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Studies on Metabolism of Thiamine in Rats. I. Cleavage of Thiamine Molecule *in Vivo*

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It was found that in the rat about 50% of orally administered thiamine was decomposed into 4-methyl-5β-hydroxyethylthiazole (HT) and pyrimidyl derivative. It was demonstrated that 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) was formed from thiamine as one of pyrimidyl derivatives. However, the amount of excreted HMP was extremely small as compared to that of excreted HT. It was concluded that the main cleavage of thiamine was not a direct hydrolysis into HT and HMP.

In the previous communication to the Editor[2] it was reported that the presence of ^{35}S-4-methyl-5β-hydroxyethylthiazole(^{35}S-HT) was demonstrated in urine of rat, mouse, rabbit and guinea pig after oral administration of ^{35}S-thiazole-labeled thiamine. Especially in the case of rat, about 50% of the administered dose was excreted as ^{35}S-HT in the urine within 24 hours. If HT was formed by hydrolysis of thiamine, 2-methyl-4-amino-5-hydroxymethylpyrimidine(HMP) would be produced as a counterpart from the pyrimidine moiety of the vitamin. The results presented in this paper concern the radioactive metabolites found in the urine after the oral administration of ^{3}H-methylene-labeled or ^{35}S-labeled thiamine to the experimental animals. It was found that the recovery of the radioactivity excreted in the urine after the oral administration of ^{3}H-thiamine was much less as compared to that after the administration of ^{35}S-thiamine from the same route. From the ratio of ^{3}H/^{35}S, and of ^{3}H-HMP/^{35}S-HT in blood and organs after the oral administration of a mixture of ^{3}H-thiamine and ^{35}S-thiamine, the behavior of the pyrimidine moiety seemed different from that of the thiazole moiety. Since Shintani[3] and Imai, *et al.*[4] reported that both of HMP and HT were metabolized *in vivo*, the metabolic fate of HMP and HT in rat was also studied with ^{3}H-HMP and ^{35}S-HT.

Experimental

Preparation of Labeled Compounds——^{3}H-2-Methyl-4-amino-5-aminomethylpyrimidine(^{3}H-II) was prepared by the catalytic hydrogenation of 2-methyl-4-aminopyrimdine-5-carbonitrile (I), (100 mg of I, 20 mg of palladium and 2.0 ml of 5.8%-HCl—methanol) with 2 curies of tritium gas according to the procedure[5] for the preparation of ^{3}H-phenylalanine. A specific activity of ^{3}H-II was 1.5 mCi/mg, and about 86% pure. ^{3}H-II was used without purification for the next reactions.

^{3}H-Thiamine(^{3}H-III) was prepared from ^{3}H-II according to the procedure of Matsukawa[6] (sp. act., 0.86 mCi/mg). ^{3}H-2-Methyl-4-amino-5-hydroxymethylpyrimidine(^{3}H-IV) was prepared from ^{3}H-II according to the procedure of Andersag[7] and purified by recrystallization from water (sp. act., 0.21 mCi/mg). The preparation of ^{35}S-thiazole-labeled thiamine was described in the previous paper.[2] ^{35}S-4-Methyl-5β-hydroxyethylthiazole(^{35}S-V) was prepared by the bisulfite cleavage of thiamine as described by Williams,

1) Location: *Todashi, Saitama.*
2) T. Meshi and Y. Sato, *Chem. Pharm. Bull.* (Tokyo), **14**, 1444 (1966).
3) S. Shintani, *Yakugaku Zasshi*, **77**, 781 (1957).
4) Y. Imai, Z. Suzuoki, and A. Kobata, *J. Biochem.*, **48**, 341 (1960).
5) Y. Sato, T. Takahashi, and T. Meshi, *Radioisotopes*, **10**, 488 (1961).
6) T. Matsukawa and T. Iwazu, *Yakugaku Zasshi*, **70**, 28 (1950).
7) H. Andersag and K. Westphal, *Chem. Ber.*, **70**, 2035 (1937).

et al.[8] (sp. act., 3.5 μCi/mg). The purity of all the labeled compounds was determined by paper chromatography and scanning of the chromatograms (most of the compounds were more than 98% pure). The solvent system for chromatography was butanol—acetic acid—water (4:1:5).

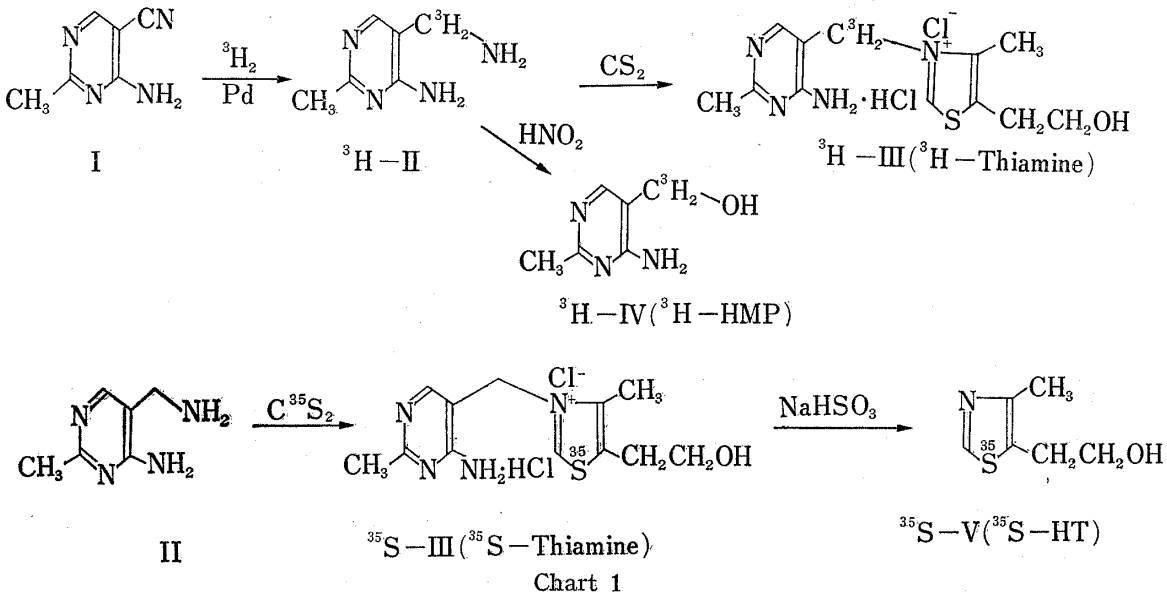

Chart 1

Animal Experiments——The female Wister rats weighing about 200 g, which were maintained on a usual laboratory chow, were used. The labeled compounds were dissolved in distilled water. Oral doses per rat were 0.2 mg/0.2 ml for ^{3}H–thiamine hydrochloride and ^{35}S–thiamine hydrochloride, 1.0 mg/0.5 ml for ^{3}H–HMP, and 5.0 mg/0.5 ml for ^{35}S–HT. Intravenous injection was also carried out for ^{3}H–thiamine hydrochloride and ^{35}S–thiamine hydrochloride. After administration of various labeled compounds, the rats were housed in cages constructed to permit the separate collection of urine and feces. The radioactive compound in the urine sample were separated by paper chromatography on Toyo filter paper No. 51 in a system of butanol—acetic acid—water (4:1:5, v/v). When a mixture of an equal amount of ^{3}H–thaimine and ^{35}S–thiamine was orally administered, the blood and tissue samples were collected. Blood, liver, kidney and small intestine (duodenum and jejunum) were weighed and homogenized with 5 volumes of cold 75%–alcohol. After centrifugation, an 0.2 ml aliquot was counted by the liquid scintillation counter. The remaining portion was concentrated *in vacuo* and then applied to the filter paper for chromatography.

Identification of Urinary Metabolites——The urinary metabolites were identified both by paper chromatography and cocrystallization with authentic compounds. The radioactive areas of chromatograms corresponding to the known authentic samples were extracted with water, and to the extract was added the appropriate cold carrier. The mixture was crystallized three times from a suitable solvent and the specific activity of each product was determined.

Measurement of Radioactivity——A Tri-Carb liquid scintillation spectrometer Model 314 A was used for assay of radioactivity. Radioassay of paper chromatograms was carried out according to the modified procedure of Cayen, *et al.*[9] The chromatograms were cut into 5 or 10 mm segments, and each segment was extracted with one ml of distilled water in a scintillation counting vial, adding 15 ml of the scintillation fluid, and counted. The scintillation fluid was made by dissolving 7.0 g of 2,5–diphenyloxazole and 50 mg of 1,4–bis–2–(5–phenyloxazolyl)benzene in one liter of 50%–ethanol—toluene. The counting data were corrected for quenching by adding to a sample a known amount of ^{3}H–thiamine or ^{35}S–thiamine as an internal standard.

Results

Identification of ^{3}H-HMP in Urine

Identification of ^{35}S–HT in the urine of rat administered ^{35}S–thiamine was described in the previous paper.[2] When the urine of rat administered ^{3}H–thiamine was subjected to paper chromatography, five radioactive components were observed normally. As shown

8) R.R. Williams, R.E. Waterman, J.C. Keresztesy, and E.R. Buchman, *J. Am. Chem. Soc.*, **57**, 536 (1935).
9) M.N. Cayen and P.A. Anastassiadis, *Anal. Chem.*, **15**, 84 (1966).

Table I. Percentages of ^{3}H and ^{35}S Radioactivity in Urine of Rat Present as Radioactive Metabolites after Oral and Parenteral Administration of ^{3}H–Thiamine and ^{35}S–Thiamine

Rf value[a]	Corresponding compound	^{3}H–Thiamine oral admin. (29.2%)[c] (6)[d] (%)	^{3}H–Thiamine *i.v.* admin. (64.0%)[c] (2)[d] (%)	^{35}S–Thiamine[b] oral admin. (58.0%)[c] (6)[d] (%)
0.0 —0.15	thiamine phosphates	25.5[f] (7.5)[h]	33.0(21.2)[h]	1.5(0.9)[h]
0.20—0.30	thiamine	51.0[g] (14.9)[h]	62.0(39.7)[h]	6.5(3.8)[h]
0.35—0.40	HMP	16.5 (4.8)[h]	2.5(1.6)[h]	0.5
0.50—0.60		3.5	1.5	0.5
0.65—0.75		1.0	0.5	7.0
0.80—0.90	HT	0	0	84.0(48.7)[h]
	THO[e]	2.5	0.5	—

a) The chromatogram was developed with a solvent system of butanol—acetic acid—water (4:1:5, v/v).
b) The data were quoted from the previous paper.[2]
c) These values indicate percent recovery of administered radioactivity.
d) The values indicate number of experiments.
e) THO represents the labile tritium eliminated *in vivo* from ^{3}H–thiamine.
f) This percentage represents a mixture of ^{3}H–thiamine phosphates and ^{3}H–pyrimidyl derivative.
g) This percentage represents a mixture of ^{3}H–thiamine and ^{3}H–pyrimidyl derivative.
h) These values indicate percentage recovery of administered radioactivity.

in Table I, the percentages of the radioactivity in the area with *Rf* values of 0.35—0.45 corresponding to that of authentic ^{3}H–HMP were greatly different between oral and parenteral administrations. This indicated that an unknown material was formed in the digestive tract. The radioactive material in this area was extracted with distilled water and re-chromatographed in three different solvent systems. The unknown compound and authentic ^{3}H–HMP had the same mobility as shown in Table II. Furthermore, 100 mg of non-labeled HMP

Table II. Range of *Rf* Values for the Unknown Compound and for 2-Methyl-4-amino-5-hydroxymethylpyrimidine (HMP) in Various Systems

Solvent system	*Rf* values	
	Unknown compound	HMP
Butanol—acetic acid—water (4:1:5, v/v)	0.35—0.40	0.38—0.40
Propanol—water—acetate buffer pH 5 (65:20:15, v/v)	0.46—0.51	0.50—0.52
Butanol saturated with water	0.57—0.62	0.59—0.62

was added to the extract of the chromatograms, and crystallized three times from water. Specific activities of first, second and third product were 1260 cpm/mg, 1120 cpm/mg, and 1130 cpm/mg, respectively.

From these results, it was concluded that HMP was formed from thiamine in rat. However, it was noticed that the amount of ^{3}H–HMP excreted in the urine was extremely small as compared to that of ^{35}S–HT, as shown in Table I.

Behaviors of Pyrimidine- and Thiazole-Moieties of Thiamine

The ratios of $^{3}H/^{35}S$, and of ^{3}H–HMP/^{35}S–HT in the small intestine, liver, kidney, and blood were determined one hour after the oral administration of a mixture of ^{3}H– and ^{35}S–thiamine. As shown in Table III, the ratios of $^{3}H/^{35}S$ in blood, liver and kidney were below one, but only in the small intestine the ratio was above one. These data indicated that the behavior of the pyrimidine moiety was different from that of the thiazole moiety in the

Table III. The Ratio of ^{3}H–HMP and ^{35}S–HT in Blood and Organs One Hour after Oral Administration of Equal Amount of ^{3}H–Thiamine and ^{35}S–Thiamine

	Total ^{3}H (%/g)[a]	Total ^{35}S (%/g)[a]	Ratio $^{3}H/^{35}S$	^{3}H–HMP (%)[b]	^{35}S–HT (%)[b]	Ratio ^{3}H–HMP/^{35}S–HT
Intestinal wall	3.85	2.20	1.75	1.5	26.0	0.06
Blood	0.04	0.18	0.22	13.5	82.0	0.16
Liver	0.17	0.28	0.62	15.0	42.5	0.35
Kidney	0.15	0.59	0.25	18.5	79.5	0.23

a) Values in these columns indicate percent recovery of administered radioactivity.
b) Values in these columns indicate the ratios of ^{3}H–HMP/total ^{3}H and the ratios of ^{35}S–HT/total ^{35}S.
The data in this Table indicate the mean values of four animals.

animal body. On the other hand, the ratios of ^{3}H–HMP/^{35}S–HT were much smaller than one both in the blood and in all other organs studied. Especially in the small intestine, where the cleavage of thiamine was thought to occur, the ratio was the smallest.

Urinary Excretion and Metabolism of ^{3}H-HMP and ^{35}S-HT

Since it was demonstrated by Shintani[3] and Imai, *et al.*[4] that both HMP and HT were metabolized in the animal body, it was necessary to ascertain whether the thiamine molecule was hydrolyzed directly into HT and HMP. It was found that most of the administered radioactivity were excreted in the urine within 24 hours after oral administration of ^{3}H–HMP or ^{35}S–HT, as shown in Table IV. When ^{3}H–HMP was orally administered into rats, more

Table IV. Urinary Radioactive Metabolites 24 Hours after Oral Administration of ^{3}H–2–Methyl–4–amino–5–hydroxymethylpyrimidine (^{3}H–HMP) and ^{35}S–4–Methyl–5β–hydroxyethylthiazole (^{35}S–HT)

Rf values[a]	Corresponding compound	^{3}H–HMP (73.8%)[b] (%)[c]	^{35}S–HT (89.5%)[b] (%)[c]
0.0 —0.16	unknown	2.5	
0.20—0.30	unknown		0.5
0.35—0.40	HMP	84.5	
0.40—0.45	unknown		0.5
0.65—0.75	ThA[d]		14.0
0.80—0.90	HT		85.0
	THO[e]	13.0	
		100.0	100.0

A dose of 1 mg of ^{3}H–HMP or 5 mg of ^{35}S–HT was orally administered into rat.
a) The chromatograms was developed with a solvent system of butanol—acetic acid—water (4:1:5, v/v).
b) Percent recovery of administered radioactivity.
c) Figures in these columns represent percentages of radioactive metabolites in urine of rat.
d) *Rf* value of 4–methylthiazole–5–acetic acid (ThA) obtained by oxidation of HT with potassium permanganate, was 0.73.
e) THO represents the labile tritium eliminated *in vivo* from ^{3}H–HMP.
The data in this Table indicate the mean values of four animals.

than 80% of the excreted radioactivity was found to be present as unchanged ^{3}H–HMP. However, Shintani[3] reported that a large portion of injected HMP were oxidized to 2–methyl–4–amino–5–pyrimidinecarboxylic acid (PCA) in rabbit. To examine the amount of tritiated water (THO) produced by oxidation of ^{3}H–HMP *in vivo*, an aliquot of 24 hours urine sample was evaporated under infra–red lamp, and the amount of the lost radioactivity was determined. It was found that 13% of the radioactivity excreted in the urine was present as THO.

Thompson[10] reported that the biological half life of body water in rat was 3.3 days. Therefore, it was assumed that most of the unexcreted radioactivity were retained as THO in the body of rat. Consequently, as shown in Table IV, 62% (84.5×73.8%) of administered ^{3}H–HMP was excreted unchanged. On the other hand, when ^{35}S–HT was orally administered into rats, 85% of the excreted radioactivity was ^{35}S–HT. However, Imai, *et al.*[4] reported that more than 95% of the injected ^{35}S–HT were recovered as ^{35}S–4–methylthiazole–5–acetic acid (ThA) in the urine of rat. The results of the present study showed that only 14% of the excreted radioactivity was present as ^{35}S–ThA. Imai, *et al.*[4] described that HT was readily extractable, but ThA was hardly extractable with $CHCl_3$. When 24 hours urine sample was adjusted to pH 6.0 and then extracted with $CHCl_3$, more than 70% of the total radioactivity was transferred into chloroform. This indicated that most of the radioactivity in the urine was not ^{35}S–ThA but ^{35}S–HT. Consequently, 76% (85.0×89.5%) of administered ^{35}S–HT was excreted unchanged, as shown in Table IV. From the results, it was clear that more than 50% of both HMP and HT administered were excreted unchanged in the urine within 24 hours.

Discussion

Kawasaki, *et al.*[11] reported that HMP was isolated from human urine. The present results also showed that ^{3}H–HMP was excreted in the urine of rats after the oral administration of ^{3}H–thiamine. However, the amount of ^{3}H–HMP excreted in 24 hours urine is only 4.8% of the administered radioactivity, about one–tenth of the amount of ^{35}S–HT (48.7%), as shown in Table I. The great difference in urinary excretion of ^{3}H–HMP and ^{35}S–HT might be due to the fact that the metabolic fate of HMP is greatly different from that of HT or that the thiamine molecule, *in vivo*, is not directly hydrolyzed to HMP and HT. The former assumption could be excluded, since most of the administered ^{3}H–HMP and ^{35}S–HT were found to be excreted unchanged in the urine within 24 hours (Table IV). Therefore, it is more probable that the cleavage of thiamine might not be a direct hydrolysis into HMP and HT. If a direct hydrolysis of thiamine occurs in rat, the ratio of HMP/HT at the site of cleavage must be close to one. However, when a mixture of equal amount of ^{3}H–thiamine and ^{35}S–thiamine was orally administered, the ratio of ^{3}H–HMP/^{35}S–HT in the small intestine, where the decomposition of thiamine was thought to occur, was less than 0.1 (Table III). From this fact, it seems reasonable to assume that the cleavage of thiamine is not a direct hydrolysis into HMP and HT. The ratio of ^{3}H/^{35}S only in the small intestine is larger than one even though the ratio of ^{3}H–HMP/^{35}S–HT in the small intestine is much smaller than one. This indicates that a different pyrimidyl derivative is produced together with HT by the cleavage of thiamine. When we compare the amounts of radioactivity of the paper chromatograms in the area of *Rf* 0.0—0.15 (thiamine phosphates) and in the area of *Rf* 0.20—0.30 (thiamine), we find that amounts of radioactivity in the both areas after oral administration of ^{3}H–thiamine are much larger than those after oral administration of ^{35}S–thiamine (Table I). This indicates that not only thiamine phosphates or thiamine, but also other pyrimidyl derivative are present. When the extracts from *Rf* 0.0—0.15 area and *Rf* 0.20—0.30 area were treated with 1N HCl, and then oxidized with potassium ferricyanide, most of the former extract were unchanged, but a large part of the latter extract was converted to thiochrome and other compounds. It was assumed that two kinds of pyrimidyl derivative were produced by the cleavage of thiamine. On the other hand, from the presence of ^{3}H–HMP in the urine of rats after oral administration of ^{3}H–thiamine, it could not be ruled out that a small amount of thiamine might be directly hydrolyzed into HT and HMP.

10) R.C. Thompson, *J. Biol. Chem.*, **200**, 731(1953).
11) C. Kawasaki and K. Okada, *Vitamins*, **13**, 255 (1957).

Since the ratio of ^{3}H–HMP/^{35}S–HT is much smaller in the small intestine than those in blood, liver and kidney (Table III), it seems possible that HMP is a secondary product from a primary pyrimidyl derivative. It is clear that the main cleavage of thiamine is not a direct hydrolysis into HT and HMP. From the facts described above, the metabolic pathways of thiamine in rats may be represented as shown in Chart 2.

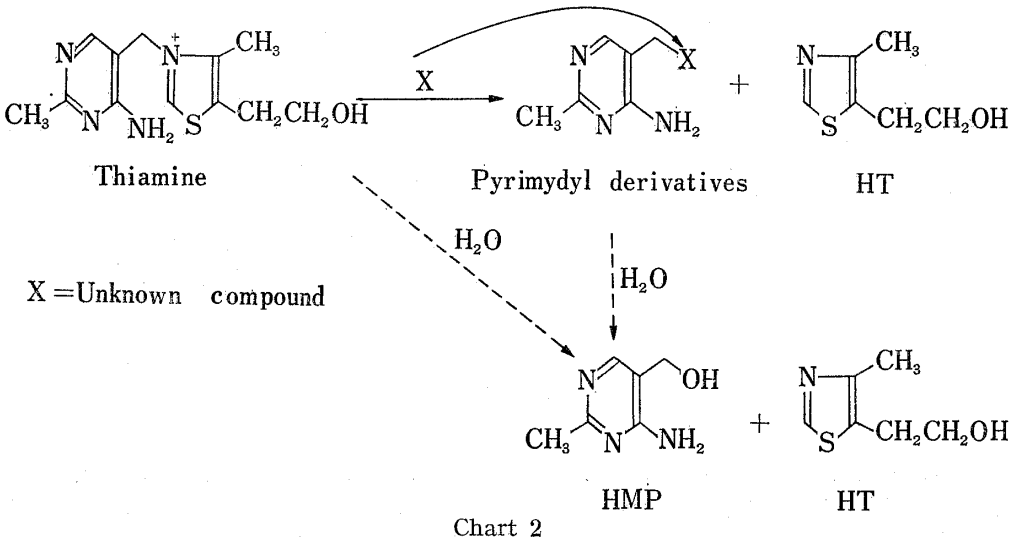

Chart 2

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