Isolation and Identification of 3-(2'-Methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic Acid (Thiamine Acetic Acid) and 2-Methyl-4-amino-5-formylaminomethylpyrimidine as Metabolites of Thiamine in the Rat*

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SUMMARY

The compounds 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (thiamine acetic acid) and 2-methyl-4-amino-5-formylaminomethylpyrimidine have been identified as metabolites of thiamine in the rat.

Evidence is presented which indicates that 5-(2-hydroxyethyl)-4-methylthiazole is also an important metabolite of thiamine in the rat.

EXPERIMENTAL PROCEDURES

Materials—Thiamine acetic acid was synthesized according to the method of Neal (7). Thiazole-2-14C-thiamine acetic acid was prepared by incubating thiazole-2-14C-thiamine with the enzyme thiamine dehydrogenase (8). The labeled thiamine acetic acid was purified by ion exchange chromatography of the incubation mixture on a column of Amberlite CG-50 as described previously (7). The compound 5-(2-hydroxyethyl)-4-methylthiazole was prepared by reaction of thiamine with sodium bisulfite (9). This reaction yields 2-methyl-4-amino-5-pyrimidinesulfonic acid and 5-(2-hydroxyethyl)-4-methylthiazole. This latter compound can be isolated by extracting the reaction mixture with chloroform and vacuum-distilling the chloroform extract. The boiling point of 5-(2-hydroxyethyl)-4-methylthiazole is 135° at 7 mm. The compound 4-methylthiazole-5-acetic acid was synthesized by the method of Cerecedo and Tolpin (10).

The compound 2-methyl-4-amino-5-formylaminomethylpyrimidine was synthesized according to the method of Matsukawa and Yurugi (11). The yield was low, however, and the following method was developed. One-half gram (3.6 mmoles) of 2-methyl-4-amino-5-aminomethylpyrimidine, obtained by cleavage of thiamine with liquid ammonia, was dissolved in 5 ml of 90% formic acid, and the mixture was refluxed for 10 min. The reaction mixture was cooled and acetone (5 ml) was added. The cloudy solution was warmed until it was clear, and then it was set aside at room temperature overnight. The crystalline material that appeared was recrystallized from acetone-water (1:1). Yield, 190 mg, 2-methyl-4-amino-5-formylaminomethylpyrimidine, m.p. 219°.

C₉H₁₈N₄O₂
Calculated: C 50.60, N 33.73, H 6.02
Found: C 50.45, N 33.50, H 5.98

Thiazole-2-14C-thiamine was purchased from Nuclear-Chicago and pyrimidine-2-14C-thiamine from Mallinckrodt Chemical Works. Ion exchange chromatography was performed on Amberlite CG-50 (H⁺, 200 to 400 mesh, Mallinckrodt), gel filtration
RESULTS AND DISCUSSION

If thiamine were metabolized in the rat by a "thiaminase" enzyme, the products would be 5-(2-hydroxyethyl)-4-methylthiazole and some form of the pyrimidine moiety. Although 5-(2-hydroxyethyl)-4-methylthiazole has been reported to be present in the urine of rats administered 1 mg of thiamine by a single intraperitoneal injection (12), we have not been able to detect its presence under similar conditions. The presence in urine of 4-methylthiazole-5-acetic acid (5, 6) implies, however, that a "thiaminase"-like cleavage of thiamine has taken place. When 5-(2-hydroxyethyl)-4-methylthiazole is administered to rats in large amounts (100 mg per day), it is almost totally metabolized to 4-methylthiazole-5-acetic acid (13). We reasoned, therefore, that the failure to detect this compound might be caused by total metabolism of any 5-(2-hydroxyethyl)-4-methylthiazole formed to 4-methylthiazole-5-acetic acid.

Accordingly, we designed an experiment to determine whether 5-(2-hydroxyethyl)-4-methylthiazole is a metabolite of thiamine in the rat. In this experiment, we administered simultaneously 1 mg (2 μCi) of thiazole-2-14C-thiamine and 200 mg of unlabeled 5-(2-hydroxyethyl)-4-methylthiazole per day to each of two rats for 2 days. The urine and feces were collected separately. The pooled urine was subjected to ion exchange chromatography as described in Fig. 1. Peak B, which contains 4-methylthiazole-5-acetic acid, was reduced in volume and set aside at 4°C overnight. Under these conditions, the relatively insoluble 4-methylthiazole-5-acetic acid crystallizes from aqueous solution. The infrared spectrum of the crystallized product from Peak B was found to be identical with that of authentic 4-methylthiazole-5-acetic acid.

Thin layer chromatography of an aliquot of Peak C, with Solvent System D revealed the presence of unmetabolized 5-(2-hydroxyethyl)-4-methylthiazole. Peak C was then subjected to liquid-chromatography on Sephadex G-10 (Pharmacia), and thin layer chromatography on microcrystalline cellulose (Avicell, FMC Corporation, Newark, Delaware).

Animals and Treatment—Female Sprague-Dawley rats were used in these studies. Animals were housed in cages that allowed for the separate collection of urine and feces. Food and water were supplied ad libitum. In the experiments in which expired gases were collected, the animals were maintained in a cage especially constructed for this purpose. Those animals receiving physiological intakes of thiamine (80 μg per day) were maintained on a thiamine-deficient diet (Nutritional Biochemicals), supplemented by daily intubations of thiamine. The animals maintained on higher levels of thiamine were fed a stock laboratory ration and given thiamine at the appropriate level either by intubation or by placing the thiamine in the drinking water. Urine was collected as previously described (1).

Spectrophotometric Determinations—In the determinations of the concentration of thiamine acetate and 5-(2-hydroxyethyl)-4-methylthiazole in solution, the optical densities of the solutions of thiamine acetate were determined at 247 nm and of 5-(2-hydroxyethyl)-4-methylthiazole at 260 nm. In the cases in which the specific activities of these compounds were also to be calculated, the radioactivity of an aliquot of the solution was determined by liquid scintillation counting and the specific activity was calculated.

Chromatographic Procedures—In a typical experiment, 500 ml of urine from rats administered 14C-labeled thiamine were concentrated to 40 ml under vacuum at 40°C and centrifuged to remove the salts that had precipitated. The concentrate was applied to a column of Amberlite CG-50 (2.5 × 70 cm) which had been prepared as described previously (1). The column was developed by downward flow of a linear gradient of distilled water to 0.35 M pyridine acetate in 2000 ml; 12-ml fractions were collected at a flow rate of 120 ml per hour. The radioactivity in these fractions was determined by planchet counting.

The radioactivity in a 0.5-ml aliquot of each fraction was determined by gas flow counting. The metabolites of thiamine isolated from urine by ion exchange chromatography were sometimes chromatographed on columns (1.5 x 105 cm) of Sephadex G-10 that had been equilibrated with 0.01 N HCl. These columns were developed with 0.01 N HCl, and 5-ml fractions were collected at a flow rate of 60 ml per hour. The radioactivity in these fractions was determined by planchet counting.

Thin layer chromatography was performed on 0.5-mm layers of microcrystalline cellulose. The solvent systems used were n-propanol-acetate buffer (1:1, pH 5.0)-water (70:10:20, v/v) (Solvent System A); 1-propanol-0.1 N HCl (67:33, v/v) (Solvent System B); chloroform-methanol-aqueous 10% NH4 (w/v) (65:34:3.5, v/v) (Solvent System C); hexane-chloroform-methanol (70:20:10, v/v) (Solvent System D); pyridine-ethyl acetate-acetic acid-water (50:50:10:30, v/v) (Solvent System E); and n-butanol-ethanol-water (80:20:20, v/v) (Solvent System F).

Melting points were determined on a Thomas-Hoover (Philadelphia, Pennsylvania) capillary melting point apparatus. Infrared spectra were obtained with a Perkin-Elmer (Norwalk, Connecticut) model 337 spectrophotometer, and the mass spectra were determined on a LKB (Rockville, Maryland) model 9000 mass spectrometer. Microanalysis was performed by the Galbraith Laboratories (Knoxville, Tennessee).

Thin-layer chromatography was performed on 0.5-mm layers of microcrystalline cellulose. The column was eluted by downward flow of a linear gradient of distilled water to 0.35 M pyridine acetate in 2000 ml; 12-ml fractions were collected at a flow rate of 120 ml per hour. The radioactivity in a 0.5-ml aliquot of each fraction was determined by gas flow counting.

Urine was collected as previously described (1). If thiamine were metabolized in the rat by a "thiaminase" enzyme, the products would be 5-(2-hydroxyethyl)-4-methylthiazole and some form of the pyrimidine moiety. Although 5-(2-hydroxyethyl)-4-methylthiazole has been reported to be present in the urine of rats administered 1 mg of thiamine by a single intraperitoneal injection (12), we have not been able to detect its presence under similar conditions. The presence in urine of 4-methylthiazole-5-acetic acid (5, 6) implies, however, that a "thiaminase"-like cleavage of thiamine has taken place. When 5-(2-hydroxyethyl)-4-methylthiazole is administered to rats in large amounts (100 mg per day), it is almost totally metabolized to 4-methylthiazole-5-acetic acid (13). We reasoned, therefore, that the failure to detect this compound might be caused by total metabolism of any 5-(2-hydroxyethyl)-4-methylthiazole formed to 4-methylthiazole-5-acetic acid.

Accordingly, we designed an experiment to determine whether 5-(2-hydroxyethyl)-4-methylthiazole is a metabolite of thiamine in the rat. In this experiment, we administered simultaneously 1 mg (2 μCi) of thiazole-2-14C-thiamine and 200 mg of unlabeled 5-(2-hydroxyethyl)-4-methylthiazole per day to each of two rats for 2 days. The urine and feces were collected separately. The pooled urine was subjected to ion exchange chromatography as described in Fig. 1. Peak B, which contains 4-methylthiazole-5-acetic acid, was reduced in volume and set aside at 4°C overnight. Under these conditions, the relatively insoluble 4-methylthiazole-5-acetic acid crystallizes from aqueous solution. The infrared spectrum of the crystallized product from Peak B was found to be identical with that of authentic 4-methylthiazole-5-acetic acid.

Thin layer chromatography of an aliquot of Peak C, with Solvent System D revealed the presence of unmetabolized 5-(2-hydroxyethyl)-4-methylthiazole. Peak C was then subjected to liquid-
liquid extraction with diethyl ether for 12 hours. Thin layer chromatography revealed that all (8 mg) of the 5-(2-hydroxyethyl)-4-methylthiazole had migrated into the ether layer. The infrared spectrum of the material in the ether layer proved to be identical with that of authentic 5-(2-hydroxyethyl)-4-methylthiazole. The 5-(2-hydroxyethyl)-4-methylthiazole isolated in this manner had a specific activity of 435 cpm per pmole. The amount of 4-methylthiazole-5-acetic acid isolated from the urine in this experiment (320 mg) indicated that the unlabeled 5-(2-hydroxyethyl)-4-methylthiazole rather than thiamine was the major precursor of this compound under the conditions of the experiment. The isolated 4-methylthiazole-5-acetic acid had a specific activity of 504 cpm per pmole. The specific activity of the 5-(2-hydroxyethyl)-4-methylthiazole and 4-methylthiazole-5-acetic acid and the amount of 5-(2-hydroxyethyl)-4-methylthiazole metabolized to 4-methylthiazole 5-acetic acid in this experiment clearly indicate that 5-(2-hydroxyethyl)-4-methylthiazole is a major metabolic product of thiamine in the rat. The higher specific activity of the 4-methylthiazole-5-acetic acid also indicates, however, that this latter compound is derived from thiamine by a route which bypasses 5-(2-hydroxyethyl)-4-methylthiazole.

In this experiment, the feces contained 2.0 mg of 5-(2-hydroxyethyl)-4-methylthiazole and 28 mg of 4-methylthiazole-5-acetic acid. The total recovery of the administered 5-(2-hydroxyethyl)-4-methylthiazole in terms of unmetabolized compound and that metabolized to 4-methylthiazole-5-acetic acid was 41%. These data indicate that 5-(2-hydroxyethyl)-4-methylthiazole or 4-methylthiazole-5-acetic acid or both are further metabolized to products as yet unidentified.

Isolation and Identification of Thiamine Acetic Acid as a Metabolite of Thiamine—The data from the experiment just described indicated the existence of an additional precursor for 4-methylthiazole-5-acetic acid other than 5-(2-hydroxyethyl)-4-methylthiazole. One of the intermediates in the metabolism of thiamine by a soil microorganism is thiamine acetic acid (7). This is the compound in which the hydroxethyl side chain of thiamine is oxidized to an acid. Thiamine acetic acid is the major source of 4-methylthiazole-5-acetic acid in this microorganism (7). Accordingly, the urine of rats was examined for thiamine acetic acid. Thiamine acetic acid is eluted from an Amberlite CG-50 column in the position of Peak C1 (Fig. 1). This peak of radioactivity from the urine of rats administered thiazole-2-"C-thiamine was reduced in volume, and a portion was lyophilized material corresponding to Peak C1, Fig. 1. The mixture, which was dissolved in a minimum amount of boiling water, was set aside at 4° overnight. A portion of the crystals of thiamine acetic acid was collected, and the specific activity was determined as described under “Experimental Procedures.” Thiazole-labeled thiamine acetic acid was administered to rats at a level of 70 pg per rat per day. Subsequent examination of the urine revealed that about 10% had been metabolized to 4-methylthiazole-5-acetic acid. The remainder of the radioactivity in urine was accounted for by unmetabolized thiamine acetic acid. The chromograms were run on thin layers (0.5 mm) of microcrystalline cellulose by the ascending technique. The solvent systems used are described under “Experimental Procedures.” Thiamine acetic acid was visualized under an ultraviolet light. The unknown was detected by autoradiography.

| Compound                  | RF value in solvent system |
|--------------------------|----------------------------|
|                          | A  | B  | C  | E  | F  |
| Thiamine acetic acid     | 0.44| 0.33| 0.15| 0.09| 0.07|
| Unknown                  | 0.40| 0.32| 0.12| 0.12| 0.05|

| Crystallization | Radioactivity (cpm/µ mole) |
|----------------|---------------------------|
| 1              | 5430                      |
| 2              | 1240                      |
| 3              | 1250                      |
| 4              | 1200                      |
Identification of Thiamine Metabolites in Rat

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**Fig. 2.** The most abundant ions from the mass spectra of (a) authentic 2-methyl-4-amino-5-formylaminomethylpyrimidine and the compound isolated from the bacterial culture and (b) the unknown isolated from rat urine. All spectra were obtained using the direct sample introduction probe.

It is not generally known, but not been possible to determine the relative contribution of these two compounds to the 4-methylthiazole-5-acetic acid excreted in the urine.

Isolation and Identification of 2-Methyl-4-amino-5-formylaminomethylpyrimidine as a Metabolite of Thiamine—About 60% of the radioactivity in Peak C, Fig. 1, from rats maintained on physiological levels (60 μg per day) of either thiazole- or pyrimidine-labeled thiamine was found to be accounted for by an ultraviolet-absorbing compound with an Rf of about 0.55 in Solvent System A. Thus, the compound contained all or portions of both the pyrimidine and thiazole moieties. This same compound was also seen in animals administered thiamine acid acetate. This indicated that the hydroxyethyl side chain of the thiazole moiety was either missing or oxidized to an acid. The compound did not give a positive thiochrome reaction, and it was not markedly affected when dissolved in a solution of sodium bisulfite. These data indicated that the structure of thiamine had been extensively modified.

At this point, it was noted that an unidentified compound which was a minor metabolic product of the metabolism of thiamine by a microorganism (14) and which was eluted from Amberlite CG-50 in a peak equivalent to Peak C, Fig. 1, had the same Rf as the unknown in Solvent Systems A, B, C, E, and F.

A sufficient amount of this bacterial metabolite was available so that it could be purified by crystallization and examined by mass spectrometry. The mass spectrum of the bacterial metabolite is shown in Fig. 2a. What appears to be the molecular ion of the compound occurs at m/e 166. The most prominent ion in the spectrum is m/e 137. There is also a prominent ion at m/e 122. The ion at m/e 137 could correspond to a loss of CHO from the molecular ion, and the ion at m/e 122 is typical of the intact pyrimidine moiety of thiamine (15). These data indicated that the compound might be 2-methyl-4-amino-5-formylaminomethylpyrimidine.

Consequently, this compound was synthesized as described under "Experimental Procedures." The infrared and mass spectra of the synthesized compound proved to be identical with those of the bacterial metabolite.

An attempt was made to obtain a mass spectrum of the metabolite from rat urine. Oral administration of large amounts of labeled thiamine to rats (40 mg per day) led to the accumulation of relatively large amounts of the unknown compound in urine. The metabolite in these urines was partially purified by chromatography on columns of Amberlite CG-50. It was further purified by chromatography of the equivalent of Peak C, Fig. 1, on a column of Sephadex G-10 as described under "Experimental Procedures." The radioactive peak from the Sephadex G-10 column was lyophilized to dryness. The residue remaining after lyophilization was dissolved in a small amount of hot 0.1 N HCl and cooled; acetone was added until the solution became cloudy, and the material was set aside at 4°C overnight. Approximately 5 mg of crystals were collected by centrifugation and washed with a small amount of acetone. Although the mass spectrum (Fig. 2b) of this partially purified material resembled that of the synthetic 5-formylaminomethylpyrimidine (Fig. 2a), it was not an exact duplication. One reason for this discrepancy was the presence of higher molecular weight impurities in the sample of the unknown. Repeated attempts to further purify this material with the use of Amberlite CG-50, Sephadex G-10, and thin layer chromatography were not successful. Although the mass peaks at m/e 122, 99, and 69 are more abundant relative to the parent peak than in synthetic 5-formylaminomethylpyrimidine, the major peaks, both in the mass at which they appeared and in their relative heights, indicated that the metabolite from rat urine was the 5-formylaminomethylpyrimidine.

In order to obtain further data as to the identity of the unknown, 50 μg of synthetic 2-methyl-4-amino-5-formylaminomethylpyrimidine and about 7,000 dpm of the unknown, which had a specific activity of approximately 10,000 dpm per μg, were applied to thin layers of cellulose as spots 1 cm in diameter. This amount of the unknown compound gave no evidence of ultraviolet absorption when examined under ultraviolet light. A mixture of 50 μg of synthetic 2-methyl-4-amino-5-formylaminomethylpyrimidine and an amount of the unknown containing approximately 7,000 dpm was spotted between and just touching...
Fig. 3 summarizes what is known concerning the metabolism of thiamine (I) in the rat. There appear to be two precursors for 4-methylthiazole-5-acetic acid (IV). One of the precursors, 5-(2-hydroxyethyl)-4-methylthiazole (III), is probably formed by the action of a "thiaminase"-like enzyme which also yields 2-methyl-4-amino-5-hydroxymethylpyrimidine (V). The 5-(2-hydroxyethyl)-4-methylthiazole is then oxidized to 4-methylthiazole-5-acetic acid by what is probably a two-step reaction. The second precursor is thiamine acetic acid (II) formed by the oxidation of thiamine. In a microorganism (8), thiamine is oxidized to thiamine acetic acid by a single enzyme without the release of the intermediate aldehyde. Whether the oxidation is catalyzed by one or two enzymes in rats remains to be determined. It is proposed that the thiamine acetic acid is then metabolized to 4-methylthiazole-5-acetic acid and 2-methyl-4-amino-5-hydroxymethylpyrimidine by a "thiaminase"-like enzyme.

The 5-hydroxymethylpyrimidine, which is postulated to be formed in the two "thiaminase" reactions, does not accumulate in the urine of animals on physiological intakes of thiamine. This compound, however, can be isolated from the urine of animals administered large oral doses of thiamine. In addition, when the 5-hydroxymethylpyrimidine is administered to rats, it is metabolized to 2-methyl-4-amino-5-pyrimidinecarboxylic acid (VI) (3). These data indicate that the 5-hydroxymethylpyrimidine is an intermediate in the metabolism of thiamine. The oxidation of the 5-hydroxymethylpyrimidine to the 5-pyrimidinecarboxylic acid is probably a two-enzyme process.

It is not possible on the basis of the present data to speculate on the metabolic route from thiamine to 2-methyl-4-amino-5-formylaminomethylpyrimidine and to sulfate. The metabolic route by which carbon 2 of the thiazole moiety is released as CO₂ is also not yet established, although it appears that the 5-formylaminomethylpyrimidine may be one of the important precursors. In adult rats on physiological intakes of thiamine (60 μg/day), 4-methylthiazole-5-acetic acid accounts for about 35% of the metabolites of the thiazole moiety of thiamine other than thiamine itself excreted in the urine. The relative amounts of this compound derived from thiamine acetic acid and 5-(2-hydroxyethyl)-4-methylthiazole are yet to be determined. The compound 2-methyl-4-amino-5-formylaminomethylpyrimidine accounts for about 11% and thiamine acetic acid for about 4% of the metabolites of both the pyrimidine and thiazole moieties of thiamine excreted in the urine. About 15 to 20% of the pyrimidine moiety of thiamine is excreted in the urine in the form of 2-methyl-4-amino-5-pyrimidinecarboxylic acid (3). Another 10% of the carbon 2 of the thiazole moiety is metabolized to CO₂ in rats maintained on physiological levels of thiamine (16). Thus, about 60% of that portion of the thiazole moiety containing carbon 2 and about 35% of the pyrimidine moiety can be accounted for by the metabolites shown in Fig. 3. The remaining

1 W. W. White, III, W. H. Amos, Jr., and R. A. Neal, unpublished observation (1970).
percentages are distributed among some 20 to 25 as yet unidentified metabolites.

That all of these metabolites are of mammalian origin rather than products of the intestinal microflora is supported by several findings. Neal and Pearson (3) demonstrated the formation of 2-methyl-4-amino-5-pyrimidinedecarboxylic acid from pyrimidine-$\text{H}^{14}$-thiamine by germ-free rats. Suzuki et al. (6) also used germ-free rats to demonstrate that 4-methylthiazole-5-acetic acid was not a product of the microbial degradation of thiazole-$\text{H}^{14}$-thiamine. It has also been found that the pattern of metabolites in the urine of animals maintained on a diet containing 1% succinylsulfathiazole is both qualitatively and quantitatively similar to that in the urine of animals not receiving the drug.2

We have examined human urine for the presence of thiamine acetic acid and of 2-methyl-4-amino-5-formylaminomethylpyrimidine. In both cases, a small amount of the labeled metabolite was added to approximately 1 liter of human urine, and the urine was subjected to the same ion exchange chromatography and thin layer chromatography procedures as described for rat urine. On the basis of the amounts of these compounds reisolated by these procedures, it appears certain that both metabolites are also present in human urine. The compounds 2-methyl-4-amino-5-pyrimidinecarboxylic acid (3) and 4-methylthiazole-5-acetic acid (17) have also been detected in human urine. Thus, the qualitative pattern of thiamine metabolism in humans appears similar to that in rats.

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