 1: Autoradiograph of radiolytic decomposed and purified DL-/methyl-^{14}C/carnitine.
sample 1: radiolytic decomposed DL-/methyl-^{14}C/carnitine, activity: 72.1 nCi/µl
samples 2-9: purified DL-/methyl-^{14}C/carnitine, fractions 163-170 (Fig. 2, peak 8), activities: 0.54; 1.71; 6.4; 14.9; 17.3; 16.7; 7.3; 0.95 nCi/µl
TLC: silica gel G; phenol/butanol/ammonia (25 %); 50/50/20 (v/v);
AR: AF 4; exposure time 72 h
each sample 3 µl and 1 µl

For the purification of this sample and the separation of the

radiolytic products from each other and their quantitative evaluation, ion exchange chromatography on Dowex 50 WX8 (200-400 mesh, H^+) was used (12). 6.1 mg DL-/methyl-^{14}C/carnitine were dissolved in water (0.5 ml) and applied to a column (33 x 2 cm; 50 ml). First this was eluted with water and 103 fractions (each 10 ml) were collected. The nonionic and anionic radiolytic products were found up to fraction 55 (Fig. 2, peaks 1-5). Then the zwitterionic and cationic products were eluted by a gradient of hydrochloric acid (0 - 2.0 mol/l). In total 370 fractions were collected and quantified. The fractions 270-370, having less activity than 1 nCi/µl, were not drawn in Fig. 2. The remaining activity was dissolved from the column with 300 ml hydrochloric acid (2.0 mol/l) and determined to be only 9.2 µCi (0.9 % of the applied activity). The main peak (Fig. 2, No. 8) was the radiochemically pure carnitine (Fig. 1, samples 2-9), as detected by TLC/AR in several TLC-systems (13).

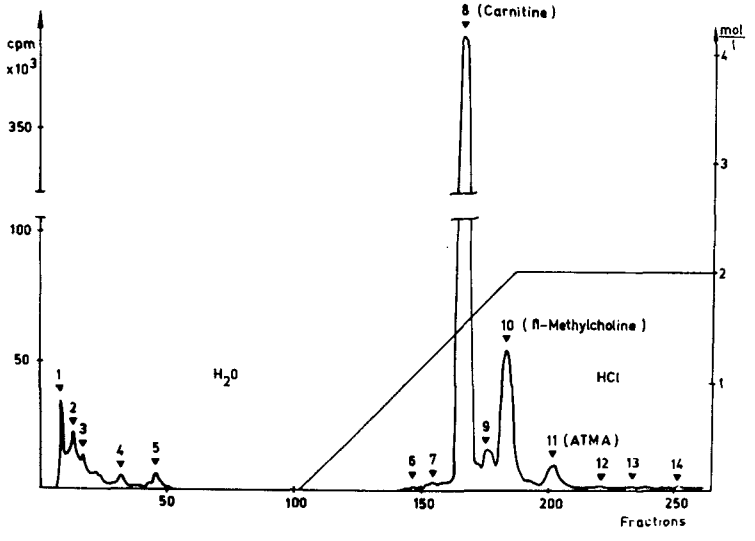


Fig. 2: Separation of DL-/methyl-^{14}C/carnitine from its radiolytic products by means of ion exchange chromatography on Dowex 50 WX8 (200-400 mesh) and gradient elution with hydrochloric acid (0-2 mol/l).

In the majority of cases the radioactivity of the other peaks was found in a single spot (Fig. 3, substances 2 and 4). The fractions belonging to one peak and consisting of one labelled compound were unified, evaporated to dryness and dissolved in 1.0 ml water for further identification.

From the applied activity given on the column, only 60.5 % was detected in the labelled carnitine (Table 1). In all the other 13 peaks, checked by TLC/AR, 21.3 % was found, the rest was the non-checked underground, which was not further examined.

Tab. 1: Distribution of the original carnitine activity on the nonvolatile radioactive decomposition products after ion exchange chromatography.

Peaks	Fractions	Activity (ipm x 10^5/ml)	$\frac{\text{Activity in the peak}}{\text{Total applied activity}} \times 100$
1	8; 9	58.5	2.17
2	13	22.1	0.82
3	17	13.5	0.50
4	32	5.7	0.21
5	46	6.1	0.23
6	147	1.4	0.05
7	155	3.1	0.11
8	162 - 170	1 633.6	60.55
9	174 - 178	70.1	2.60
10	180 - 190	319.2	11.83
11	198 - 205	56.1	2.08
12	221 - 223	5.5	0.20
13	231 - 235	8.8	0.33
14	250 - 252	3.7	0.14

On the basis of the percentage decomposition, we calculated the magnitude of the self-decomposition coefficient G (-M), indicating the sensitivity of the substance to self-decomposition by radiation.

$$P = 100 \left(1 - e^{- f \cdot E \cdot G \cdot s \cdot t \cdot 6.14 \cdot 10^{-16}} \right) \quad (14)$$

$$G = - \frac{\ln (1 - 0.01\ P)}{f \cdot E \cdot t \cdot s \cdot 6.14 \cdot 10^{-16}} = \underline{4.2}$$

P: percentage decomposition at the time t = 39.45 %
t: time of storage ^{14}C-ß-radiation = $144 \cdot 10^6$ s
E: mean energy of ^{14}C-ß-radiation = $45 \cdot 10^3$ eV
s: specific activity at the time t_o = 32.1 mCi/mmol
f: fraction of the radiation energy absorbed = 0.93

The value f is very problematical. In the case of the weak ß-energy of ^{14}C, f can be assumed to be unity in a rough simplification (14). In analogy to the structure-related choline, we used the proposed value of 0.93 (15).

The group of trimethylammonium compounds is known to be very sensitive to radiation. Several results exists on the radiolytic decomposition of /methyl-^{14}C/choline. In one of the first descriptions of self-decomposition, choline chloride in the crystalline form was extensively decomposed in vacuo to 63 % in a time shorter than one year (16). The reported G values vary widely depending on the experimental conditions and the evaluation (17). G values received by ^{60}Co-γ-radiation differ from those of ^{14}C-ß-radiation, e.g. choline chloride has with 1.25 MeV-γ- radiation a G = 175, but with ^{14}C-ß-radiation a G = 1250 (18). The sensitivity of choline to radiation depends also on the anion. With ^{60}Co-γ-radiation G = 354 was published for choline chloride, G = 92 for the bromide and G = 2.5 for the iodide (19). Under the same conditions the G value for ß-methylcholine chloride was only 6.9 and substitution of the hydroxyl group of choline by an hydrogen atom (trimethylethylammonium chloride) decreased the

radiolytic rate 100 times (18,20).

The stability of carnitine hydrochloride in comparison with choline chloride was tested with ^{60}Co and a G = 14 was calculated on the basis of precipitation with reineckate or tetraphenylboron (19). Information about the products of decomposition was not given. Recently in certain lots of DL-/carboxy-^{14}C/carnitine a radioactive contaminant posessing approximately 5 % of the total radioactivity has been found (8), after ion exchanger passage the carnitine contained less than 0.05 % radioactive impurities.

Because of their biochemical importance we looked for ß-methylcholine and ATMA (secondary product of L-carnitine dehydrogenase of bacteria, EC 1.1.1.108) among the radiolytic products of our DL-/methyl-^{14}C/carnitine. In the previously published TLC-systems (9) the decomposition products of peak 10 and 11 run as these trimethylammonium bases. After addition of unlabelled ß-methylcholine to peak 10, the spots with iodine vapor detection were congruent. The same result was achieved with unlabelled ATMA as an inner standard for the substance in peak 11 (Fig. 3). Because the R_f-values of the two trimethylammonium bases only weakly differ from each other, the formation of a characterizing derivative corresponding to the hydroxyl or carbonyl group was necessary. Identification as 2,4-dinitrophenylhydrazone was chosen for ATMA (9). After the addition of unlabelled ATMA, the crystals of its 2,4-dinitrophenylhydrazone were filtered off, washed, recrystallized and then examined by TLC/AR (Fig. 3, sample 3). The radioactivity of peak 11 was found in the 2,4-dinitrophenylhydrazone of ATMA. ß-Methylcholine was identified by means of its

n-butyrylester. The activity of peak 10 was found in the spot of n-butyryl-ß-methylcholine (Fig. 3, sample 5).

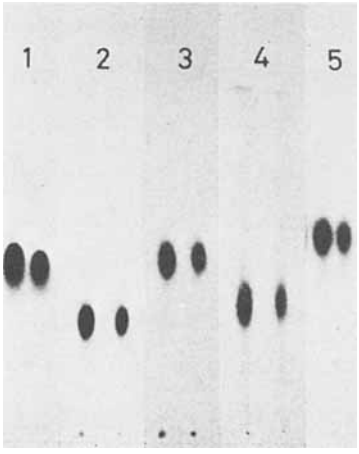
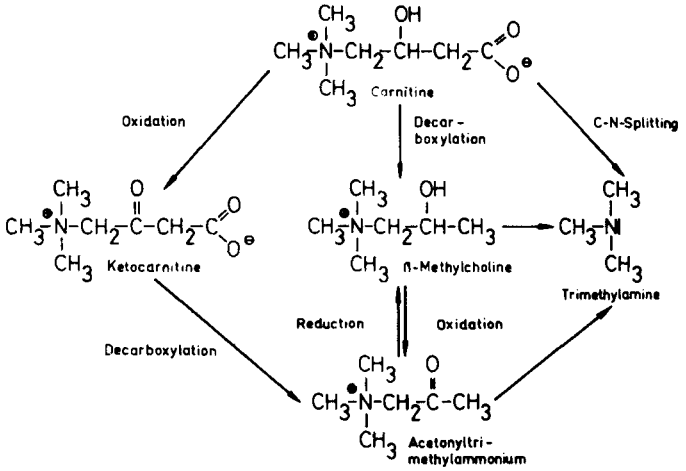


Fig. 3: Autoradiographic identification of the two radiolytically formed trimethylammonium bases.
sample 1: purified DL-/methyl-^{14}C/carnitine (Fig. 2; peak 8)
sample 2: /methyl-^{14}C/ATMA (Fig. 2; peak 11)
sample 3: 2,4-dinitrophenylhydrazone of /methyl-^{14}C/ATMA
sample 4: /N-methyl-^{14}C/ß-methylcholine (Fig. 2; peak 10)
sample 5: n-butyryl-/N-methyl-^{14}C/ß-methylcholine
TLC: silicagel G; acetone/methanol/hydrochloric acid; 90/10/10
AR: TF 13; exposure time 72 h
each sample 3 µl and 1 µl

Trimethylamine is the main product of the self-decomposition of choline (18, 21). We have to assume that volatile trimethylamine was formed from ß-methylcholine and ATMA, as well as from the labelled carnitine, since these two substances were themselves decomposed by radiolysis. Because the desiccator was opened several times during the storage period, the trimethylamine could not be detected by our method. If carnitine was first oxidized to ketocarnitine by radiation, the following

spontaneous decarboxylation to ATMA of the ß-ketonic acid formed is a well-known reaction of the unlabelled substance. These possible reactions for the decomposition of carnitine are given in the following scheme:



The two nonvolatile radiolytically formed trimethylammonium bases may interfere in biochemical or pharmacokinetical experiments with endogenous physiologically formed carnitine metabolites. The self-decomposition examined here in the solid state could take place in solution too, although the radioactive concentration is then lower. The radiolytic rate could be increased by the formation of hydroxyl radicals from water and chain reaction mechanisms (14, 22). On the other hand it can be of practical relevance, that from a radiolytically decomposed preparate of /methyl-^{14}C/carnitine, which has to be purified before use in any case, radiochemically pure ^{14}C-labelled acetonyltrimethylammonium and ß-methylcholine can be produced in the same experiment with the same procedure simultaneously with the pure carnitine.

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