CCA-49

547.466.8:539.163:577.12

# On the Metabolism of β-Methionine-Methyl-$C^{14}$ *in vivo*

*D. Keglević-Brovet, A. Kornhauzer, B. Ladešić and S. Iskrić*

*in collaboration with:*

*N. Allegretti and L. Rabađija**

*Tracer Laboratory, Institute »Ruđer Bošković«, Zagreb, Croatia, Yugoslavia*

Received January 19, 1957

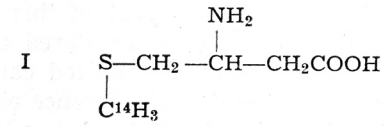
β-Amino-γ-methyl-$C^{14}$-thiobutyric acid (β-methionine-methyl-$C^{14}$) fed in a single dose to rats caused a negligible activity of the carbon dioxide expired. Most of the activity was found in the urine due to the unchanged compound administered, as well as to its oxydation product β-amino-γ-methyl-$C^{14}$-sulfoxibutyric acid (β-methionine sulfoxide). Choline and creatinine isolated from the body tissues and creatinine isolated from the urine showed a low activity. It is assumed therefore that under such conditions only a small fraction of β-methionine transfers its methyl group to the methyl acceptors.

As a result of the investigations of du Vigneaud and his school, the process of transmethylation *in vivo* has become a well established fact. There are few compounds occuring in nature which have been identified as biological donors of methyl groups. Besides methionine and choline, betaine[1] and dimethylpropiothetin[2] were found to be able to transfer the methyl group under biological conditions *in toto*. From the compounds, not occuring in nature, monoethylcholine, dimethylthetin and methylethylthetin[3] have been shown to be capable to perform the methylation of homocystine in vivo.

In general three lines of evidence have been established for the identification of the methyl donors[4]:

1. the compound must be lipotropic,
2. the compound must support growth in animals on a diet devoid of methionine but supplemented by homocystine,
3. the compound if administered must transfer its methyl group to such substances which are known to obtain this group by means of the transmethylation reaction.

From this point of view, it seemed to us of interest to observe the metabolic fate of β-amino-γ-methylthiobutyric acid (β-methionine), labelled in the methyl group by $C^{14}$:



I

* Department of Biology, Institute »Ruđer Bošković«, Zagreb

The starting material was β-amino-γ-benzylthiobutyric acid obtained by the method of Balenović and Fleš[5, 6] in a series of reactions from the natural amino acid cystine. The method for the preparation of labelled β-methionine was the same as that described by Balenović and al.[7] for the inactive compound, with some suitable modifications for the tracer work. The synthesis of the labelled compound has already been described elsewhere[8].

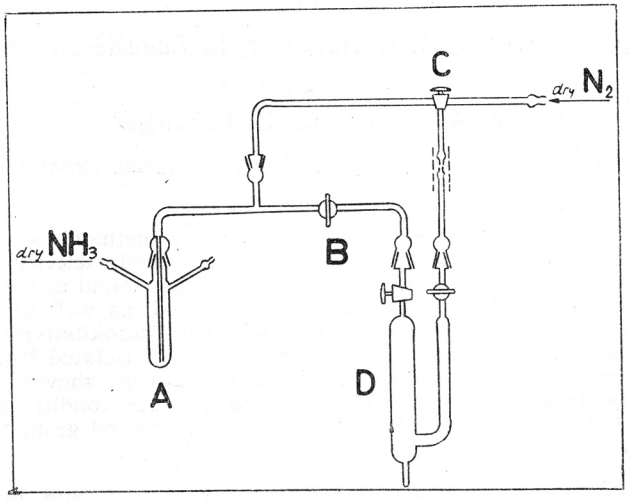



Fig. 1.

β-Methionine was also prepared by Birkofer and his collaborators[9, 10] from optically inactive materials in two ways. The same authors[11] also prepared optically inactive β-amino-γ-benzylthiobutyric acid.

To obtain the first information about the metabolism of β-methionine-methyl-$C^{14}$, 98.8 mg. (0.662 mMole) of this substance was given in a single dose to three rats, which were then placed in a metabolism cage. The carbon dioxide expired and the urine excreted, as well as creatine and choline from body tissue were checked for their radioactivity.

Du Vigneaud and his collaborators[12, 13] showed in their papers on the oxidation of the methyl group of α-methionine that a considerable amount of the methyl groups is oxidized and expired as radio carbon dioxide. Doubling the α-methionine content of the diet (from 0.6% resp. 0.08 mMole to 1.2% resp. 0.16 mMole in a single dose) the rate of oxidation increased approximately 9-fold during the first six hours after administration (from 2.35% to 20.3%).

As can be seen from the experimental part of this paper there was no significant oxidation of the methyl group administered as β-methionine. The barium carbonate samples obtained from the expired carbon dioxide showed negligible activities. There was no significant difference either in the activities between the samples of the first and the latter hours. It follows therefore, that β-methonine is not oxidized by the living body.

The urine was by far the most active part of the products examined. About 83% of the whole activity administered was excreted in urine. From the paper chromatography of urine samples it could be seen that two new spots appeared after the administration of β-methionine (Figs. 2 and 3). The first spot was identified as the original β-methionine (Fig. 4), whilst the second spot was accounted to β-amino-γ-methylsulfoxibutyric acid (β-methionine sulfoxide) (II) because of its radioactivity and smaller Rf value then β-methionine. We prepared, therefore, β-methionine sulfoxide from β-methionine, following in general the procedure for the preparation of α-methionine sulfoxide given by Toennis and Kolb[14]. In fact the Rf values, of synthetically prepared β-methionine sulfoxide agreed with those of the product excreted in the urine of the rats to which β-methionine was administered (Fig. 5).

The urine chromatogram obtained from the rats fed with β-methionine was further checked for radioactivity by applying the autoradiography technique. Two dark spots appeared on the X-ray film (Fig. 6), corresponding to the dark spot of radioactive β-methionine (Fig. 7) and radioactive β-methionine sulfoxide (Fig. 8), respectively.

From the isolated methylacceptors, choline showed to be the most active, the one obtained from the water soluble fraction being more active than the other obtained from the ether soluble fraction. Comparing the activities of the samples per milliequivalent of methyl group, the choline contained about three times as much radioactivity as the methyl group belonging to the creatine from the body tissues.

A far greater part of the whole activity had been incorporated into the body by feeding the rats with α-methionine[12, 15]. It is, however, of interest that in general the same range of activities was found in the above mentioned methylacceptors after administration of β-methionine as after the administration of α-methionine. It may, therefore, be of interest to administer β-methionine to animals under the strict conditions required for testing the compounds for their property of being a methyl donor or not.

## EXPERIMENTAL

*β-Amino-γ-methyl C$^{14}$-thiobutyric acid (β-methionine)* (I)

Methyl iodide C$^{14}$ (0.1 mC — 2,6 mg.) was purchased from the Radiochemical Centre Amersham. The content of the breakseal tube was diluted with the corresponding quantity of inactive methyl iodide by applying the high vacuum distillation in the manifold. The diluted methyl iodide C$^{14}$ was distilled into the cold finger of the trap D (Fig. 1), one arm of which was attached to the manifold. The trap, still immerged in liquid air, was then attached to the condensation apparatus (Fig. 1), the stopcock being closed. The condensation apparatus used was similar to that described for the synthesis of α-methionine-C$^{14}$ [16].

β-Amino-γ-benzylthiobutyric acid[5] (200 mg.; 0,89 mMole) was placed in the flask A (Fig. 1) and dry anhydrous ammonia introduced under cooling till about 20 ml. of ammonia was collected. With stopcock B still closed, dry nitrogen was bubbled through the mixture and the minimum amount of sodium necessary to produce a permanent blue colour was gradually added through a side arm of the flask A. The three-way stopcock C was converted to D when the mixture in flask A had been dissolved. The stopcocks of trap D were cautiously opened and nitrogen introduced till normal pressure was achieved. Then B was opened. The trap D was warmed to the room temperature and then slowly to 70°C. After the

nitrogen had swept all the methyl iodide out, a nitroprusside test was performed and if positive, inert methyl iodide was added to the flask A in small portions as already described[16].

The ammonia was evaporated in the current of nitrogen and the residue dissolved in water, neutralized and treated on Amberlite IR-100 as described for the preparation of the inactive compound[7]. The yield was 93 mg. (70%). After two recrystallizations from 96% ethanol, the radioactivity of the sample remained unchanged, giving a specific activity of 0.71 μC/mg. (Radiochemical yield 60%). One dimensional paper chromatography gave a single spot with ninhydrin resp. p-benzochinone[17] in butanol-ethanol-water (80 : 20 : 20) (Rf = 0.26), as well as in butanol-acetic acid — water (80 : 20 : 20) (Rf = 0.36).

*β-Amino-γ-methylsulfoxibutyric acid (β-methionine sulfoxide)* (II)

32.2 mg. (0.216 mMole) of β-methionine was oxidized by hydrogen peroxide (0.025 ml. conc. hydrochloric acid, + 0.3 ml. methanol + 0.03 ml. 30% hydrogen peroxide, room temp., 30 minutes), by adapting the procedure for the preparation of α-methionine sulfoxide[14]. The following modifications were made: a) the addition of water was omitted in oxidation mixture and b) the obtained β-methionine sulfoxide hydrochloride was passed after being diluted by water to 50 ml. through Amberlite IR—4B. Water was evaporated *in vacuo* and the oily residue dissolved in methanol and treated with charcoal. The solution was concentrated to dryness and the oil dried *in vacuo* over night. After that it was dissolved in abs. methanol (1 ml.), precipitated with aps. ether till turbidity occurred and allowed to stand in a refrigerator for 48 hours. Thereby 20 mg (56%) of colourless crystals were obtained. The substance which proved to be extremely hygroscopic was twice recrystallized from abs. methanol — abs. ether, m. p. 160.5 — 161,5°.

One dimensional paper chromatography gave a single spot with ninhydrin in butanol — ethanol — water (80 : 20 : 20) (Rf = 0.06), as well as in butanol-acetic acid-water (80 : 20 : 20) (Rf = 0.09).

For the analysis the compound was dried at high vacuum for 8 hours at 100°

*Anal.* 7.342 mg. subst.: 9.673 mg. $CO_2$, 4.521 mg. $H_2O$
$C_5H_{11}NO_3S$ (165.206) calc'd: C 36.35; H 6.71%
found: C 35,96; H 6,82%

*Feeding experiments*

*Material*

98.8 mg (0.662 mMole) of chromatographicaly pure β-amino-γ-methyl-$C^{14}$-thiobutyric acid (β-methionine-methyl-$C^{14}$) with a total activity of 76 μC (114.8 μC/mMole, 0.769 μC/mg.) was used. The total activity of administered β-methionine expressed as barium carbonate was $2.23 \times 10^6$ counts per minute (c. p. m.).

*Determination of Radioactivity*

All the measurements were made with the use of a thin mica window G.-M. counter. The samples of »infinite thickness«[18] were mounted on 1 or 2 sq. cm. polythene discs and compared with the $C^{14}$ Amersham standards of the same size, after corrections for background and self absorption were made.

In order to have a better survey of the relations between the administered activity on one side and the activity of the metabolites on the other, samples of β-methionine as well as those of excreted urine and choline and creatinine derivatives were degraded to carbon dioxide by wet combustion[19, 21]. Barium carbonate thus obtained was counted in duplicate, as infinitely thick standard 2.32 sq. cm. plates[22]. The sodium carbonate obtained by the wet combustion of a sample of β-methionine (1.594 mg.) was diluted with inactive sodium carbonate (53 mg.), precipitated as barium carbonate and two barium carbonate plates mounted and counted alternatively ($35.97 \times 10^3$ c. p. m.).

*Methods*

The metabolic studies were undertaken with three young adult female albino rats, weighing 180 — 190 g. Prior to the experiment they had been kept on a protein

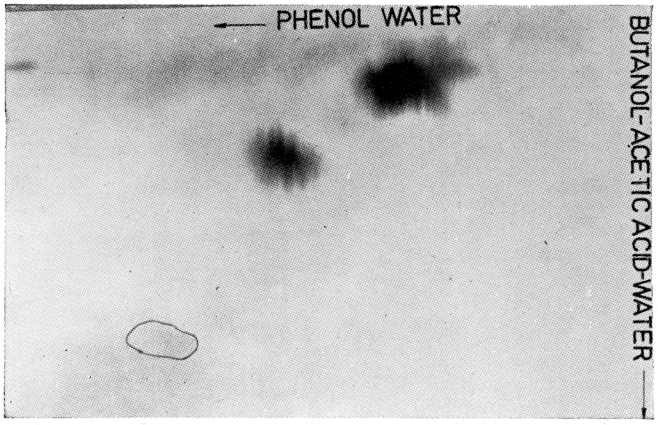



Fig. 2. Chromatogram of normal rat urine.

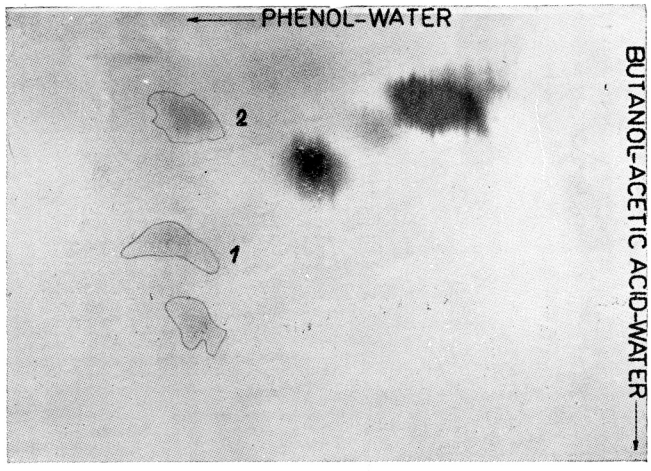



Fig. 3. Chromatogram of the urine of rats which were fed β-methionine-methyl-$C^{14}$.
1. β-Methionine. 2. β-Methionine sulfoxide.

[To face page 18

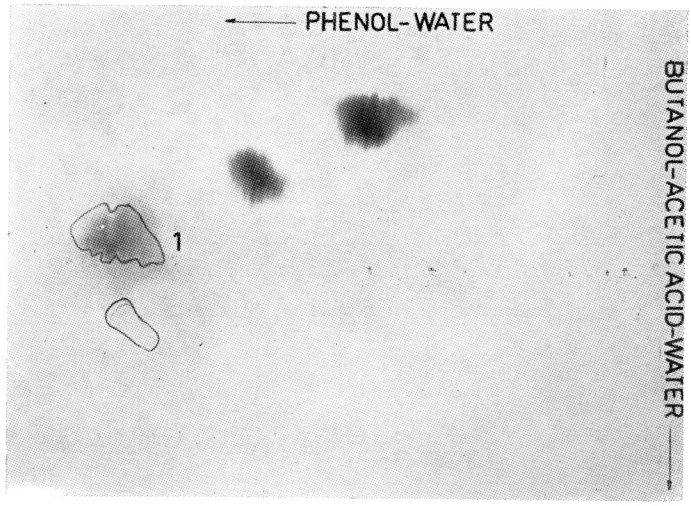



Fig. 4. Chromatogram of normal rat urine with β-methionine-methyl-$C^{14}$ added.
1. β-Methionine.

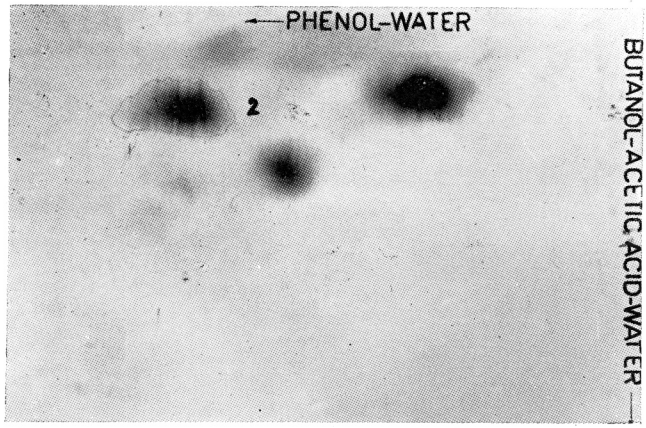



Fig. 5. Chromatogram of normal rat urine with β-methionine-methyl-$C^{14}$-sulfoxide added.
2. β-Methionine sulfoxide.

[To face page 18

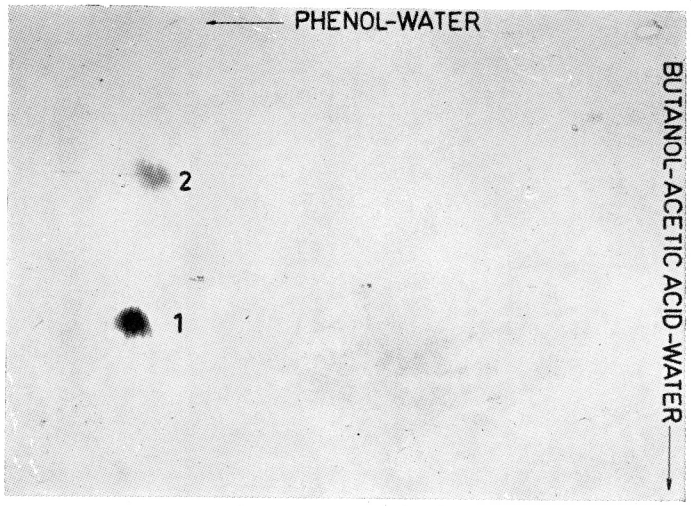



Fig. 6. Radioautogram of the urine of rats which were fed β-methionine-methyl-$C^{14}$
1. β-Methionine. 2. β-Methionine sulfoxide.

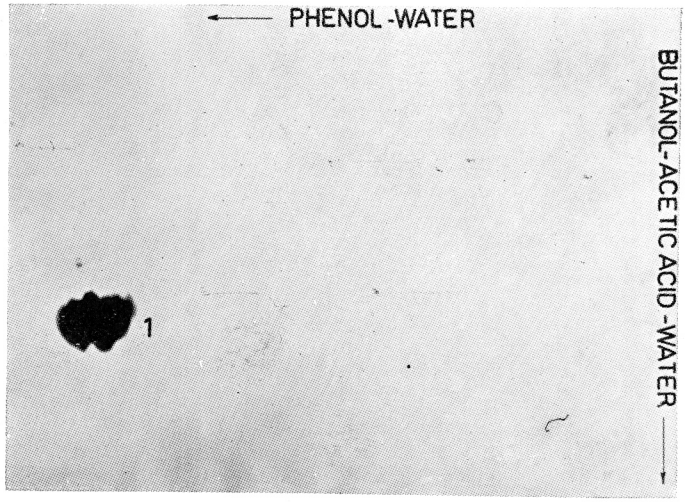



Fig. 7. Radioautogram of normal rat urine with β-methionine-methyl-$C^{14}$ added.
1. β-Methionine.

[To face page 18

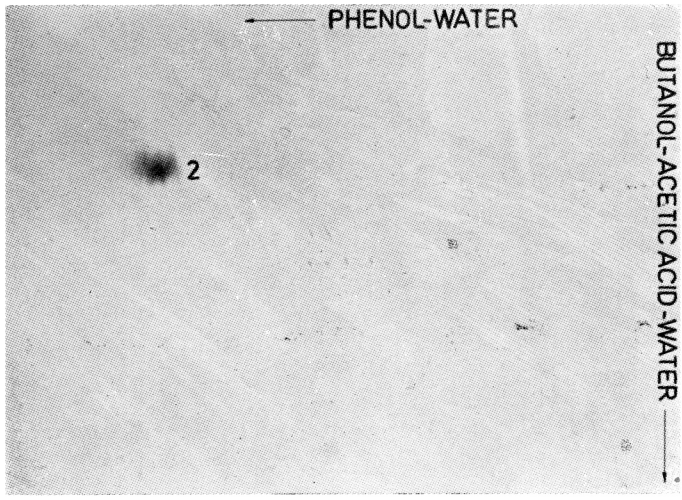



Fig. 8. Radioautogram of normal rat urine with β-methionine-methyl $C^{14}$-sulfoxide added.
2. β-Methionine sulfoxide.

[To face page 18

low diet for ten days. On the 11-th day 98.8 mg. of β-methionine, dissolved in 6 ml. of water was fed by a stomach tube, each animal receiving 2 ml. of the radioactive solution.

The animals were immediately placed in a metabolism cage, constructed for the continuous and quantitative collection of carbon dioxide[12]. Food was withheld during the experiment. Water was available ad libitum.

The expired carbon dioxide was collected in *N* or. 2*N* sodium hydroxide solution ($CO_2$ free) at 3, 6, 12, 24, 36 and 48 hour intervals. The sodium hydroxide solution from each interval was diluted to 1 litre and the barium carbonate samples (50—60 mg. — 2.32 sq. cm.) were prepared in duplicate in the same way as described by du Vigneaud and al.[12]. All the samples showed a negligible activity. The samples of 3, 6 and 12 hour periods gave after several hours of counting a constant of about 2 c. p. m. above background, corresponding to about 8 c. p. m/mMole of $CO_2$ expired. As in the 3, 6 and 12 hour periods 14.020, 13.015 resp 30.050 mg. of barium carbonate (total 57.085 mg., 289.2 mMole) was collected, the activity expired as $CO_2$ in the first 12 hours was about 0.1% of the whole activity administered, expressed as c. p. m., (activity from $CO_2$ collected in 12 hours: $289{,}2 \times 8 = 2.314$ c. p. m.; total act. of the administered compound: $2.23 \times 10^6$ c. p. m.).

The samples of 24, 36, and 48 hour periods showed no activity at all. Thus the efficiency of our mica G.—M. counter was not sufficient to count the expired radioactivity.

At the end of 52 hours the animals were sacrificed under anaesthesia. The 52 hours urine collected under toluene was quantitatively removed and water added up to 80 ml. Aliquot amounts of the whole volume were used for the determination of the whole urine activity, for filter paper chromatography and for the creatinine isolation.

Three 0,8 ml. portions of urine were evaporated to dryness, oxidized by the wet combustion method and counted as standard barium carbonate plates of infinite thickness. The activity thus obtained was $1.86 \times 10^6$ c. p. m. for the whole urine collected, representing 83% of the whole activity administered.

The paper chromatography of urine samples was carried on Whatman No. 1 filter — paper sheets, a two dimensional descending phenol — water and butanol-acetic acid — water (80 : 20 : 20), solvent system being used. The chromatograms were developed with ninhydrin. As can be seen by comparison of Figs. 2 and 3, two new spots appeared in the urine of animals receiving β-methionine. Both spots gave a pale violet blue colour with ninhydrin, typical for β-amino acids[9], and both were radioactive. The lower spot (Rf values: 0.75 — phenol; 0.22 — butanol) was identified as β-methionine by comparing it with a chromatogram of normal urine, obtained under the same conditions, containing an added amount of pure β-methionine, Fig. 4. Applying the same method we identified the upper spot (Rf values: 0.76 — phenol; 0.06 — butanol) as β-methionine sulfoxide, Fig. 5.

The paper chromatograms were then exposed for three days to X-ray films. Radioautography of urine chromatogram obtained from the animals which were fed β-methionine showed two dark spots Fig. 6. They correspond to the dark spot of radioactive β-methionine, Fig. 7, and radioactive β-methionine sulfoxide, Fig. 8, obtained by exposing the chromatograms of Fig. 4 and Fig. 5 to X-ray films.

Creatinine was isolated from the urine (75 ml.) as potassium creatinine picrate[23]. In order to check the purity of the creatinine absorbed on Fuller's earth, all the washings were chromatographed in two samples in a butanol — ethanol — water (80 : 20 : 20) solvent system and sprayed with ninhydrin and picric acid[24]. The purity of the creatinine potassium picrate was controlled by the Jaffe colorimetric reaction. The substance was thrice recrystallized from water and twice from 90% ethanol till a constant specific activity of $6.9 \times 10^{-3}$ μC/mMole was obtained.

*Anal:* 6.560 mg. subst.: 7.487 mg. $CO_2$, 1.172 mg. $H_2O$
$C_{16}H_{12}N_9O_{15}K$ (609.424) calc'd: C 31.53; H 1.99%
found: C 31.15; H 2.00%

*Isolation of choline and creatinine from the tissues.*

After the contents of the gastrointestinal tract had been washed out, the whole bodies of the rats were frozen, finely ground and extracted several times with boiling ethanol. Following the procedure of du Vigneaud and al.[25], choline was isolated as the Reinecke salt from the ether soluble fraction and separately from the water soluble fraction. The Reinecke salts were decomposed, choline, precipitated as chloroplatinate and recrystallized several times from 50% ethanol to constant specific activity.

I. Choline from ether soluble fraction:

a) Reinecke salt:

*Anal:* 7.074 mg. subst.: 5.786 ml. *N*/50 HCl (Microkjeldahl)
$C_9H_{20}N_7OS_4Cr$ (422.556) calc'd: N 23.20%
found: N 22.90%

b) Choline chloroplatinate:

*Anal:* 6.923 mg. subst.: 2.210 mg. Pt
$C_{10}H_{28}N_2O_2Cl_6Pt$ (616.312) calc'd: Pt 31.68%
found: Pt 31.92%

II. Choline from water soluble fraction:

Choline chloroplatinate:

*Anal:* 5.623 mg. subst.: 1.775 mg. Pt
$C_{10}H_{28}N_2O_2Cl_6Pt$ (616.312) calc'd: Pt 31.68%
found: Pt 31.57%

The pure choline chloroplatinate obtained from the ether soluble fraction was then converted by wet combustion into carbon dioxide and counted as barium carbonate plates. The activities of the choline derivatives plated directly (expressed as $\mu$C/mMole) as well as those converted to barium carbonate (expressed as c. p. m./mMole) are summarized in the Table I.

TABLE I

*Incorporation of β-Methionine-methyl-$C^{14}$ into labile methyl groups.*
*Single dose administered:* 98.8 *mg.* — 76 $\mu$C — $2.23 \times 10^6$ *c. p. m.* — $3.37 \times 10^6$ *c. p. m. per mMole.*

| Compound | $\mu$C per mMole $\times 10^{-3}$ | c. p. m. per mMole | c. p. m. per m. eq. of methyl | RSA* |
|---|---|---|---|---|
| Choline (water sol. fract.) | | | | |
| as Reinecke salt | 25.3 | | | |
| as chloroplatinate | 25.5 | | | |
| Choline (ether sol. fract.) | | | | |
| as Reinecke salt | 21.3 | | | |
| as chloroplatinate | 19.0 | 319 | 106 | 0.00316 |
| Creatinine (from tissue) | | | | |
| as potassium picrate | 2.3 | | | |
| as pure creatinine | 1.6 | 38 | 38 | 0.00112 |
| Creatinine (from urine) | | | | |
| as potassium picrate | 6.9 | | | |

*Relative specific activity = (c. p. m. per mMole of isolated compound) / (c. p. m. per mMole of administered compound) $\times$ 100.

Creatine from the tissue was isolated from the water soluble fraction as creatinine potassium picrate and recrystallized several times from water resp. 90% ethanol to constant specific activity.

*Anal:* 6.472 mg. subst.: 7.410 mg. $CO_2$, 1.250 mg. $H_2O$
14.083 mg. subst.: 10.261 ml. *N*/50 HCl (Microkjeldahl)
$C_{16}H_{12}N_9O_{15}K$ (609.424) Calc'd: C 31.53; H 1.99; N 20.68%
found: C 31.24; H 2.16; N 20.40%

The picrate was oxidized by wet combustion and counted as barium carbonate plates in quadruple. The pure creatinine was isolated from its picrate salt[26] and recrystallized from 96% ethanol to constant specific activity. The activities are summarized in Table I.

*Acknowledgments.* The authors are indebted to the Microchemical Laboratory of the Institute »Ruđer Bošković« (supervision prof. L. Filipović) for the microanalyses. They also wish to acknowledge the valuable technical assistance of Miss Đurđa Brebrić.

REFERENCES

1. V. du Vigneaud, S. Simmons, J. P. Chandler and M. Cohn, *J. Biol. Chem.* **165** (1946) 639.
2. F. Challenger and M. I. Simpson, *Biochem. J.* **41** (1947) XI.
3. G. A. Maw and V. du Vigneaud, *J. Biol. Chem.* **176** (1948) 1037.
4. A. White, P. Handler, E. L. Smith and De Witt Stetten Jr., *Principles of Biochemistry* (1954) 544, Mc Graw-Hill Book Comp.
5. K. Balenović and D. Fleš, *J. Org. Chem.* **17** (1952) 347.
6. K. Balenović, N. Bregant, B. Gašpert, I. Jambrešić and V. Tomašić, *Arhiv kem.* **27** (1955) 207.
7. K. Balenović, D. Fleš and I. Jambrešić, *Croat. Chem. Acta,* **28** (1956) 303.
8. D. Keglević-Brovet, *Int. Conf. on the Peaceful Uses of Atomic Energ.,* August (1955), Coll. Vol. XII P/967.
9. L. Birkofer und I. Storch, *Chem. Ber.* **87** (1954) 571.
10. L. Birkofer und I. Hartwig, *Chem. Ber.* **87** (1954) 1189.
11. L. Birkofer und A. Birkofer, *Chem. Ber.* **89** (1956) 1226.
12. C. G. Mackenzie, J. P. Chandler, E. B. Keller, J. R. Rachele, N. Cross and V. du Vigneaud, *J. Biol. Chem.* **180** (1949) 99.
13. C. G. Mackenzie, J. R. Rachele, N. Cross, J. P. Chandler and V. du Vigneaud, *J. Biol. Chem.* **183** (1950) 617.
14. G. Toennis and J. J. Kolb, *J. Biol. Chem* .**128** (1939) 399.
15. E. B. Keller, J. R. Rachele and V. du Vigneaud, *J. Biol. Chem.* **177** (1949) 733.
16. D. B. Melville, J. R. Rachele and E. B. Keller, *J. Biol. Chem.* **169** (1947) 419.
17. S. Iskrić, *Arhiv kem.* **24** (1952) 83.
18. G. Popjak, *Biochem J.* **46** (1950) 560.
19. D. D. Van Slyke and J. Folch, *J. Biol. Chem.* **136** (1940) 509.
20. J. H. Weisburger, E. K. Weisburger and H. P. Morris, *J. Am. Chem. Soc.,* **74** (1952) 2399.
21. A. Lindenbaum, J. Schubert and W. D. Armstrong, *Anal. Chem.* **20** (1948) 1120.
22. F. C. Henriques, G. B. Kistiakowsky, C. Margnetti and W. G. Schneider, *Ind. Eng. Chem. Anal. Ed.* **18** (1946) 349.
23. K. Bloch and R. Schönheimer, *J. Biol. Chem.* **131** (1939) 111.
24. S. R. Arnes and H. A. Risley, *Proc. Soc. Exptl. Biol. and Med.* **69** (1948) 267.
25. V. du Vigneaud, M. Cohn, J. P. Chandler, J. R. Schenck and S. Simmonds, *J. Biol. Chem.* **140** (1941) 625.
26. G. L. Foster, R. Schönheimer and D. Rittenberg, *J. Biol. Chem.* **127** (1939) 319.

## IZVOD

### O metabolizmu β-metionina-metil-$C^{14}$ *in vivo*

*D. Keglević-Brovet, A. Kornhauzer, B. Ladešić i S. Iskrić*

*u suradnji s*

*N. Allegretti i L. Rabadija*

β-Amino-γ-metil-$C^{14}$-tiomaslačna kiselina (β-metionin-metil-$C^{14}$) dan je štakorima u jednoj dozi. Izdisani ugljični dioksid pokazivao je neznatnu aktivnost. Najveći dio aktivnosti nađen je u urinu kao nepromijenjena supstanca i kao njezin oksidacioni produkt β-Amino-γ-metil-$C^{14}$-sulfoksimaslačna kiselina (β-metionin sulfoksid). Holin i kreatinin izolirani iz tkiva, te kreatinin izoliran iz urina bili su slabo aktivni. Prema dobivenim rezultatima zaključujemo, da u prilikama, u kojima se radilo: 1) živi organizam ne oksidira β-Metionin u ugljični dioksid, 2) da se glavni dio spoja izlučuje u urinu nepromijenjen, kao i u svojoj oksidacionoj formi, 3) radioaktivni ugljik metilne grupe β-Metionina u maloj je količini ugrađen u molekule metil-akceptora.

RADIOIZOTOPNI LABORATORIJ,
INSTITUT »RUĐER BOŠKOVIĆ«
ZAGREB

Primljeno 19. siječnja 1957.