METABOLIC CONVERSION OF CYCLOCARBO- THIAMINE INTO THIAMINE¹

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In the preceding paper, it was demonstrated that a new thiamine derivative, cyclocarbothiamine, raises thiamine levels in the blood and tissues much more than does thiamine when administered either orally or intravenously (1). These findings, considered together with its full thiamine activity *in vivo*, suggest that this compound may be readily transformed into thiamine in the body. This paper presents the detailed study on this transformation, examined *in vivo* as well as *in vitro*.

EXPERIMENTAL

1. Transformation of Cyclocarbothiamine into Thiamine *in vitro*

Blood—Nine ml of rabbit blood, citrated, was mixed with 1.0 ml of physiological saline containing 100 μ g of cyclocarbothiamine and incubated at 37°. At intervals, aliquots of 1.0 ml were removed and immediately added to 10 ml of 0.1 N H₂SO₄. Thiamine was determined by the method of Fujiwara *et al.* (2,3). For the study of the distribution of thiamine formed, 5.0 ml of the incubation mixture was centrifuged at 3000 rpm for 10 minutes, the separated residue of erythrocytes was washed twice with 3.0 ml of the saline and washings were combined with the plasma fraction. The plasma plus washings as well as the residual erythrocytes was assayed for thiamine in the same manner.

In order to see how plasma and erythrocytes affect the compound individually, the two fractions were also studied separately. One ml of physiological saline containing 100 μ g of cyclocarbothiamine was mixed with 5.0 ml of either plasma or erythrocytes and made up to 10 ml with the saline.

Subsequent procedure was the same as described above. When erythrocyte residue was used, the distribution of thiamine was examined simultaneously.

Tissue extracts—Male Wistar strain rat, weighing 150 g, was killed by decapitation and the liver, kidney and small intestine were immediately excised. The tissues were homogenized in 9 volumes of Krebs-Ringer phosphate buffer (pH 7.4) at 0° using a Teflon homogenizer and the homogenates were centrifuged at 5000 rpm for 15 minutes in the cold.

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Five ml of each supernatant was mixed with an equal volume of the same buffer containing 100 μ g of cyclocarbothiamine and incubated at 37°. Aliquots of 1.0 ml were used for the determination of thiamine.

2. Measurement of Carbon Dioxide Liberated during the Conversion of Cyclocarbothiamine into Thiamine

Carbon dioxide was measured by the conventional Warburg technique. Into the main compartment of a Warburg flask was pipetted 2.0 ml of 0.05 *M* phosphate buffer, pH 7.4, containing 3 μ moles of cyclocarbothiamine. The reaction was started by adding 0.2 ml of heparinized rabbit blood plasma from a side bulb and the flask was shaken at 37° in air. After 10 or 20 minutes, 0.3 ml of 6 *N* H₂SO₄ was tipped in from the second side bulb and the change in gas volume was measured.

Similar experiment was done with 0.2 ml of 20 % KOH in the center well under otherwise identical conditions and the difference between these two measurements was regarded as due to carbon dioxide evolved. Control experiment without cyclocarbothiamine was run simultaneously to correct for the carbon dioxide originally present in the plasma. After reading was taken, the reaction mixture was appropriately diluted with 0.1 *N* H₂SO₄ for the determination of thiamine.

3. Attempt to Detect Cyclocarbothiamine in the Blood and Urine Following Its Oral or Intravenous Administration to Rabbits and Human Subjects

Rabbits—The compound was administered to male rabbits, weighing approximately 2.5 kg, either orally or intravenously under exactly the same conditions as described previously (1).

One ml of blood sample obtained by cardiac puncture was immediately added the same volume of 0.6 *N* HClO₄ and the mixture was kept in an ice bath for to 10 minutes. After the precipitate was removed by centrifugation, the supernatant (0.05 ml) was spotted on Toyo Roshi No. 50 paper and subjected to paper chromatography with the solvent system *n*-butanol: acetic acid: water (4:1:5) by the ascending technique.

Five-hour urine collected by the use of a catheter was directly spotted on the paper and treated similarly. After drying, the paper was cut into ten pieces, each 0.1 *Rf* unit wide, and each piece was moistened with 1 *N* NaOH and 2 *N* HCl, successively, to convert cyclocarbothiamine quantitatively into thiamine. Each strip treated was placed into 6 ml of MaciasR medium (4), which was then sterilized at 100° for 10 minutes and inoculated with sterile saline suspension of *Lactobacillus fermenti*. After incubation at 37° for 20 hours, the filter paper was removed and bacterial growth was measured turbidimetrically with Hitachi Photoelectric Photometer Model EPO-B using filter 61.

Human Subjects—Healthy male subjects, about 25 years of age, received 2 tablets, each containing 25 mg of cyclocarbothiamine.

Three-hour urine was collected in the presence of one ml of acetic acid and subjected to bioautographical study as described above.

RESULTS

1. Formation of Thiamine *in vitro*

As is shown in Fig. 1, cyclocarbothiamine was rapidly transformed into thiamine

when incubated with whole blood. Further examination revealed that the activity for this conversion was higher in the plasma than in the erythrocyte (Fig. 2).

FIG. 1 *Transformation of Cyclocarbothiamine into Thiamine in the Blood (1)* Cyclocarbothiamine was incubated with rabbit blood *in vitro* and formation of thiamine, together with its distribution among blood components, was examined on aliquot samples. Detailed procedure is described in the text.
■, erythrocyte; □, plasma.

FIG. 2 *Transformation of Cyclocarbothiamine into Thiamine in the Blood (2)* Cyclocarbothiamine was incubated with either plasma (○) or erythrocytes (×) and formation of thiamine, together with its distribution in case of the latter, was followed. Detailed procedure is described in the text.
■, erythrocyte; □, plasma.

Interestingly, marked difference was observed in the distribution of thiamine, depending on the presence or absence of plasma. When the incubation was made in its presence, thiamine was detected mainly outside the erythrocyte (Fig. 1), whereas in its absence most of the thiamine was found inside the erythrocyte (Fig. 2).

This presents an apparent contrast to the results obtained *in vivo*, where

FIG. 3 *Distribution of Thiamine Among Blood Components After Oral or Intravenous Administration of Cyclocarbothiamine to Rabbits* Thiamine was determined after Takadiastase treatment. Dose: 10 (oral) or 5 (intravenous) mg equivalent of thiamine per kg body weight. ■, erythrocyte; □, plasma.

thiamine was always found in the erythrocyte in larger amounts in spite of the presence of the plasma (Fig. 3).

Of the three tissues examined, the liver showed the highest activity of producing thiamine (Fig. 4). This activity must be enzymatic in nature, since no thiamine was formed when the tissue extracts were previously heated at 90° for 20 minutes. Incidentally, cyclocarbothiamine does not change into thiamine during the assay procedure of the latter, as demonstrated independently.

FIG. 4 Transformation of Cyclocarbothiamine into Thiamine Catalyzed by Rat Tissue Extracts

○, liver; ×, kidney; △, small intestine.

Experimental details are described in the text.

2. Stoichiometry between Formations of Thiamine and Carbon Dioxide

Manometric measurement showed that carbon dioxide was produced in an amount equimolar to thiamine (Table 1).

TABLE 1

Stoichiometry between Formations of Thiamine and Carbon Dioxide during in vitro Conversion of Cyclocarbothiamine into Thiamine

The reaction mixtures contained in a final volume of 2.2 ml: 100 μ moles of phosphate buffer, pH 7.4, 3 μ moles of cyclocarbothiamine and 0.2 ml of rabbit blood plasma. The reaction was carried out at 37° and carbon dioxide was measured manometrically. The details are given in the text.

Reaction time	CO ₂ evolved	Thiamine formed
<i>min</i>	<i>μmoles</i>	<i>μmoles</i>
10	2.12	2.08
	2.11	2.33
20	2.53	2.73
	2.85	3.00

3. Transformation of Cyclocarbothiamine into Thiamine in vivo

Rabbits—Bioautographical study revealed that not a trace of cyclocarbothiamine existed unchanged in blood samples taken as early as 30 minutes after oral or intravenous administration (Fig. 5). Nor was it detected as such in urine samples (Fig. 6).

FIG. 5 Bioautograms of Thiamine Compounds in the Blood Following Oral or Intravenous Administration of Cyclocarbothiamine to Rabbits

Solvent: *n*-BuOH: CH₃COOH: H₂O (4:1:5, upper phase) Test organism: *Lactobacillus fermenti*. Dose: 20 (oral) or 5 (intravenous) mg equivalent of thiamine per kg body weight.

Detailed procedure is described in the text. Bioautograms of authentic samples were obtained with the supernatant of deproteinized blood sample to which were added appropriate amounts of respective compounds.

FIG. 6 Bioautograms of a Thiamine Compound in Urine Following Oral or Intravenous Administration of Cyclocarbothiamine to Rabbits

The dose was the same as in Fig. 5. Analyses were done on five-hour urine samples.

The only compounds which supported the growth of *Lactobacillus fermenti* were thiamine and its phosphates.

Human Subjects—A trace amount of cyclocarbothiamine was detected unaltered in three-hour urine samples (Fig. 7).

FIG. 7 Bioautograms of Thiamine Compounds in Urine Following Oral Administration of Cyclocarbothiamine to Human Subjects

Analyses were done on three-hour urine samples. Dose: 50 mg per subject.

DISCUSSION

The experimental findings in this paper amply demonstrate the easy metabolic conversion of cyclocarbothiamine into thiamine. The factor which is responsible for this conversion seems to be widely distributed in the body and must be enzymatic in nature, the detailed study of which is in progress.

An apparent discrepancy was noted between *in vitro* and *in vivo* experiment in the distribution of thiamine formed from cyclocarbothiamine in the blood. A larger amount of thiamine was found in the erythrocyte following administration of the compound *in vivo*, whereas the greater part was present in the plasma fraction in the case of incubation experiment *in vitro*. The cause of this phenomenon remains to be clarified.

Kawasaki *et al.* pointed out that the erythrocyte membrane does not allow the free passage of thiamine, while modified thiamine compounds can penetrate into the blood cell easily (5). Consequently, once thiamine is formed from these compounds within the erythrocyte, it is trapped there and does not flow out freely. This is believed to be one of the reasons why modified thiamine compounds lead to higher thiamine levels in the blood. In the light of this finding, persistence of high thiamine contents in the blood brought about by cyclocarbothiamine *in vivo* can be explained by its greater distribution in the blood cell.

A glance at the chemical structure of cyclocarbothiamine suggests that the carbonyl segment of the cyclic thiocarbonate moiety will be split off as carbon dioxide during the process of its transformation into thiamine. The quantitative measurement of carbon dioxide proved this to be the case. Hence, the following metabolic schema has been established.

SUMMARY

Metabolic conversion of cyclocarbothiamine into thiamine was studied *in vivo* as well as *in vitro*.

1. The compound was easily transformed into thiamine when incubated with rabbit blood.

The supernatant obtained from liver and kidney homogenate of rats also changed cyclocarbothiamine into thiamine. No such conversion occurred with heated supernatant.

2. Manometric measurement of carbon dioxide liberated during the conversion showed that it was produced in an amount equimolar to thiamine.

3. Bioautography with *Lactobacillus fermenti* revealed that not a trace of cyclocarbothiamine remained unchanged in blood samples taken 30 minutes after oral or intravenous administration to rabbits. Nor was it detected as such in urine.

However, a trace amount of the compound was detected unaltered in three-hour urine samples following oral administration of 50 mg to human subjects.

4. The following metabolic schema has been established.

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