Antithiamine Effect of Ethyldeoxythiamine¹

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Ethyldeoxythiamine having a CH_3CH_2 -radical in place of CH_3 at C-2 position of pyrimidine moiety of deoxythiamine was synthesized by applying the method of ethylthiamine synthesis. The following chemical and physical properties were similar to those of deoxythiamine and thiamine: stability to heat, formation of thiochrome form with BrCN . The biological activities of this compound was first tested by two kinds of microorganisms which require thiamine, *Lactobacillus fermenti* and *Kloeckera apiculata*. Ethyldeoxythiamine showed thiamine antagonistic activity at the same level as deoxythiamine to both organisms. Inhibition indices of ethyldeoxythiamine for growth and for thiamine uptake were as follows: growth 280 (*L. fermenti*), 380 (*Kl. apiculata*); thiamine uptake 10 (*L. fermenti*), 50 (*Kl. apiculata*).

A series of systematic studies on thiamine antagonists which modified the hydroxyethyl group on C-5 position of the thiazole moiety of thiamine have been reported by the authors (1-4). Some of these compounds show an effective anticoccidial activity and two of them, deoxymethylthiamine and chlorothiamine, are now used commercially as anticoccidants. The authors chose deoxythiamine as an experimental material for the more detailed studies and have reported its mechanism of action on several enzymes in the microorganism and animals (5-7). Deoxythiamine having an ethyl group in place of the hydroxyethyl group competes mainly with thiamine in the thiamine uptake systems, leading to the growth inhibition of thiamine requiring microorganisms. The thiamine antagonistic effects of deoxythiamine on

Lactobacillus fermenti and *Kloeckera apiculata* are the strongest among the series of compounds reported by the authors. To elucidate the relationship between the chemical structure and the antithiamine activity, several modifications of deoxythiamine have been studied (8, 9). This paper describes the antithiamine effects of ethyldeoxythiamine which has

¹ This paper was partly presented at the 89th Meeting of the Pharmaceutical Society of Japan held in 1969. Following abbreviations are used: deoxythiamine, 3,2'-methyl-4'-aminopyrimidyl-(5')-methyl-4-methyl-5-ethyl thiazolium chloride hydrochloride; ethyldeoxythiamine, 3,2'-ethyl-4'-aminopyrimidyl-(5')-methyl-4-methyl-5-ethyl thiazolium chloride hydrochloride; deoxymethylthiamine, 3,2'-methyl-4'-aminopyrimidyl-(5')-methyl-4,5-dimethyl thiazolium chloride hydrochloride; chlorothiamine, 3,2'-methyl-4'-aminopyrimidyl-(5')-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride, ethyldiamine, 2-ethyl-4-amino-5-aminomethyl-pyrimidine.

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an ethyl group in place of the methyl group on C-2 position of the pyrimidine moiety as a part of the series of modifications of deoxythiamine.

EXPERIMENTAL

1. Chemicals

Ethyldeoxythiamine; The pyrimidine moiety (ethylidiamine) of ethyldeoxythiamine was synthesized from propioamide and 2-methoxyethoxymethyl-3-ethoxypropionitrile according to the method reported by Ogawa and Kasahara (10). Ethyldeoxythiamine was synthesized through the condensation and cyclization of ethyldiamine, CS₂ and monochloride of methylpropioketone applying Matsukawa's thiamine synthesis method through thiothiamine (11). mp 225–233 (decomposed). Anal. Calcd. for C₁₃H₂₀N₄SCl₂ · 1½H₂O: C 43.09, H 6.40, N 15.47. Found: C 43.09, H 6.23, N 15.72. 2-methoxyethoxymethyl-3-ethoxypropionitrile was a generous gift from Sankyo Research Laboratories. Thiamine-¹⁴C-HCl was a generous gift from Takeda Chemical Industries. Other reagents were of analytical grade.

2. Determination of Ethyldeoxythiamine

Ethyldeoxythiamine was determined in a similar manner to thiamine determination; To 5 ml of ethyldeoxythiamine solution including 1–5 µg, 3 ml of BrCN and 3 ml of 30 % NaOH were added. To this mixture, 15 ml of isobutanol were added and mixed well to extract the thiochrom form of ethyldeoxythiamine. After standing for a while, the isobutanol layer was transferred into test tubes and water was removed with Na₂SO₄. The fluorescence was measured by Hitachi FPL-2 fluorometer.

3. Growth Inhibition Test of Microorganisms

Organisms—As thiamine requiring organisms, *Lactobacillus fermenti* 36 (ATCC 9338), a strain of lactic acid bacteria, and *Kloeckera apiculata* (IFO 0630), a strain of yeasts were used.

Stock of Organisms—The medium for lactic acid bacteria (peptone 1 %, glucose 1 %, Na-acetate 1 %, yeast extract 0.5 %, agar 2 %) was used for the stock of *Lactobacillus fermenti* and malt extract slant agar medium (malt extract 10 %, agar 2 %) was used for that of *Kloeckera apiculata*. Each organism was subcultured every 2 weeks and stored in refrigerator.

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Preparation of Inocula—Stock culture of *Lactobacillus fermenti* was inoculated to 2 ml of Macias R medium (12) containing 1 µg of thiamine and the cells were harvested after preculture for 24 hr at 37°, washed twice with sterile saline solution and diluted up to 5 times volume with sterile saline. *Kloeckera apiculata* was incubated at 30° in Hoff-Jørgensen's medium (13). The final dilution was 50 times.

Bioassay Method—To 4 ml of basal medium containing a constant amount of thiamine (0.1 µg) and varying amounts of ethyldeoxythiamine, one drop of the cell suspension mentioned above was inoculated. After 24 hr incubation, the growth of cells was measured using a Hitachi 101 spectrophotometer and readings of optical density at 610mµ were obtained. Macias R medium and Hoff-Jørgensen's medium were used for *Lactobacillus fermenti* and *Kloeckera apiculata*, respectively.

4. Experiment on Uptake of Thiamine by Organisms

Lactobacillus fermenti grown in basal medium containing 50 µg of thiamine/4 ml for 24 hr at 37° or *Kloeckera apiculata* grown in basal medium containing 6 µg of thiamine/4 ml for 24 hr at 30° were harvested, washed twice with saline and suspended in saline adjusted to an optical density of 0.6 at 610 mµ. A number of 8 ml suspensions (including 4.2 mg of dry weight cells) were centrifuged. Four milliliters of basal medium containing a constant amount of thiamine-¹⁴C-HCl and varying amounts of ethyldeoxythiamine were added to the separated cells. Each mixture was incubated for 30 min on a shaker at 37° in the case of *Lactobacillus fermenti*, and 30° in the case of *Kloeckera apiculata*. After the incubation, the cells were separated by centrifugation, washed twice with water and suspended in 4 ml of 0.1 N HCl. The cell suspensions were then heated in a water bath

at 100° for 30 min. After centrifuging, the supernatants were used for the determination of thiamine-¹⁴C-HCl.

5. Determination of Thiamine-¹⁴C-HCl

One half milliliter of supernatant was used for the measurement of radioactivity of thiamine-¹⁴C-HCl by Aloka Radiation Counter Model TDC-2.

RESULTS

1. Inhibitory Effect of Ethyldeoxythiamine on the Growth of *Lactobacillus fermenti*

As shown in Fig. 1, the growth of *Lactobacillus fermenti* showed inhibition in the presence of 20 μ g of ethyldeoxythiamine with 0.1 μ g of thiamine and it was completely inhibited by 80 μ g of ethyldeoxythiamine. The inhibition index for the 50 % growth inhibition was 280 and this was almost same as that for deoxythiamine (inhibition index 200).

2. Inhibitory Effect of Ethyldeoxythiamine on the Growth of *Kloeckera apiculata*

The growth of *Kloeckera apiculata* was inhibited by ethyldeoxythiamine in proportion to the amount of ethyldeoxythiamine. The inhibition index was about 380 and similar to that of deoxythiamine 310 (Fig. 2).

FIG. 1 Growth inhibitory effect of ethyldeoxythiamine on *Lactobacillus fermenti*

●—●, Deoxythiamine; ○—○, ethyldeoxythiamine. 0.1 μ g of thiamine was added to each test tube.

FIG. 2 Growth inhibitory effect of ethyldeoxythiamine on *Kloeckera apiculata*

●—●, Deoxythiamine; ○—○, ethyldeoxythiamine. 0.1 μ g of thiamine was added to each test tube.

FIG. 3 Effect of ethyldeoxythiamine on uptake of thiamine by *Lactobacillus fermenti*

●—●, Deoxythiamine; ○—○, ethyldeoxythiamine. 2 μ g of thiamine-¹⁴C-HCl were added to each test tube.

3. Effect of Ethyldeoxythiamine on Uptake of Thiamine by *Lactobacillus fermenti*

In the presence of 2 μ g of thiamine-¹⁴C-HCl, 20 μ g of ethyldeoxythiamine caused about 75% of inhibition for thiamine uptake and 80 μ g caused about 100 % inhibition (Fig. 3). The inhibition index at 50 % inhibition was about 10 and it was almost the same as that of deoxythiamine (inhibition index 9).

4. Effect of Ethyldeoxythiamine on Uptake of Thiamine by *Kloeckera apiculata*

As shown in Fig. 4, ethyldeoxythiamine

FIG. 4 Effect of ethyldeoxythiamine on uptake of thiamine by *Kloeckera apiculata*

●—●, Deoxythiamine; ○—○, ethyldeoxythiamine. 2 μg of thiamine-¹⁴C-HCl were added to each test tube.

inhibited the uptake of thiamine by *Kloeckera apiculata* and the inhibition index was about 50 (deoxythiamine 45).

DISCUSSION

Applying Ogawa and Kasahara's ethyldiamine synthesis method (10) and Matsukawa's thiamine synthesis method (11), ethyldeoxythiamine was synthesized. This compound was oxidized quantitatively with BrCN to form its thiochrome form. By using this reaction, the determination of this compound was performed. This compound was heat stable in acidic solution and converted to thiochrome form with BrCN in the similar manner as deoxythiamine and thiamine. Thiamine antagonistic effects on growth and on uptake of thiamine in *Lactobacillus fermenti* and *Kloeckera apiculata* were at almost the same levels as those of deoxythiamine. Judging from these results, substitution of CH₃ radical at C-2 position of pyrimidine ring for CH₃CH₂ seems not to lead to much difference in the antithiamine activity. Similar findings have been reported by Schults (14) and Aramaki (15) on ethylthiamine, an ethyl analogue of thiamine, which shows almost

the same activity as thiamine. However, several reports have offered opposite view points on this compound. Mano and Kaziyo (16,17) reported that ethylthiamine inhibits thiaminokinase in rats and yeast. Deoxythiamine competes with thiamine at the thiamine uptake systems and not at the enzyme system (6,7). It seems that ethyldeoxythiamine competes with thiamine at the thiamine uptake system in the same manner as deoxythiamine. As expected, anticoccidial activity of ethyldeoxythiamine was observed to be similar to those of deoxythiamine and amprolium. The authors will present detailed data on the anticoccidial activity of deoxythiamine analogues in another paper.

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