RADIOLYSIS OF D(+)-CARNITINE BY ^{60}Co-γ-RADIATION AND FORMATION OF L(+)-ß-METHYLCHOLINE

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Summary

The radiolysis of D(+)-carnitine by ^{60}Co-γ-radiation was examined to obtain optically active ß-methylcholine. It was found that the radiolysis leads to a number of trimethylammonium bases but to no other betaines. The acids formed were not investigated. The bases produced were separated by ion exchange chromatography. (+)-ß-Methylcholine and acetonyltrimethylammonium could be identified by means of common analytical methods (IR, NMR, MS and MA) applied to the fractions. The amounts of methylamines formed by irradiation, and determined by gas chromatography, were very small. Racemization of the D(+)-carnitine did not occur during irradiation, L(-)-carnitine was not found when an enzymatical determination method was used. The fact that (+)-ß-methylcholine was formed from D(+)-carnitine is pharmacologically important, because acetyl-L(+)-ß-methylcholine has a strong interaction with muscarinic receptors.

Key words: radiolysis, carnitine, ß-methylcholine, pharmacology, trimethylammonium compounds

INTRODUCTION

L(-)-carnitine (L(-)-3-hydroxy-4-N,N,N-trimethylaminobutyrate) is the essential carrier for the penetration of long chain fatty acids through the inner membrane of mitochondria. Therefore L(-)-

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carnitine is the limiting factor of the ß-oxidation in the cells of animals and humans (1). Because of its importance in the medical sciences (carnitine is used for treating carnitine deficiency syndromes (2)) the metabolism of carnitine has been intensively studied. One of the main questions in the development of carnitine deficiency syndromes is: are there catabolic pathways in normal or sick organisms and to which compounds is carnitine decomposed?

Investigating the self-decomposition of DL/methyl-^{14}C/carnitine (3) we obtained DL-/N-methyl-^{14}C/ß-methylcholine as one of the decomposition products. But the degradation of L(-)-carnitine to the optically active ß-methylcholine isomer is pharmacologically much more interesting because of the stereospecific muscarinic activity of this trimethylammonium base in vivo (4). Since one cannot determine the optical rotation of the /methyl-^{14}C/ß-methylcholine formed from L(-)-/methyl-^{14}C/carnitine, we looked for a method to decarboxylate L(-)- or D(+)-carnitine while maintaining its configuration. Therefore we have investigated the decomposition of D(+)-carnitine by means of ^{60}Co-γ-radiation and the nature of the compounds formed.

MATERIAL AND METHODS

Chemicals

The optical isomers of carnitine were obtained by resolution of the DL-carnitine hydrochloride (MERCK, Darmstadt, FRG) by the method of STRACK and MUELLER (5). Methyl-^{14}C-labelled carnitine, -ß-methylcholine and -acetonyltrimethylammonium (ATMA) were independently synthesized or obtained by separating the self-decomposition products of DL-/methyl-^{14}C/carnitine (3). The ion exchangers were purchased from SERVA, Heidelberg, FRG (DOWEX 50 WX8), MERCK, Darmstadt, FRG (MERCK IV) and VEB Chemiekombinat Bitterfeld, GDR (Wofatit SBK) resp.

Apparatus

The sample irradiation was carried out in a Co-60 unit (ZfI Leipzig) at dose rates of 1.5 Gy·s^{-1}. The radiation doses were mea-

sured by means of various dosimeter systems (a chlorobenzene-ethanol, a FRICKE, and a semiconductor dosimeter). The temperature during irradiation was about 40°C.
The radioactivity of the ^{14}C-labelled samples from the ion exchange chromatography was measured by liquid scintillation counting (LSC) in a TRI-CARB 300C (PACKARD INSTRUMENTS,Inc.,USA). The methylamines were determined by means of a gas chromatograph model GCHF 18.3 (VEB Chromatron, Berlin), equipped with a steel column (3m) and packed with 20 % polyethylene glycole 600 and 1 % polyethylene imine on porolith (60-80 mesh) (VEB Berlin-Chemie, GDR) and a flame ionization detector.

Sample preparation

For the formation of ß-methylcholine, 8 samples (5 samples of about 1g, 3 of about 5g) of chromatographically pure D(+)-carnitine ($/\alpha/_{546}^{20}$ = + 37.1°) were put into ampoules, evacuated and irradiated with different total doses of 0.1 - 2 MGy [1]. The use of D(+)-carnitine permits a convenient enzymatical examination of the L(-)-carnitine possibly formed by racemization. After opening the ampoules (with exception of the ampoules reserved for determining the methylamines), the dark coloured substances were dissolved in water and passed through an anion exchanger column (50 ml Wofatit SBK, OH^--form) to absorb the anions, which were not investigated further. Then the solutions were passed very slowly (4 drops/min.) through a column filled with the weak cation exchanger MERCK IV (about 10 ml) to separate the betaines from the bases. Only when a very weak cation exchanger was applied were the bases bound at the ion exchanger, but not the betaines. The first eluates were evaporated to dryness, carefully dried and weighed. The bases bound at the exchanger were subsequently eluted with 100 ml of 2 % hydrochloric acid, washed with about 100 ml water and the solutions were treated in the same way as the betaine fraction. The solutions were examined by thin layer chromatography (TLC).

Ion exchange chromatography

For isolating ß-methylcholine from the numerous substances of the base fraction an ion exchange chromatography was carried out by using a glass column (30 x 2 cm; 50 ml) and the cation exchanger

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Table 1: Radiolysis rates of D(+)-carnitine after separation from radiolytic products.

No	dose /MGy/	sample weight /g/	amounts of betaines /mg/	bases /mg/	sum /mg/	loss (total) /mg/	D-carnitine destr. /%/	G(-M)-value
1	0.1	1.000	931	21	952	48	6.9	41.30
2	0.2	1.031	948	28	976	55	7.8	23.34
3	0.3	1.000	913	33	946	54	8.7	17.36
4	0.4	1.208	1065	51	1116	92	9.8	14.62
5	0.5	1.000	893	48	941	59	10.7	12.81
6	0.5	5.000	4539	140	4679	321	9.2	11.04
7	1.0	4.713	4093	253	4346	367	13.2	7.87
8	2.0	4.982	3789	420	4209	773	23.9	7.16

DOWEX 50 WX8 (H^+-form). The total fraction of the bases of sample 6 (0.5 MGy) was given on the column and small amounts of DL-/methyl-^{14}C/ß-methylcholine and /methyl-^{14}C/ATMA were added as tracers for the non-radioactive substances. To remove non-ionic substances, first water was pumped through the column (samples 1-50). Then hydrochloric acid (0-2N) in a non-linear gradient was used to elute the following samples, which were collected in a sample collector. 500 samples were collected, of 5 ml each. The radioactivity was measured in each sample (10 µl) by LSC.

Determination of L(-)-carnitine

The L(-)-carnitine was determined enzymatically by the DTNB-method (6). In this method L(-)-carnitine reacts with acetyl-CoA thereby forming acetyl-L(-)-carnitine and CoA under the influence of carnitine acetyltransferase (EC 2.3.1.7). The CoA reacts with DTNB (5,5'-Dithiobis(2-nitrobenzoic acid); Ellmann's reagent) to form a yellow substance, which was measured photometrically at 412 nm.

GC-determination of methylamines

Two ampoules of D(+)-carnitine (0.2 and 0.4 MGy) were opened in a closed volume. From the head space definite amounts (0.25, 0.5 and 1 ml) were injected into the gas chromatograph [2]. Carbon dioxide formed during the irradiation could not be determined simultaneously.

[2] We are indebted to Dr. R. Kliche, Sektion Tierproduktion und Veterinärmedizin der Karl-Marx-Universität Leipzig for the determination of the methylamines.

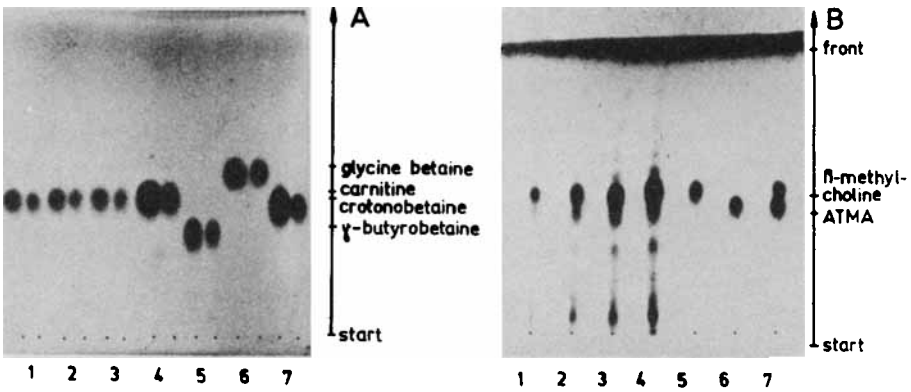


Fig. 1: Thin layer chromatograms of the betaine fraction (A) and the base fraction (B) after separation by anion exchanger Wofatit SBK and cation exchanger MERCK IV.

A) peaks 1-3: betaines from the irradiation samples 6,7,8 (0.5, 1.0, 2.0 MGy)
conc. 2 ug/ul; each sample 3 µl and 1 µl
peaks 4-7: reference substances:
4: carnitine betaine, conc. 5 µg/µl; 2 µl and 1 µl
5: γ-butyrobetaine, conc. 3 µg/µl; 2 µl and 1 µl
6: glycine betaine, conc. 5 µg/µl; 2 µl and 1 µl
7: crotonobetaine, conc. 4 µg/µl; 2 µl and 1 µl

B) peaks 1-4: bases (eluates from MERCK IV) from the samples 4, 6, 7, 8;
conc. 1 % of the total amount; 1µl
peaks 5-7: reference substances:
5: ß-methylcholine, conc. 5 µg/µl; 1 µl
6: ATMA, conc. 5 µg/µl; 1 µl
7: ß-methylcholine and ATMA mixed; 1 µl

TLC: silica gel G (MERCK), acetone/methanol/hydrochloric acid (25 %); 90/10/10 (v/v)

RESULTS

By irradiating the samples of D(+)-carnitine betaine with increasing doses of 0.1 - 2 MGy , the carnitine was decomposed to trimethylammonium bases and methylamines, dependent on the doses (Tab. 1). The G-values were calculated according to the formula:

$$G\ (-M) = \frac{\text{wt. \% decomp. of the compd. x } 6.023 \times 10^{23}}{\text{mol. wt. x Gy x } 10^{-2} \times 6.24 \times 10^{13}} \qquad (7)$$

The examination of the TLC of the betaine fraction showed that no other betaines were formed (e.g. γ-butyrobetaine or crotonobetaine) (Fig. 1A). Thus, it was possible to use the complete betaine frac-

tion for determination of the decomposition rate. In contrast, the base fraction (eluate of MERCK IV) consisted of numerous substances, the mean peak was ß-methylcholine (Fig. 1B).

By ion exchange chromatography the interesting bases ß-methylcholine and ATMA were separated. The radioactivity was found in two peaks: in the samples 230-280 and 301-323 for ß-methylcholine and ATMA resp. (Fig. 2). The identity of the substances in these samples was examined by TLC (Fig. 3).

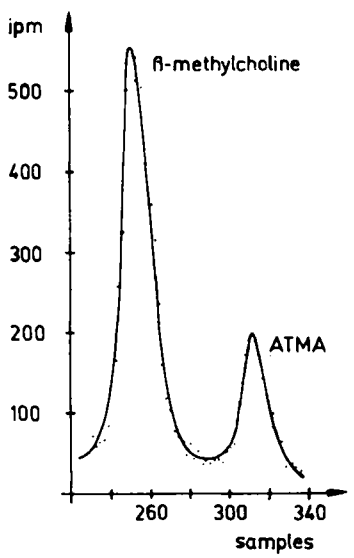


Fig. 2: Distribution of the radioactivity of the samples separated by ion exchange chromatography.

The autoradiographs from TLC evidenced the identity of the two compounds with the reference substances. The samples with the same compound were united, evaporated and recrystallized (isopropanol). We obtained 69.3 mg of ß-methylcholine chloride (about 50 % of the total fraction) and 16.2 mg of ATMA chloride. From both substances the IR, NMR and mass spectra and the micro analyses were made. The optical rotation of ß-methylcholine was determined to be $/\alpha/_D^{20} = +27.3^\circ$ (l=1; c=3.5) and $/\alpha/_{546}^{20} = +28.5^\circ$ resp.

The IR spectra showed for ß-methylcholine a wide OH-absorption band (3100-3700 cm^{-1}) and for ATMA a sharp CO-absorption at 1730 cm^{-1}. Both compounds showed the NR_3^+-absorption frequency, ß-methylcholine at 980 cm^{-1} and ATMA at 950 cm^{-1}.

The ^{1}HNMR spectra showed the following chemical shifts (DMSO-d_6): ATMA: δ =3.38 (s,9H; $(CH_3)_3N$); δ =4.76 (s,N-CH_2); δ =2.37 (s,CH_3) ppm; ß-methylcholine: δ =3.36 (s,9H; $(CH_3)_3N$); δ =5.52, 5.57 (d, N-CH_2) $J_{1,2}$=4.5 Hz; δ =4.25-4.63 (m, CH); δ =1.31, 1.38 (d,CH_3) ppm (HMDS as internal standard).

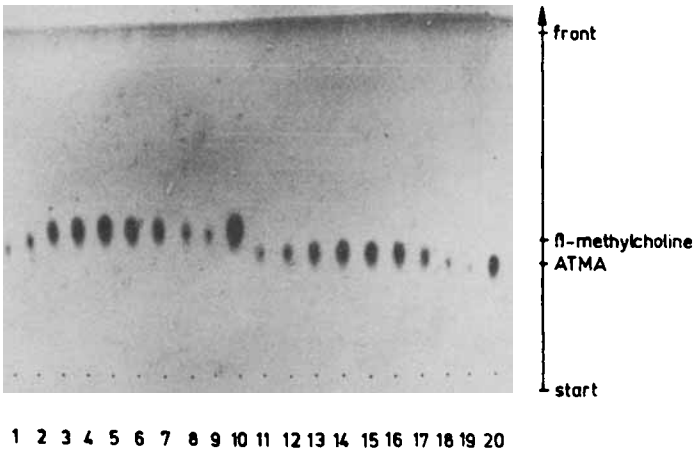


Fig. 3: Thin layer chromatogram of the ion exchange chromatography samples.

peaks 1-9: samples 240,243,246,248,250,255,260,265,270 (samples evaporated, then dissolved in 500 µl water); 1 µl

peaks 11-19: samples 295,300,304,308,312,316,320,324,330 (samples evaporated, then dissolved in 200 µl water); 1 µl

peak 10: ß-methylcholine as reference: 8 µg/µl; 1 µl
peak 20: ATMA as reference: 6 µg/µl; 1 µl

TLC: silica gel G (MERCK), acetone/methanol/hydrochloric acid (25 %); 90/10/10 (v/v)

In the mass spectra the dimethylimine fragment at m/e=44 as base peak for both compounds could be seen. The main fragmentation pathway was the elimination of methyl chloride and the loss of dimethylamine. In another pathway ß-methylcholine split off water, whereas in ATMA the bond between the trimethylamino and the acetonyl group split.

It could be possible that a racemization of the D(+)-carnitine occurs by radiation in addition to the decarboxylation. In this case we would obtain a wrong value for the optical rotation of the ß-methylcholine formed. Therefore we examined the amount of L(-)-carnitine in two radiolyzed betaine fractions (0.2 and 2 MGy) in high concentrations (5 mMol/ml) by means of the enzymatical assay. There was no L(-)-carnitine in any of the samples ($<$ 1 nmol/ml). After the qualitative proof of methylamines by TLC we determined the quantity of methylamines in two samples by means of gas chromatography. The amounts of methylamines were very low ($<$ 1 % of the total fraction) (Tab. 2).

Tab. 2: Methylamines formed by irradiation of D(+)-carnitine (nmol/sample)

	0.2 MGy sample	0.4 MGy sample
Trimethyl-amine	65.4	140.2
Dimethyl-amine	20.4	33.1

Monomethylamine was not found. Trimethylamine and dimethylamine were separated with this column successfully. The methylamines investigated appeared in the gas chromatogram with different intensities. The peak areas of trimethylamine and dimethylamine were in relation of 2.75 to 1 with the same amount injected.

DISCUSSION

It is a well known fact that the stability of trimethylammonium compounds is low against ionizing radiation (9). The stability depends on the structure of the substance. Simple compounds, such as /methyl-^{14}C/trimethylethylammonium chloride only have a G-value of 2 (10), but by substituting an OH-group for an H in the ß-position (forming choline chloride) the G-value increases more than 100 times (10,11). The /methyl-^{14}C/choline chloride is the compound with one of the most striking decomposition rates observed. Besides the substituents, the decomposition of /methyl-^{14}C/choline depends on the anion (12). Carnitine has likewise an OH-group in the ß-position, but it is a betaine, in contrast to cholins. However the former examination of its stability against radiation has been done on the hydrochloride. For this compound a G-value of 14 has been found for both ^{14}C-ß-radiation and ^{60}Co-γ-radiation (12). No information was given about the products formed by decomposition.

The G-values calculated in our experiments for the decomposition of D(+)-carnitine by ^{60}Co-γ-irradiation show no correlation with the G-value obtained by the self-decomposition of DL-/methyl-^{14}C/carnitine (3). It is obviously not possible to compare the G-values obtained from the self-decomposition of a labelled com-

pound and the G-value obtained from an external irradiation (13). Moreover, it is assumed that the high G-values are caused by traces of water contained in the samples irradiated. Further the G-values for the decomposition of D(+)-carnitine at various radiation doses differ over a wide range (Tab. 1) and show no linear relationship between the decomposition and the radiation doses. The G (-M)-values decrease with increasing doses of radiation. Obviously a stabilizing effect of the increasing amounts of radiation products occurs in the remaining D(+)-carnitine. Moreover these G-values are much higher than the G-values of self-decomposition of /methyl-^{14}C/carnitine. It was assumed by EVANS (13) that a compound with a G-value >10 is decomposed frequently by a chain reaction mechanism.

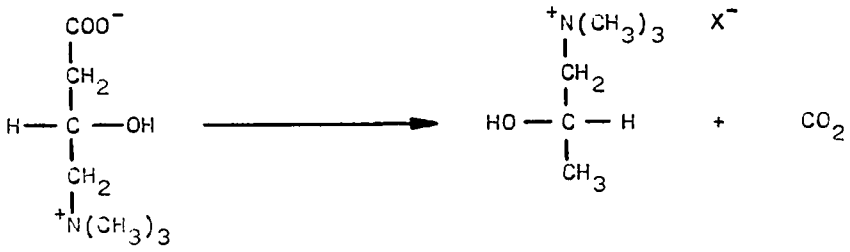
In our paper on the self decomposition of DL-/methyl-^{14}C/carnitine betaine we found labelled DL-/methyl-^{14}C/ß-methylcholine and /methyl-^{14}C/ATMA. It is of great importance to know the configuration of the ß-methylcholine formed of an optically active carnitine isomer. The possibility that L(-)-carnitine decomposes *in vivo* to ß-methylcholine under the influence of the enzyme carnitine decarboxylase (EC 4.1.1.42) (14) is under discussion (15, 16). This fact is of pharmacological interest. In the case that acetyl-L(+)-ß-methylcholine is formed from acetyl-L(-)-carnitine, a strong interaction with muscarinic receptors is to be expected.

The configuration of ß-methylcholine has been evidenced by BECKETT et al. (8) and ELLENBROEK et al. (17) via an independent route of synthesis from L(+)-lactic acid:

L(+)-Lactic acid L(+)-ß-Methylcholine

By our procedure optically active (+)-ß-methylcholine was ob-

tained as well. Theoretically L(+)-ß-methylcholine must be formed from D(+)-carnitine according to the equation:



D(+)-Carnitine betaine L(+)-ß-Methylcholine

The decarboxylation of D(+)-carnitine leads to L(+)-ß-methylcholine. Consequently, decarboxylation of acetyl-L-carnitine must lead to acetyl-D-ß-methylcholine, a compound of relatively low muscarinic activity.

In conclusion: If the carnitine decarboxylase is active in the body, only the inactive ß-methylcholine isomer could be formed from the carnitine pool or from the L-carnitine therapeutically used for substitution in patients with carnitine deficiency syndromes.

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