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Author(s)	Suzue, Ryokuero
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Studies on the Degradation of Thiamine. (I)

The Degradation of Thiazole

Ryokuero SUZUE*
(Hayaishi Laboratory)

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Degradation of 4-methyl-5-β-hydroxyethylthiazole was demonstrated with soil bacteria. In this reaction, seven μ moles of oxygen were consumed per one μ mole of thiazole, and four μ moles of carbon dioxide were evolved. The sulfate ion was also detected as $BaSO_4$ by precipitation with $BaCl_2$.

With the cell-free enzyme system, one μ mole of oxygen was consumed and a new metabolic intermediate was detected. This substance was identified as 4-methylthiazole-5-acetic acid.

This enzyme was purified about 20 fold and its properties were studied. The maximum enzymic activity was observed by the addition of NAD and FAD.

It may be concluded that this reaction was probably a two steps reaction which needed NAD and FAD as coenzymes.

INTRODUCTION

The metabolic fate of thiamine has been studied on numerous occasions in various species of animals and bacteria. Despite the fact that thiamine is known to be hydrolyzed to its thiazole and pyrimidine moieties by thiaminase, further metabolism of them are not known yet[1]. A number of metabolites were found in the urine which were derived from either thiazole-2-C^{14}-thiamine or thiazole-S^{35}-thiamine[2,3,4]. Only three of these were identified as thiamine disulfide, thiochrome and the thiazole moiety of thiamine, but further metabolites were not identified yet.

This paper describes the isolation of a bacterium from soil by the technique of enrichment culture, degradation of 4-methyl-5-β-hydroxyethylthiazole (the thiazole moiety of thiamine) by the soil bacterium and by its cell free extract, identification of the intermediate as 4-methylthiazole-5-acetic acid, purification of the enzyme which dehydrogenates the thiazole moiety of thiamine and studies of its properties.

MATERIALS AND METHODS

Growth of bacteria. *Pseudomonas sp.*, isolated from the soil, was found to grow in a synthetic medium which contained thiazole as the sole carbon and nitrogen sources[5,6,7].

Cells were grown on the medium of the following basal composition: 0.2% K_2HPO_4, 0.1 % thiazole and 0.05 % $MgSO_4$ in 500 ml. of water. In such synthetic

* 鈴江　緑衣郎

media, the growth yields were low. When a large amount of cells was required, *Pseudomonas* was cultured for 16 hours at 30° in the following medium; 0.1 % thiazole, 0.15 % K_2HPO_4, 0.05 % KH_2PO_4, 0.05 % dried yeast extract and 0.3 % polypeptone (Wako Pure Pharmacy Co.) in 1.5 liters of water. The cells were harvested by centrifugation and stored below 0° in a deep-freezer until needed.

Oxygen uptake and carbon dioxide evolution. The oxygen consumption and the CO_2 evolution were measured by the conventional Warburg technique.

Preparation of cell free extracts. Ten grams of the frozen *Pseudomonas* cell were suspended in 25 ml. of phosphate buffer (0.05 *M.*, PH 7.5) and disrupted for 10 minutes by a sonic oscillator. The supernatant, after 60 minutes of centrifugation at 100,000×*g.*, was used as cell free extract.

Determination of enzyme activities and protein content. Activities of the thiazole dehydrogenating enzyme system were routinely determined by solvent extraction with radioactive 2-C^{14}-thiazole. Five mg. of the enzyme protein, 600 mμ moles of NAD, 300 mμ moles of thiazole (15,000 c. p. m.) and 25 μ moles of phosphate buffer pH 7.5 (total volume was 0.5 ml.) were incubated at 37° for 60 minutes. At the end of the reaction, 0.1 ml. of 2 per cent perchloric acid was added to the reaction mixture and the precipitated proteins were centrifuged off. To 0.3 ml. of the supernatant solution, 0.3 ml. of 10 per cent K_2CO_3 was added and again centrifuged. To 0.3 ml. of the supernatant solution, 0.5 ml. of water saturated chloroform was added and the mixture was shaken vigorously. Aliquots (0.1 ml. and 0.05 ml.) of the water layer were transferred to aluminium planchets and their radioactivities were counted by the Nuclear Chicago Gas Flow Counter with the thin mica window. The enzyme activity was expressed in terms of the counts per minute in 0.1 ml. of the water layer. This method is based on the fact that in alkaline solutions thiazole is completely extracted to the chloroform layer, whereas 4-methylthiazole-5-acetic acid remains in the water layer.

Protein in the enzyme preparation was determined by the method of Warburg and Christian[8]

2-C^{14}-thiazole. The synthesis was carried out according to the procedure of Williams and Ronzio[9]. The radioactive specific activity of thiazole obtained was 146,000 c.p.m. per μ mole and it showed a single spot when analyzed by paper chromatography with several different solvent systems.

4-Methyl-thiazole-5-acetic acid. 4-methyl-thiazole-5-acetic acid was prepared by the method of Cerecedo *et al*[10].

Dragendorff and Tollen's reagents. Dragendorff and Tollen's reagents were prepared by the method of Zaffaroni *et al.*[11].

Chemical materials. Thiazole and γ-chloro-γ-acetopropanol were generously furnished by Drs. Suzuoki and Yurugi, Takeda Pharmacy Co.

C^{14}-thiourea was purchased from the Daiichi Pure Chemicals Co. Ltd. and its specific activity was 1.4 mc/m moles.

EXPERIMENTAL AND RESULTS

Degradation of thiazole by the growing cells. Bacteria were inoculated on 500 ml. of phosphate buffer, pH 8.3, containing 0.1 per cent thiazole in an one liter

Erlenmeyer flask. Every three days, 10 ml. of aliquots were taken off from this culture medium, centrifuged and the supernatant solution was diluted 50 times with water. The amount of thiazole remained was determined by the absorbancy at 250 mμ at which thiazole had the maximum absorption. For 20 days, thiazol was decomposed completely. The optical density of bacteria at 660 mμ was increased from 0.013 to 0.150 during the above period.

Production of sulfate ions was recognized by the precipitation as $BaSO_4$ at the same time. However, no free ammonium ion was detected by Nessler's reagent.

Oxygen uptake and carbon dioxide evolution. The induced cells were suspended in 0.1 *M* phosphate buffer, pH 8.3. The compartment of a Warburg flask contained 1.5 ml. of the cell suspension in 0.1 *M* phosphate buffer, pH 8.3. The side arm of the flask contained either 1.0 or 0.5 μ mole of thiazole in 0.2 ml. The oxygen consumption and the CO_2 evolution were measured at 30°.

As shown in Fig. 1, the oxygen consumption essentially completed after 3 hours. By that time, seven μ moles of oxygen were consumed per μ mole of thiazole, and 4 μ moles of CO_2 were evolved. When thiamine was used as the substrate, the consumption of oxygen was not detected.

As shown in Fig. 2, when one μ mole of thiazole was incubated with the cell free extract, 1 μ mole of oxygen consumption was observed. And no CO_2 evolution was detected.

Identification of reaction product. The identification of reaction products was carried out in the following manner: fifty micromoles of 2-C^{14}-thiazole (4-methyl-5-β-hydroxyethylthiazole), (488,000 c.p.m.) were incubated with 15 mg. of the purified enzyme in 5 ml. of 0.25 *M* potassium phosphate buffer, pH 7.5, at 37° with constant shaking. The reaction was stopped after 2 hours by the addition of 3.0

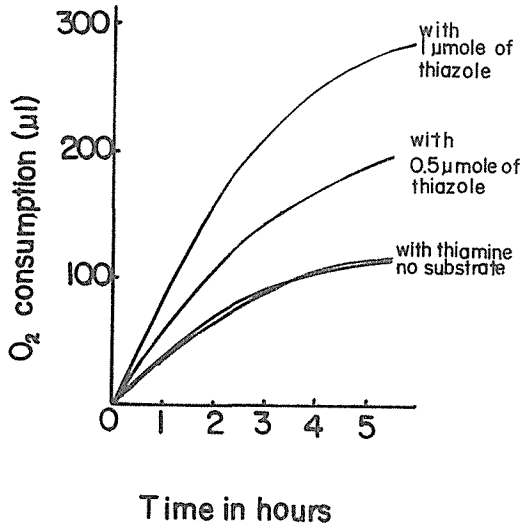

Fig. 1. Oxygen uptake during the degradation of thiazole by whole cells. The reaction vessels contained cells (about 0.5 mg. of dry weight) suspended in 1.5 ml. of 0.1 *M* phosphate buffer (pH 8.3). Oxygen uptake was measured manometrically after the addition of 5 μ mole or 1 μ mole of thiazole.

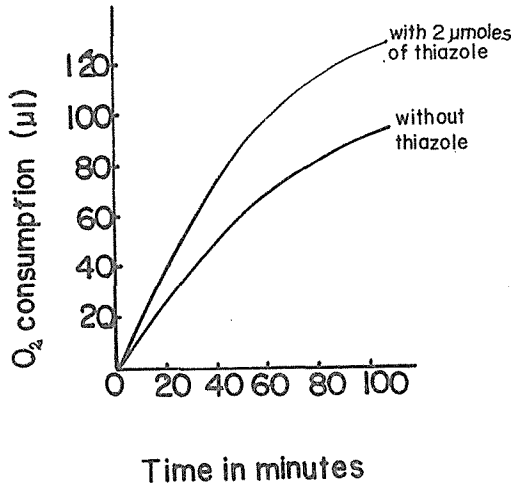

Fig. 2. Oxygen uptake during the degradation of thiazole by cell free extract. The reaction vessels contained cell free extract (about 40 mg. of protein) in 1.5 ml. of 0.25 *M* phosphate buffer (pH 7.5). Oxygen uptake was measured manometrically after the addition of 2μ moles of thiazole.

ml. of 60 % perchloric acid. After centrifugation for 10 minutes at 8,500×*g*., 2 *N* KOH was added to adjust the pH to 7.0. The resulting solution was applied to Whatman No. 31 double thick paper. The ascending paper chromatography was carried out using water saturated secondary butanol as the solvent and the products were detected by quenching under the mineral light. The product obtained by the above method showed an intense spot (Rf=0.35). (Rf value of thiazole = 0.90) Area of the spot on the chromatogram was cut and the material was eluted with water. Analysis of the eluate showed that there was a maximum absorption

Table 1. Constant specific activities of product.

	Yield (mg.)	Total count (c.p.m.)	Specific activity
Original mixture	20.0	30350	44.7
		6900	43.8
CG-50 fraction		8700	49.0
		4400	43.9
Lyophilized mixture	16.6		40.7
1st crystallization			
Supernatant			40.7
Crystal	14.6		36.5
2nd crystallization			
Supernatant			45.3
Crystal	9.8		42.4
3rd crystallization			
Supernatant			44.6
Crystal	6.3		42.8

The original mixture contained 18 mg. of 4-methylthiazole-5-acetic acid and 2 mg. of thiazole decomposed product.

Specific activity was expressed as c. p. m. per one density unit of thiazole acetic acid solution at 250 mμ.

The lyophilized mixture was crystallized from hot water.

peak at 250 mμ as thiazole. The Dragendorff reaction was also positive. But the ninhydrin and Tollen's tests appeared to be negative. The eluate from the paper chromatogram was passed through the CG-50 column (H^+) with water. The percolate from the column was then collected and lyophilized. The amount of the product was 2.95 mg. and its radioactive count was 121,800 c.p.m.

Eighteen mg. of the synthesized 4-methylthiazole-5-acetic acid and 2 mg. of the product were mixed and recrystallized with hot water several times. As shown in Table 1, the specific radioactivity of the crystalline material thus obtained was found to be constant. The melting point of the product was 183° which was close to that of the synthetic 4-methylthiazole-5-acetic acid (185°). The product showed no depression in melting point when admixed with the synthetic sample. The Rf value of the product (0.35), upon chromatographed with water saturated secondary butanol as the solvent, was the same as that of the synthetic thiazole acetate.

The above results suggest this product to be 4-methylthiazole-5-acetic acid.

Isolation and purification of enzyme. The cells of *Pseudomonas* were suspended in the twice volume of 0.02 *M* potassium phosphate buffer, pH 7.5 and were disrupted in a Kubota 10 kc sonic oscillator for 10 minutes. The cell debris was then removed by centrifugation at 15,000×*g*. for 30 minutes. A one-fourth volume of 5 % streptomycin solution was added slowly to the supernatant while stirring, and the mixture was centrifuged again. The pH of the supernatant (26 ml.) was adjusted to 5.4 by 1.0 *N* acetic acid and then treated with 2.6 ml. of calcium phosphate gel (about 19.0 mg. of dry matter per ml). After leaving the mixture at 0° for 30 minutes, the gel was discarded by centrifugation, and 27 ml. of the gel were added to the supernatant solution at pH 4.8. The supernatant fluid was removed by centrifugation and the enzyme was extracted with 35 ml. of 0.5 *M* potassium phosphate buffer, pH 7.5. To 35 ml. of the eluate 6.08 gm. of ammonium sulfate were added and the precipitate was discarded by centrifugation. Upon further addition of 0.84 gm. of ammonium sulfate to the supernatant solution, the

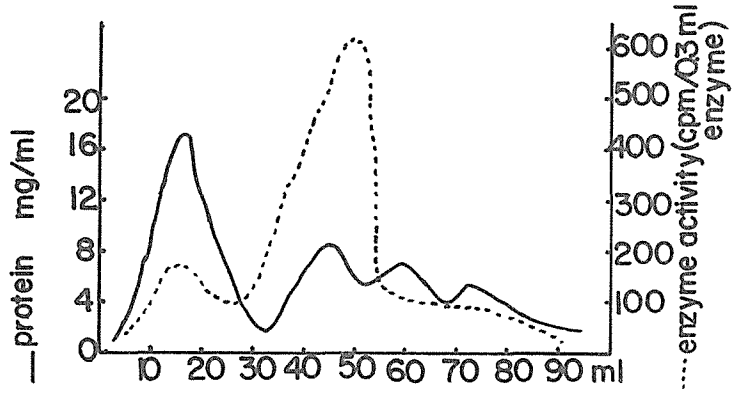

Fig. 3. Chromatography of thiazole dehydrogenating enzyme on DEAE-cellulose. Approximately 230 mg. of the enzyme were applied on the column. Detailed conditions are described in the text. The activity was expressed with c.p.m. of thiazole acid formed by 0.3 ml. of effluent per 60 minutes.

—— mg. of protein; ····· enzyme activity.

Table 2. Purification of thiazole dehydrogenating enzyme from *Pseudomonas sp.*

Purification step	Total volume (ml.)	Total protein (mg.)	Specific activity (units/mg. protein)	Total activity
Crude extract	20.5	753	23.9	17390
Streptomycin supernatant	25.5		46.6	45890
Calcium phosphate gel eluate	35.0	375	122.5	45000
Ammonium sulfate fractionation	11.0	233	183.0	42600
DEAE-cellulose column eluate	7.5	29.3	427.0	12500

precipitate was collected and was dissolved in 10 ml. of 0.1 *M* phosphate buffer, pH 7.5. This solution was passed through a column (1.5 cm × 20 cm) of Sephadex G-25 for desalting. Eleven ml. of this enzyme solution were applied to a DEAE cellulose column (1.5 cm. × 25 cm.), which was previously equilibrated with 0.005 *M* of phosphate buffer, pH 7.5, and the column was then washed with the same buffer till no further protein could be detected in the effluent. Elution was carried out with a linear concentrations gradient of potassium phosphate buffer, pH 7.5, from 0.005 *M* to 0.5 *M* (mixing chamber, 50 ml. of 0.005 *M* buffer; reservoir, 50 ml. of 0.5 *M* buffer). A result of the DEAE cellulose chromatography is shown in Fig. 3. Fractions of 1.5 ml. each were collected and assayed for protein and enzyme activity. A typical example of the enzyme purification is shown in Table 2. As shown in this Table, specific activity of the enzyme was arisen about 20 times.

Formation of thiazole-5-acetic acid. A typical time course of the formation of thiazole-5-acetic acid from thiazole is shown in Fig. 4. With the complets system, the rate of thiazole acetate formation was linear for about the first 90 minutes.

Effect of pH. Rates of the formation of thiazole-5-acetic acid at different pH values are shown in Fig. 5. The activity was highest at about pH 7.0.

Effect of enzyme concentration. Fig. 6 illustrates the relationship between the concentration of enzyme and the amount of thiazole-5-acetic acid formed. It can be seen from the figure that the activity was linear for up to 6 mg. of the enzyme.

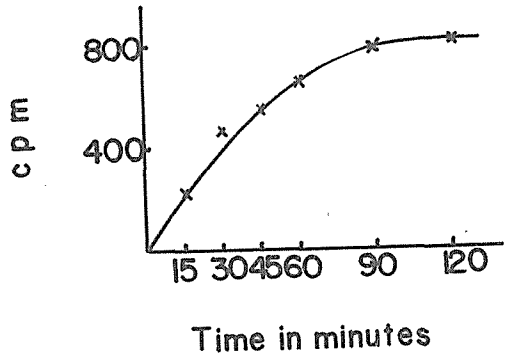

Fig. 4. Time course of the formation of thiazole acetic acid. The standard assay was carried out with 5 mg. of enzyme. The results are presented as the increase of c.p.m. of thiazole acetic acid formed.

Effect of nucleotide concentration. In Fig. 7 is shown the effect of varying NAD concentration on the formation of thiazole-5-acetic acid. As shown in Table 3, the addition of NAD + FAD showed the maximal effect. However, the addition

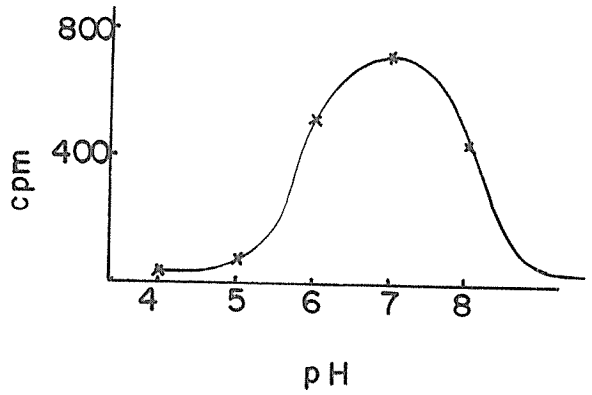

Fig. 5. Effect of pH on the activity of thiazole dehydrogenating enzyme. The standard assays for this enzyme were carried out with 5mg. of enzyme.

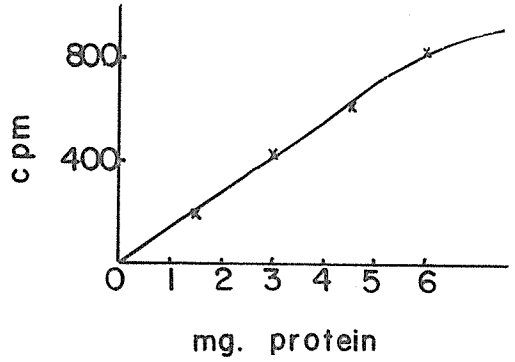

Fig. 6. Enzyme concentration versus rate of thiazole acetic acid formation. The standard assay was carried out with varying amounts of thiazole dehydrogenating enzyme. The results were presented as the increase of c.p.m. during the first 30 minutes after addition of enzymes.

Table 3. Effect of nucleotide coenzymes on thiazole dehydrogenating enzyme. (Per cent of Activity)

600 mμ moles NAD	100%
600 mμ moles NADP	12
600 mμ moles FAD	11
600 mμ moles NAD + 600 mμ moles FAD	168
600 mμ moles NAD + 600 mμ moles FMN	124

The reaction mixtures contained, in a final volume of 0.5 ml., potassium phosphate buffer, pH 7.5, 25 μ moles; thiazole, 300 mμ moles; enzyme, 5 mg.; and other additions as indicated.

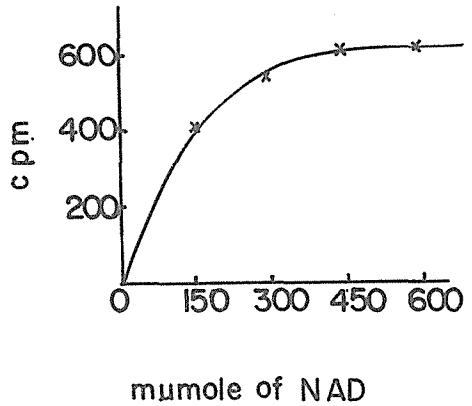

Fig. 7. Effect of NAD concentration.
Each reaction mixture contained 300 mμ moles of 2-C^{14}-thiazole (15,000 c.p.m.), 5 mg. of thiazole dehydrogenating enzyme and varying amount of NAD. The other components and assay procedure were as indicated in the standard assay. Theoretical c.p.m. for the complete degradation of thiazole to thiazole acetic acid is 1250 c.p.m.

Table 4. Effect of SH-agents and metals on thiazole dehydrogenating enzyme (per cent of activity).

No addition	100%
$10^{-4}M$ PCMB	82
$10^{-2}M$ Iodoacetate	84
$10^{-3}M$ Iodoacetate	95
$10^{-2}M$ $MgCl_2$	118
$10^{-3}M$ $MgCl_2$	109
$10^{-2}M$ $CaCl_2$	35
$10^{-3}M$ $CaCl_2$	53
$10^{-2}M$ $FeCl_2$	123
$10^{-3}M$ $FeCl_2$	98

The reaction mixtures contained, in a final volume of 0.5 ml., potassium phosphate buffer, pH 7.5, 25 μ moles; thiazole, 300 mμ moles; NAD, 300 mμ moles; puified enzyme, 5 mg., and other additions as indicated.

of NADP, FAD or FMN gave no effect.

Effect of inhibitors. The enzyme was inhibited by Ca^{++} as indicated in Table 4. This enzyme was also inhibited by PCMB and Iodoacetate but to a lesser degree than occurred by Ca^{++}.

DISCUSSION

Though Suzuoki demonstrated that S^{35}-thiazole injected in a rat was metabolized to 4-methylthiazole-5-acetic acid and excreted in urine, no further metabolism was observed[12]. In the present experiment, bacteria was found to metabolized thiazole to carbon dioxide and sulfate. Ammonium ion was not detected in the culture medium, probably because it was taken into the bacteria as the nitrogen source.

Thiazole dehydrogenating enzyme was purified about 20 fold. Since the thiazole dehydrogenating reaction is the dehydrogenation of alcohol into carboxylic acid, this reaction may consist of two steps, namely, a step from alcohol to aldehyde, and from aldehyde to carboxylic acid.

It is not clear whether this two steps reaction is caused by two enzymes or by a single enzyme. Though the separation of two enzymes is tried unsuccessfully, it is revealed that this reaction reaches to the maximum activity by the addition of both NAD and FAD, and that indicates probably two enzymes are concerned in it.

The measurement of the enzyme activity by the increase of the absorption spectrum of $NADH_2$ was hindered by unusually strong activities of $NADH_2$-oxidase.

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