ACTIVITY OF CARBETHOXYTHIAMINE AND THIAMINE PROPYL DISULFIDE ON *KLOECKERA APICULATA*¹

CHIKATARO KAWASAKI AND SUMIO SHINODA²

School of Pharmacy, Osaka University, Toyonaka

(Received March 8, 1965)

Carbethoxythiamine stimulates the growth of a thiamine-requiring mutant, *Kloeckera apiculata* IFO No. 0630, more than thiamine-HCl. To clarify the reason, various behaviors of CET toward *K. apiculata* was investigated by the authors (1, 2). Asymmetrical thiamine disulfide derivatives, such as TPD, promote the growth of *K. apiculata* like CET more than thiamine (3). In this paper, the behavior of CET toward the growth of *K. apiculata* is compared with that of TPD.

EXPERIMENTAL

1. Culture of *K. apiculata*

To 2 ml of the medium improved by Hoff-Jørgensen (5), as described by the author (4), the samples were added aseptically in a total volume of 4 ml. The cells were inoculated to the medium with a hypodermic syringe with a needle, 1/3 mm in diameter. After incubation at 30° for 20 hours, the growth of the cells was measured spectrophotometrically as the optical density at 610 mμ, using Hitachi Spectrophotometer.

2. Samples

Thiamine, CET, CBT and TPD, each as hydrochloride, were dissolved in sterilized distilled water.

3. Determination of TPD and CET

TPD was reduced to thiamine by cysteine treatment and the thiamine thus produced was determined as thiochrome by the BrCN method. CET was treated with alkali (7) and the thiamine thus produced was determined by the BrCN method.

RESULTS

1. Activities of CET, CBT and Thiamine Treated with Takadiastase

As CET and CBT are known to be easily reduced to thiamine by Takadiastase

¹ The Growth-stimulating Activity of Thiamine Derivatives on Thiamine-requiring Microorganisms. XI. Following abbreviations are used: CET, carbethoxythiamine; TPD, thiamine propyl disulfide; CBT, *S*-carbobutoxythiamine.

² 川崎近太郎, 篠田純男.

(6, 7), both derivatives were treated with Takadiastase to be converted into thiamine and the activities were compared with that of thiamine as follows. To 2 ml of the medium, CET, CBT or thiamine, equivalent to 0.02–0.1 μg thiamine, is added and, in addition, Takadiastase B (thiamine was removed by adsorption on acid clay) is added to give a final concentration of 250 mg per 100 ml in a total volume of 4 ml. The mixtures were incubated at 37° for 24 hours. Thereafter they were heated at 100° for 15 minutes for sterilization of the bacteria contaminated in Takadiastase solution and after cooling, the bacteria were inoculated (Fig. 1, A). The control test was made in the same way but without Takadiastase treatment (Fig. 1, B).

FIG. 1 Activity of CET, CBT and Thiamine after Takadiastase Treatment on the Growth of *K. apiculata*

A, inoculation was made after incubation at 37° for 24 hr with 250 mg Takadiastase per 100 ml

B, inoculation was made without Takadiastase treatment.

○, thiamine; □, CET; △, CBT.

Without Takadiastase treatment, the activity showed in the decreasing order of CET, thiamine, CBT, but after conversion to thiamine by Takadiastase all showed the same activity.

2. Activity of TPD Treated with Cysteine

To 2 ml of the medium, cysteine hydrochloride was added up to a final concentration of 50 mg per 100 ml. After sterilization the samples were added in a total volume of 4 ml. The mixtures were heated at 60° for 30 minutes to reduce TPD to thiamine. After inoculation, the whole were incubated at 30° for 20 hours (Fig. 2, A). The control tests were made with inoculation of the medium, but without heating at 60° for 30 minutes (Fig. 2, B). TPD showed, however, differing from CET, stronger stimulation of the growth than thiamine, though TPD had

FIG. 2 Activity of TPD and Thiamine Treated with Cysteine on the Growth of *K. apiculata*

A, inoculation was made after treatment with 50 mg cysteine per 100 ml at 60° for 30 min.

B, inoculation was made without cysteine treatment.

□, TPD (A); ■, TPD (B); ○, thiamine (A); ●, thiamine (B).

been converted to thiamine extracellularly. TPD equivalent to 1 μ g thiamine produced 102 % thiamine after cysteine treatment under the same condition as described above. Therefore, TPD given in Fig. 2, A is assumed to have completely converted to thiamine after cysteine treatment. Cysteine is known to inhibit the growth of *K. apiculata*, but it was very slight at this concentration.

3. Growth of the Cells Accumulated with TPD or Thiamine

CET was taken up by the cells in the saline. If the cells thus treated were diluted with the medium and were incubated, it showed the activity greater than thiamine treated similarly (2). The same test was made with TPD as follows. To the cell suspension in saline (containing 30 mg dry weight of cells), TPD or thiamine, corresponding to 10 μ g thiamine, was added in a total volume of 5 ml. The mixtures were heated at 30° for 30 minutes. With this treatment, TPD or thiamine added to the suspension was completely accumulated in the cells and TPD was reduced to thiamine. A part of the suspension was centrifuged and the cells were washed twice with saline and suspended in the medium (Fig. 3, A). The other part of the suspension was diluted immediately with saline (Fig. 3, B) without centrifugation. The suspensions containing the vitamin, equivalent to 0.01–0.08 μ g thiamine, were cultured at 30° for 20 hours. TPD, accumulated in the cells being

FIG. 3 Growth of *K. apiculata* Accumulated TPD or Thiamine

A, after accumulation of the vitamin in saline, the cells are collected and washed and diluted with the medium

B, after accumulation of the vitamin in saline, the cells are diluted with the medium without centrifugation.

○, thiamine; ●, TPD.

suspended in saline, showed the same activity as thiamine irrespective of the treatment of centrifugation and washing, a behavior quite different from CET.

4. Activity of TPD and Thiamine on *K. apiculata* (Effects of the Amounts of the Cells Inoculated)

That TPD showed the same activity on the growth of *K. apiculata* as thiamine may be due to the fact that more cells were inoculated than in the ordinary culture. On the other hand, it was observed in the culture of *Lactobacillus fermenti* 36 that the difference of the activities between TPD and thiamine was the more marked, the less bacteria were inoculated (8). The effects of the amounts of the inoculated *Kloeckera* were therefore investigated as follows. To the medium containing TPD or thiamine equivalent to 0.05 µg thiamine, the cells 0.1 to 10³ times the amount usually employed were inoculated and the whole were cultured at 30° for 20 hours (Fig. 4). In the experiment given above (Fig. 3), the yeast about 10 to 100 times the amount usually employed was used. Within this range, TPD showed definitely greater activity than thiamine.

5. Reduction of TPD and CET to Thiamine by Cell Suspension

To the cell suspension in saline containing the bacteria (about 10 mg as dry weight) was added TPD or CET equivalent to 10 µg thiamine and the whole was

FIG. 4 Activity of TPD and Thiamine on the Growth of *K. apiculata*
(Effects of the Amounts of Inoculated Cells)

Amounts of TPD and thiamine: equivalent to 0.05 μ g thiamine per tube.

○, thiamine; ●, TPD.

incubated at pH 4.7 and 30°. After a certain period, the reaction was stopped by addition of 5 ml 10 % metaphosphoric acid, and the thiamine in the cells was extracted for 10 minutes in a boiling water bath. After centrifugation, the supernatant was assayed for free and total thiamine including TPD and CET (Table 1). Remarkable differences were observed between the conversion of TPD and CET into thiamine; TPD was completely reduced to thiamine but CET was a little reduced.

TABLE 1
Fermentation of Thiamine from TPD and CET by Cell Suspension
Amounts of TPD and CET, equivalent to 10 μ g thiamine; cells, 10 μ g (dry weight); total volume, 5 ml; 30°, pH 4.7.

	Incubation time (min)					
	0	20	40	60	80	100
TPD	<i>per cent</i>					
	11 (98) ^a	96 (94)	95 (96)	97 (96)	98 (98)	94 (96)
CET	0 (96) ^a	7 (95)	7 (99)	8 (96)	9 (100)	12 (94)

^a Total thiamine estimated including TPD or CET.

DISCUSSION

Both TPD and CET were shown to stimulate the growth of *K. apiculata* more than thiamine, but the behaviors of the two toward the yeast were different. CET and CBT showed the same activity as thiamine when they were reduced to thiamine extracellularly, whereas TPD showed the activity greater than thiamine, when it was converted to thiamine extracellularly. Both of them became active after conversion to thiamine. Therefore, the propylmercapto or carbethoxy radical which was produced during conversion to thiamine, is assumed to be effective for the growth. From the findings of the authors it is assumed that the propylmercapto group was effective extracellularly and carbethoxy radical intracellularly. But it is to be noted that CET was heat-sterilized after Takadiastase treatment. If the carbethoxy group is evaporated by heat sterilization, the above assumption may be untenable. To avoid the contamination in Takadiastase preparation, the suspension was sterilized after Takadiastase treatment, but the effect may be clarified by comparing the suspension after aseptic addition of CET-degrading enzyme. When the cells in which CET and TPD had been accumulated in saline were diluted with the medium, CET showed the activity greater than thiamine (2), but TPD was as active as thiamine. It may be due to the fact that TPD was easily reduced to thiamine by the contact with the cells in saline. It is a good contrast to the finding that TPD showed the activity greater than thiamine, when it was reduced in the medium. CET is hardly reduced to thiamine in saline, but was gradually reduced in the medium when the cells were in the accelerated growth (1, 2). It was thus concluded that TPD and CET stimulate the growth of *K. apiculata* but each of them behaves differently in the mechanism of growth-stimulation.

SUMMARY

Both thiamine propyl disulfide (TPD) and *S*-carbethoxythiamine (CET) stimulate the growth of *K. apiculata* but they show the following different behaviors.

1. When CET is reduced to thiamine extracellularly by Takadiastase, it shows the same activity as thiamine on the growth of the bacterium.
2. When TPD is reduced to thiamine by cysteine extracellularly, it shows the activity greater than thiamine.
3. When TPD accumulated in the cells in saline are diluted with the medium and incubated, they show the same activity as thiamine, but when CET is treated in the same way, it shows the activity greater than thiamine.
4. TPD is easily reduced to thiamine in the presence of the yeast suspension but CET is slowly converted to thiamine on the same treatment.

REFERENCES

1. Kawasaki, C., Hiraoka, E. and Shimamoto, T., *Vitamins*, **28**, 295 (1963).
2. Kawasaki, C. and Shinoda, S., *Vitamins* **28**, 299 (1963).
3. Kawasaki, C. and Yamada, C., *Vitamins* **28**, 290 (1963).
4. Kawasaki, C., Hiraoka, E. and Shimamoto, T., *Vitamins* **22**, 387 (1961).
5. Hoff-Jørgensen, E. and Hansen, B., *Acta Chem. Scand.* **9**, 562 (1955).
6. Kawasaki, C., Ono, Y. and Shinoda, S., *Vitamins* **25**, 520 (1962).
7. Yamamoto, R., Inazu, K. and Shima, K., *Vitamins* **25**, 478 (1962).
8. Kawasaki, C., Ichikawa, T., Shinoda, S. and Morishita, Y., *Vitamins* **30** 89 (1964).