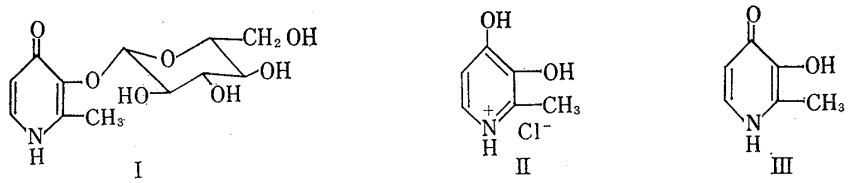

Chart 1.

The authors are greatly indebted for the microanalysis to Miss Tamie Yamagishi of this university and to the staff of Institute for Organic Microanalysis, Faculty of Pharmaceutical Sciences of Kyoto University. Thanks are also due to the staff of the Research Laboratories, Takeda Chemical Industries, Ltd., for the NMR and molecular weight measurements.

Faculty of Pharmaceutical Sciences,
Nagoya City University,
Tanabe-dori, Mizuho-ku, Nagoya

Masaiti Yasue (安江政一)
Norihisa Kawamura (河村典久)

Received July 20, 1966

[Chem. Pharm. Bull. 14(12)1444~1448(1966)]

UDC 612.392.01-084 : 612.015.3

Hydrolytic Cleavage of Thiamine in Mammalian Animals

The metabolic fate of thiamine has been studied in various species of animals, but relatively little is known of how it is metabolized in the mammalian body. Verrett, *et al.*[1] reported that oral and parenteral routes of administration did not make great differences in the metabolic pattern of ^{35}S-thiamine in rabbits.

The results of the present study showed that the metabolic pattern of ^{35}S-thiamine in rats was remarkably different between oral and parenteral routes of administration. Female Wister rats weighing 150~200 g. were used. They were housed in cages constructed to permit the separate collection of urine and feces. ^{35}S-Thiamine with a specific activity of 25 μc./mg., was prepared from $C^{35}S_2$ according to the procedure of Matsukawa.[2] A dose of 0.2 mg. of ^{35}S-thiamine was administered orally and intravenously. Twenty-four-hour urine specimens were collected in glass bottles. For separation of the urinary metabolites, paper chromatography was employed. A part of the pooled urine was spotted on Toyo filter paper No. 51 and developed with *n*-butanol-acetic acid-water(4:1:5, v/v). Radioactive scanning of paper chromatograms was accomplished by dividing the chromatograms in 10 mm. segments, extracting each segment with distilled water and counting ^{35}S radioactivity of each extract in a windowless gas-flow counter. No attempt was made to correct for recovery of the radioactivity from chromatograms and sample absorption in extracts. Scintillation counting was used to determine the recovery of the administered radioactivity from the urine. The percentages of ^{35}S radioactivity in urine represented by radioactive metabolites of ^{35}S-thiamine are indicated in Table I.

1) M.J. Verrett, L.R. Cerecedo : Proc. Soc. Exp. Med. Biol., **98**, 509 (1958).
2) T. Matsukawa, T. Iwazu : Yakugaku Zasshi, **70**, 28 (1950).

From data in Table I, it is evident that the metabolic pattern of ^{35}S-thiamine is considerably different between oral and parenteral routes of administration. The Rf value of authentic ^{35}S-thiamine is 0.24, and hence most of the radioactivity in peak no. 2 are considered to be unchanged ^{35}S-thiamine. When ^{35}S-thiamine was injected intravenously, 65.5% of the excreted radioactivity occurred in the area corresponding to thiamine, as shown in Table I. This datum is in agreement with the result of Iacono *et al.*[3] that approximately 60% of the excreted radioactivity occurred in the thiamine area when ^{14}C-thiamine was injected intraperitoneally into rats. On the other hand, when ^{35}S-thiamine was administered orally, only 6.5% of the excreted radioactivity was observed in the thiamine area, and 84.0% was found in the area corresponding to Rf value of 0.80~0.90. Since the Rf value of the unknown compound was high, it was assumed that this compound might have high lipid-solubility.

When the urine of rats receiving 10 mg of ^{35}S-thiamine was extracted with chloroform, it was found that more than 70% of the excreted radioactivity was transferred into chloroform layer. The extract did not fluoresce when treated with potassium ferricyanide, which converts thiamine and thiamine derivatives into their corresponding thiochrome derivatives, whereas it colored when treated with Dragendorff's reagent. These facts led to an assumption that the unknown compound in peak no. 6 might be thiazole moiety of thiamine.

Thiazole moiety of thiamine has been reported to occur in rat and rabbit urine by several groups of workers.[1,3~5] Recently, Ogawa[6] described that 4-methyl-5β-hydroxyethylthiazole (HT) could be identified in rabbit urine by paper chromatography. Therefore, HT was prepared from thiamine according to the procedure of Matsukawa,[7] and chromatographed in *n*-butanol-acetic acid-water (4:1:5, v/v), along with the chloroform extract of rat urine. As a result, its Rf value was identical to that of the unknown compound. The range of Rf values obtained with the unknown compound and with HT in 3 solvent systems is shown in Table II. To ascertain the unknown compound from urine to be HT, isotope dilution method was applied.

The experimental details are as follows: The pooled urine was concentrated *in vacuo* and then extracted with alcohol. To the alcohol solution was added 100 mg. of non-labeled HT and the solvent was evaporated. The residue was extracted with dry chloroform and the chloroform layer was extracted with *N*/10 hydrochloric acid. The aqueous solution was adjusted to pH 9.0 with *N* aqueous ammonia and then extracted with chloroform. The chloroform layer was dried over sodium sulfate and the solvent was evaporated. The residue was dissolved in 2 ml. of alcohol, and to alcohol solution was added the alcohol solution containing 300 mg. of picric acid. The crystallized picrate was recrystallized from alcohol and then from hot water. Specific activity of each picrate was determined. As the results, specific activities of first, second and third picrates were 9400 c.p.m./mg., 9200 c.p.m./mg., and 9150 c.p.m./mg., respectively. The slight decrease in specific activity was thought to be due to the presence of non-radioactive other picrate. From these data above described, it is evident that ^{35}S-HT is excreted in rat urine after oral administration of ^{35}S-thiamine. This compound, which is known to be a product of the action of a specific bacterial thiaminase,[8] would be generated by hydrolytic cleavage of the covalent bond between the methylene bridge and thiazole

3) J.M. Iacono, B.C. Johnson: J. Am. Chem. Soc., **79**, 6321 (1957).
4) P.T. McCarthy, L.R. Cerecedo, E.V. Brown: J. Biol. Chem., **209**, 611 (1954).
5) T. Suhara, N. Iritani: J. Vitaminol., **8**, 128 (1962).
6) R. Ogawa: Vitamins, **33**, 318 (1966).
7) T. Matsukawa, S. Yurugi: Yakugaku Zasshi, **71**, 1423 (1951).
8) A. Fujita, Y. Nose, K. Kuratani: J. Vitaminol., **1**, 1 (1954).

ring. The occurrence of the pyrimidine moiety of thiamine in urine has been reported. Kawasaki, *et al.*[9] isolated 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) from human urine after giving large doses of thiamine to test subjects. Neal, *et al.*[10] reported that 2-methyl-4-amino-5-pyrimidinecarboxylic acid, an oxidation product of HMP, was found in the urine of rats receiving ^{14}C-thiamine. The marked difference in metabolic pattern of ^{35}S-thiamine brought about by different routes of administration suggests that formation of HT from thiamine occurs in the gut. A few percent of ^{35}S-HT was detected in rat urine even after parenteral administration of ^{35}S-thiamine, as shown in Table I. This may be explained by taking account of the fact

Table I. Percentage of ^{35}S Radioactivity in Rat Urine Present as Radioactive Metabolites after Oral and Parenteral Administration of ^{35}S-Thiamine

Peak No.	Rf value	Corresponding compound	Oral admin. (6) (%)	Intravenous injection (2) (%)
1	0.0 ~0.15	thiamine phosphates[a]	1.5	25.5
2	0.20~0.30	thiamine	6.5	65.5
3	0.35~0.45	thiochrome[b]	0.5	1.5
4	0.50~0.60		0.5	1.0
5	0.65~0.75		7.0	2.0
6	0.80~0.90	HT	84.0	4.5

Values in parentheses indicate number of experiments.
a) Rf values of authentic thiamine monophosphate and thiamine diphosphate are 0.10 and 0.06, respectively.
b) Rf value of thiochrome is 0.40.

that the injected thiamine is secreted into the gut lumen.[11]

Since it is known that bacterial thiaminase occurs in the gut, there is a possibility that HT may be produced microbiologically.

However, Neal, *et al.*[10] described that this possibility would have to be excluded, since even in the urine of germ-free rats the presence of 2-methyl-4-amino-5-pyrimidinecarboxylic acid was demonstrated. The results of chromatographic analyses of the radioactivity in portal venous blood, intestinal wall, liver and kidney one hour after oral administation of ^{35}S-thiamine are shown in Table III. The percentage of

Table II. Range of Rf Values for the Unknown Compound and for 4-Methyl-5β-hydroxyethylthiazole (HT) in Various Systems

Solvent system	Rf values	
	Unknown compound	HT
n-butanol–acetic acid–water (4:1:5, v/v)	0.83~0.88	0.83~0.85
n-propanol–water–acetate buffer pH 5 (65:20:15, v/v)	0.90~0.94	0.92
n-butanol saturated with water	0.33~0.36	0.35

^{35}S-HT in portal venous blood was the highest and that in intestinal wall was the lowest. This also indicates that only in the gut HT is formed from thiamine and suggests that the transport of HT from intestinal wall into portal blood takes place extremely rapidly as compared to that of thiamine.

9) T. Kawasaki, K. Okada : Vitamins, **13**, 351 (1964).
10) R.A. Neal, W.N. Pearson : J. Nutrition, **83**, 351 (1964).
11) B. Gassmann, H.A. Ketz : Biochem. Z., **334**, 245 (1961).

Table Ⅲ. Percentage of ^{35}S Radioactivity in Blood, Intestinal Wall, Liver and Kidney Present as Radioactive Metabolites One Hour after Oral Administration of ^{35}S-Thiamine

Rf value	Portal blood (0.18%/ml.) (%)	Intestinal wall (2.2%/g.) (%)	Liver (0.28%/g.) (%)	Kidney (0.59%/g.) (%)
0.0 ~0.15	4.0	16.0	18.5	2.5
0.20~0.30 (thiamine)	5.0	56.5	12.0	3.0
0.35~0.45	2.0	1.5	1.0	1.0
0.50~0.60	1.0	0	0	0
0.65~0.75	6.0	0	26.0	14.0
0.80~0.90 (HT)	82.0	26.0	42.5	79.5

Values in parentheses indicate percent recovery of administered radioactivity. The percentages in four columns indicate the mean values of two animals.

To know whether the hydrolytic cleavage of thiamine occurs in other mammalian animals, the similar experiments were made with adult guinea pig, mouse and rabbit. As shown in Table Ⅳ, it was found that ^{35}S-HT was excreted in the urine of all of the animals studied. However, percentages of ^{35}S-HT in the urine of these animals were smaller than that in rat urine. The excretion of ^{35}S radioactivity and unchanged ^{35}S-thiamine in urine 24 hours after oral administration of ^{35}S-thiamine is shown in Table Ⅴ. Percent recovery of the administered radioactivity was the highest in rat

Table Ⅳ. Percentage of ^{35}S Redioactivity in Urine of Mammalian Animals Present as Radioactive Metabolites after Oral Administration of ^{35}S-Thiamine

Rf value	Corresponding compound	Rabbit (2) (%)	Mouse (2) (%)	Guinea pig (4) (%)
0.0 ~0.15	thiamine phosphates	9.0	8.3	20.2
0.20~0.30	thiamine	40.7	40.5	41.2
0.35~0.45	thiochrome	3.5	1.7	4.6
0.50~0.60		3.0	3.0	6.0
0.65~0.75		6.3	18.7	5.3
0.80~0.90	HT	37.5	27.8	22.7

Values in parentheses indicate number of experiments.

Table Ⅴ. Excretion of ^{35}S Radioactivity and ^{35}S-Thiamine in Urine of Mammalian Animals 24 Hours after Oral Administration of ^{35}S-Thiamine

Animal	No. of exp.	Admin. dose (μg./animal)	Recovery of ^{35}S (% of admin. ^{35}S)	Recovery of ^{35}S-thiamine (% of admin. ^{35}S)[a]
Rat	6	200	58.0	3.77
Guinea pig	4	200	9.8	4.04
Rabbit	2	2000	30.5	12.42
Mouse	2	50	29.3	11.85

a) Values in this column were obtained by multiplying the percentage of the radioactivity in the area of Rf value (0.20~0.30). which was shown in Table I and Ⅳ, by the percentage of total radioactivity in the urine, which was shown in this table.

and the lowest in guinea pig. When one compares the percent recovery of the total radioactivity and unchanged ^{35}S-thiamine in rat with that in guinea pig receiving the same dose of ^{35}S-thiamine, one finds that the total radioactivity excreted in rat urine is six times as high as that in guinea pig urine although the amount of unchanged

^{35}S-thiamine in rat urine is similar to that in guinea pig urine. This is apparently due to the fact that more than 80% of the radioactivity excreted in rat urine is present as ^{35}S-HT whereas in guinea pig urine only about 20% is ^{35}S-HT. This, in turn, indicates that the hydrolytic cleavage of thiamine occurs much more actively in rat than in guinea pig. It should be noted that only about 4% of the administered ^{35}S-thiamine was excreted as unchanged ^{35}S-thiamine in rat and guinea pig urine within 24 hours when a dose of 200 μg. of ^{35}S-thiamine was orally administered.

This data approximately agree with the report of Fukutomi[12] that 5% of the administered thiamine was excreted in rat urine within 5 days when 500 μg. of thiamine was orally administered for 4 days. To know whether or not bacterial thiaminase takes part in hydrolytic cleavage of thiamine in rat, further experiments are now in progress.

The authors wish to express their deep gratitude to Dr. K. Abe, Director of this Research Laboratory for his interest and encouragement throughout this work.

Biological Research Laboratory,
Tanabe Pharmaceutical Co., Ltd.,
Toda-cho, Kitaadati-gun, Saitama

Teruhiko Meshi (飯 照彦)
Yoshishige Sato (佐藤善重)

Received August 11, 1966

12) H. Fukutomi: Vitamins, **33**, 151 (1966).

[Chem. Pharm. Bull. 14(12)1448~1450(1966)]

UDC 547.873.07

Covalently Hydrated Oxazirino-tetrahydro-*as*-triazines

In our previous work,[1] we reported about the oxidation products of 5,6-diphenyl-*as*-triazin-3(2*H*)-one and its 2-methyl analogue with organic peracids, presenting their structures as 5,6-diphenyl-*as*-triazin-3(2*H*)-one 1-oxide mono-hydrate (Ⅱ'a) and its 2-methyl analogue (Ⅱb).

Now, we must revise their structures as 6-hydroxy-6,7-diphenyloxazirino[2,3-*f*]hexahydro-*as*-triazin-4-one (Ⅱa) and its 3-methyl analogue (Ⅱb) on the basis of further chemical and spectroscopical reinvestigations.

The mass spectrum of Ⅱa showed: m/e 283 (M^+), 162, 121 (fission A), 147, 136 (fission B, suggesting a prototropy upon electron impact), 118 and 103, the latter two being understandable as generated by dehydration of the fragment 136 and 121, respectively. The absence of $(M-16)^+$ peak[2] to be formed by deoxygenation of a N-oxide group and of $(M-18)^+$ peak to be generated by dehydration precludes a structure with a N-oxide group and a molecule of water of crystallization for the oxidation products.

Reduction of Ⅱb with lithium aluminum hydride led to 2-methyl-5,6-diphenyl-4,5-dihydro-*as*-triazin-3(2*H*)-one (Ⅲ),[3] indicating the *as*-triazine ring was retained in these oxidation products. Methylation of Ⅱa with methyl iodide in the presence of alkali

1) T. Sasaki and K. Minamoto: This Bulletin, **13**, 1172 (1965).
2) *a*) For identification of the N-oxide group by mass spectrometry, see T.A. Bryce and J.R. Maxwell: Chem. Commun., 206 (1965); *b*) We also observed, without exception, $(M-16)^+$ peak in the mass spectra of *as*-triazine N-oxides we sythesized. The data in this line will be published later.
3) H. Bilz, *et al*: Ann., **339**, 285 (1905).