Bacterial Metabolism of Thiamine

II. THE ISOLATION AND CHARACTERIZATION OF 3-(2'-METHYL-4'-AMINO-5'-PYRIMIDYLMETHYL)-4-METHYLTIAZOLE-5-ACETIC ACID (THIAMINE ACETIC ACID) AS AN INTERMEDIATE IN THE OXIDATION OF THIAMINE*

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SUMMARY

The compound 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (thiamine acetic acid) has been identified as an intermediate in the metabolism of thiamine by a soil microorganism. Evidence is presented which indicates the compound 2-oxy-3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (2-oxythiamine acetic acid) is also an intermediate in the metabolism of thiamine by this microorganism.

The previous work in this laboratory (1) established a portion of the general pathway for thiamine decomposition by cell suspensions of a soil microorganism. By use of washed cell suspensions and cell-free extracts, two additional metabolites of thiamine have been identified. The structure of one of these compounds has been shown to be 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (thiamine acetic acid). The available data indicates the structure of the second metabolite is 2-oxy-3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (2-oxythiamine acetic acid).

This paper describes the isolation and identification of thiamine acetic acid and 2-oxythiamine acetic acid.

EXPERIMENTAL PROCEDURE

Materials—The ion exchange resin Amberlite CG-50 was purchased from Mallinckrodt. Thiamine chloride hydrochloride was a product of Merck. Microcrystalline cellulose was obtained from the American Viscose Corporation, Newark, Delaware. Protamine sulfate was purchased from Sigma. Thiazole-2-14C-thiamine was obtained from Nuclear-Chicago.

Culture Procedures—The classification of the organism used in these studies has been described previously (1). The procedures used to grow the organism have also been described (1).

1 Arrangements for including this microorganism in the American Type Culture Collection have not yet been completed.

Preparation of Cell-Free Extract—One gram of lyophilized cells was suspended in 30 ml of 0.05 M sodium phosphate, pH 7.2, containing 1 X 10^-8 M EDTA and 1 X 10^-4 M mercaptoethanol (Buffer A), and was sonically treated for 10 min with a Branson W-185-C sonifier at a power setting of 85 watts. During sonic treatment, the temperature of the suspension was maintained between 5 and 10° by use of an ice bath. The suspension was then centrifuged at 20,000 X g for 30 min in a refrigerated centrifuge. In some experiments the cell-free extract was treated with protamine sulfate by adding 0.15 volume of a 2.5% solution. After 15 min at 4° the protamine sulfate-treated extract was centrifuged at 20,000 X g for 20 min.

Chromatographic Procedures—The ion exchange chromatography of thiamine and its metabolites on Amberlite CG-50 has been described previously (1). Thin layer chromatography on microcrystalline cellulose was carried out with the use of the following solvent systems: n-propyl alcohol-sodium acetate buffer (1:1, pH 5.0)-water (70:10:20, v/v) (Solvent A); n-propyl alcohol-0.1 N HCl (67:33, v/v) (Solvent B); and chloroform-methanol-aqueous 10% (w/v) NH3 (55:45:3.5, v/v) (Solvent C).

Unknown compounds were recovered from the thin layer plates by scraping the cellulose from the plates and eluting with distilled water.

Synthesis of Thiamine Acetic Acid—2-Methyl-4-amino-5-bromomethylpyrimidine hydrobromide (250 mg, 0.88 mmole) (2) and 4-methylthiazole-5-acetic acid (157 mg, 1 mmole) (3) were coupled by the method of Gravin (4). Yield: m.p. 231°, 74 mg (30%) of the bromide, hydrobromide salt of thiamine acetic acid.

C12H1602N4Br2

Calculate: C 32.87, H 3.74

Found: C 32.72, H 3.66

The radioactive thiamine acetic acid, 5-(2-hydroxyethyl)-4-...
methylthiazole, 4-methylthiazole-5-acetic acid, and 2-oxy-4-methylthiazole-5-acetic acid used in the metabolic experiments were isolated as metabolic products of thiazole-2-14C-thiamine by cell-free extracts of the microorganism by the use of ion exchange and extraction procedures previously described (1).

RESULTS AND DISCUSSION

Isolation and Characterization of Thiamine Acetic Acid—It had been reported previously (1) that small amounts of an unknown compound with an \( R_f \) of 0.34 to 0.40 in Solvent A was detectable on thin layer chromatography of an incubation mixture containing thiamine and a washed cell suspension of a soil microorganism. During preliminary experiments dealing with the purification of a "thiaminase" enzyme which metabolizes thiamine to 2-methyl-4-amino-5-hydroxymethylpyrimidine (I) and 5-(2-hydroxyethyl)-4-methylthiazole (2), it was noted that if more than 0.1 volume of the thiaminase activity was apparently precipitated and this same compound accumulated in the incubation mixture in relatively large quantities.

An aliquot of a reaction mixture consisting of the protamine sulfate supernatant and thiazole-2-14C-thiamine was spotted on a thin layer of microcrystalline cellulose and the chromatogram was developed with Solvent A. The unknown compound was isolated from the thin layer chromatogram as described under "Experimental Procedure." The ultraviolet spectra of the unknown at pH 1.0, 7.2, and 10.0 were determined and found to be identical with those of thiamine at the same pH values.

The behavior of the compound in comparison with thiamine on thin layers of cellulose was examined next. It was found that the unknown compound migrated with the same \( R_f \) as thiamine (0.37) in the acidic Solvent B, but had a lower \( R_f \) (0.20) than thiamine (0.48) in the basic Solvent C. These data indicated that the unknown compound might contain an acidic group.

In order to obtain sufficient material for further structural studies, a protamine sulfate supernatant from 3 g of lyophilized cells was prepared (see "Experimental Procedure"). The supernatant (total volume 155 ml) was incubated with 150 mg of thiazole-2-14C-thiamine (0 \( \mu \)Ci per mmole) with shaking at 37 \( ^\circ \) for 2 hours, and the protein was precipitated by adding 5 volumes of acetone. The supernatant was reduced in volume in a vacuum to about 5 ml and chromatographed on a column (1 \( \times \) 30 cm) of Amberlite CG-50.

The elution pattern of the radioactivity is shown in Fig. 1. The column fractions comprising each of the peaks of radioactivity were pooled, lyophilized to dryness, and examined for radioactive metabolites by thin layer chromatography and autoradiography. Peak A contained almost exclusively 2-oxy-4-methylthiazole-5-acetate (0.019 mmole); Peak B contained 4-methylthiazole-5-acetic acid (0.005 mmole); Peak C contained 5-(2-hydroxyethyl)-4-methylthiazole (0.021 mmole) and the unknown compound (0.230 mmole); and Peak D contained thiamine that had not been metabolized (0.169 mmole). The remainder of the residue from Peak C was dissolved in 2 ml of water, the pH adjusted to 7.5 with 0.1 \( \times \) NaOH and extracted three times with 5 ml of chloroform. This treatment removed almost all of the 5-(2-hydroxyethyl)-4-methylthiazole. The volume of the aqueous layer was reduced to about 0.5 ml on a steam bath under a stream of nitrogen and set aside at 4 \( ^\circ \). After about 2 days some light yellow crystals appeared. These crystals were isolated by filtration and recrystallized from acetone, 0.1 \( \times \) HCl (25:75, v/v); yield: 40 mg; m.p. 229 \( ^\circ \) with decomposition.

Mass spectra analysis of the unknown gave an apparent molecular ion of \( m/e \) 278. In the mass spectra of thiamine (6) there is a prominent peak at \( m/e \) 143 corresponding to the ion \( [5-(2-
-\text{hydroxyethyl})-4-methylthiazole]+ \). This peak is missing in the spectra of the unknown compound. The \( m/e \) 143 ion from thiamine loses \(-\text{CH}_2\text{OH}\) to form an ion of \( m/e \) 112 and \(-\text{CH}_3\text{OH}\) to form an ion of \( m/e \) 113. Both of these peaks are prominent in the spectra of the unknown. These data indicated the unknown was thiamine in which the hydroxethyl side chain had been oxidized to an acid. The infrared spectra of the unknown showed a prominent peak at 1740 cm\(^{-1}\) which also indicated the presence of a carboxyl group in the molecule. Therefore, thiamine acetic acid was synthesized (see "Experimental Procedure"), and the infrared and mass spectra of the unknown and synthesized compounds were compared. The spectra proved to be identical.

As with thiamine, thiamine acetic acid is converted to a fluorescent compound when treated with an alkaline solution of ferricyanide. On a molar basis, the thiamine acetic acid has 25% greater fluorescence than does thiamine.

Metabolic Studies—A series of experiments were performed to clarify further the metabolic sequence for the formation of the various metabolites of thiamine.

When thiamine was incubated with a cell suspension and the incubation mixture was examined for metabolites by thin layer chromatography before all the thiamine was transformed, an accumulation of 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine, 4-methylthiazole-5-acetic acid, 5-(2-hydroxyethyl)-4-

![Fig. 1. Ion exchange chromatography of thiamine and its metabolites on Amberlite CG-50. The procedures for the preparation and equilibration of the column have been described previously (5). The sample containing 2.7 \( \mu \)Ci (0.445 mmole) of \(^{14}C\)-thiamine and its metabolites was applied and the column (1 \( \times \) 30 cm) was eluted by downward flow with 100 ml of water followed by 250 ml of pyridine-acetic acid-water (7.5:1.5:91.0, v/v). Five milliliter fractions were collected at a flow rate of 50 ml per hour. The radioactivity in a 0.5-ml aliquot of each fraction was determined by gas flow counting.](http://www.jbc.org/content/244/19/5202)
methylthiazole, and 2-oxy-4-methylthiazole-5-acetic acid could be detected on examining the thin layer plate under ultraviolet light. There are two potential metabolic precursors for the 4-methylthiazole-5-acetic acid. These are thiamine acetic acid and 5-(2-hydroxyethyl)-4-methylthiazole. Thin layer chromatographic examination of an incubation medium containing a cell suspension and thiamine acetic acid indicated the presence of 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine, 2-oxy-4-methylthiazole-5-acetic acid, and 4-methylthiazole-5-acetic acid. Examination of an incubation mixture containing washed cells and 5-(2-hydroxyethyl)-4-methylthiazole revealed 2-oxy-4-methylthiazole-5-acetic acid as the only metabolic product. Incubation of 4-methylthiazole-5-acetic acid with a washed cell suspension also revealed 2-oxy-4-methylthiazole-5-acetic acid as the only metabolic product detectable by the use of an ultraviolet light. The rate of oxygen uptake by whole cells in the presence of 4-methylthiazole-5-acetic acid is approximately 15 times that in the presence of 5-(2-hydroxyethyl)-4-methylthiazole. Therefore, a trapping experiment was performed with the use of unlabeled 4-methylthiazole-5-acetic acid (10 µmoles) and labeled 5-(2-hydroxyethyl)-4-methylthiazole (10 µmoles) (0.200 µCi per µmole) to see if 4-methylthiazole-5-acetic acid was an intermediate in the metabolism of 5-(2-hydroxyethyl)-4-methylthiazole. The incubation was stopped before all the 4-methylthiazole acetic acid had disappeared. The 4-methylthiazole-5-acetic acid, 2-oxy-4-methylthiazole-5-acetic acid, and 5-(2-hydroxyethyl)-4-methylthiazole present in the incubation mixture were separated by ion exchange chromatography (see “Experimental Procedure”), and the specific activities of the compounds were determined by comparing the radioactivity of each compound with its absorption at 252 nm in distilled water. The 4-methylthiazole-5-acetic acid isolated in this manner was labeled and had nearly the same specific activity (0.005 µCi per µmole) as the 2-oxy-4-methylthiazole-3-acetic acid (0.003 µCi per µmole). No labeled 4-methylthiazole-5-acetic acid could be detected when labeled 2-oxy-4-methylthiazole-5-acetic acid and unlabeled 4-methylthiazole were incubated with a cell suspension. These data indicated the presence of the metabolic pathway with 4-methylthiazole-5-acetic acid and unlabeled 4-methylthiazole and unlabeled 4-methylthiazole-5-acetic acid were nearly the same. These data favor the reaction sequence outlined in a above. However, an examination of incubation media containing labeled thiamine and whole cells, a cell-free extract, or a protamine sulfate-treated cell-free extract, failed to reveal a compound with chromatographic properties compatible with the proposed intermediate 2-oxythiamine.

Thiamine reacts slowly with hydroxyl ions to form a pseudo base (7). The site of attack of the hydroxyl ion is carbon 2 of the thiazole ring to form the compound 2-hydroxy-3-(2′-methyl-4′-amino-5′-pyrimidylmethyl)-5-(2-hydroxyethyl)-4-methyl-
thiazole. The pKₐ for the formation of this pseudo base is approximately 9.0. This pseudo base can be converted back to thiamine with the addition of acid. The titration curve of thiamine acetic acid² indicates that it undergoes this same reversible pseudo base formation. Since 2-oxythiamine acetic acid was not converted to thiamine acetic acid at the pH used during the isolation (pH 5.0), the oxygen on carbon 2 of the thiazole moiety must be in the keto form. This conclusion is born out by the formation of a yellow spot when a thin layer chromatogram containing 2-oxythiamine acetic acid is sprayed with 2,4-dinitrophenylhydrazine.

The conversion of the pseudo base to the 2-keto compound would require the loss of 2 protons and 2 electrons. This requirement and the fact that the incubation as carried out at a pH (7.2), considerably below the pKₐ for nonenzymic hydroxylation of thiamine, would indicate that the introduction of oxygen at carbon 2 is enzyme-catalyzed.

In order to determine whether the formation of 2-oxy-4-methylthiazole-5-acetic acid from 4-methylthiazole-5-acetic acid was nonenzymic; this latter compound was incubated with Buffer A for 24 hours, and the reaction mixture was examined for 2-oxy-4-methylthiazole-5-acetic acid by thin layer chromatography. No 2-oxy-4-methylthiazole-5-acetic acid could be detected. Thus, the formation of 2-oxy-4-methylthiazole-5-acetic acid from 4-methylthiazole-5-acetic acid also appears to be enzyme-catalyzed.

The isolation and identification of these two new metabolites plus the information derived from the metabolic studies require that the principal routes for the metabolism of thiamine by the soil organism outlined previously (1) be revised. This revision is shown in Fig. 2. The hypothetical intermediate 2-oxythiamine, as well as 2-oxythiamine acetic acid, and 2-oxy-4-methylthiazole-5-acetic acid are drawn in the keto form. The infrared spectra of 2-oxy-4-methylthiazole-5-acetic acid (1) supports the presence of a keto group in this compound, and, as mentioned previously, 2-oxythiamine acetic acid yields a yellow spot on a thin layer chromatogram when sprayed with 2,4-dinitrophenylhydrazine. However, in all likelihood, these three compounds also exist partially in the enol form at acid pH. The metabolic sequences shown in Fig. 2 are based in part on the result of trapping experiments. The relative permeabilities of the various metabolites used in these trapping experiments into the cells have not been

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Fig. 2. Principal routes for metabolism of thiamine by a soil organism
determined. Therefore, the metabolic sequences shown can only be considered as the most probable ones.

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