Biological Activities of β -Ketovinyl Thioether Type Thiamines and Related Compounds

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β -Ketovinyl thioether type thiamines and the related compounds have been examined on thiamine activity as well as thiamine liberation *in vitro* and *in vivo*. Sixteen compounds showed more or less vitamin activity, of which the most active ones were BD 108 and BD 81 with about 70 % of the activity of thiamine HCl, while BD 101 and BD 142 showed no activity. BD 99, BD 108, and BD 112 with higher thiamine activity were converted into thiamine by incubation with glutathione. Urinary excretions of thiamine was significantly increased following intraperitoneal injection of these derivatives. A correlation was recognized between thiamine activity and urinary excretion of thiamine.

Vitamin B₁ activity of newly synthesized thiamine derivatives has been studied by a standard curative assay using thiamine deficient rats. This report describes the results obtained on eighteen derivatives of thioether type thiamine including S-(β -ketovinyl) thiamines and related compounds.

EXPERIMENTAL

Test Compounds

Table 1 lists the names and chemical structures of test compounds, which were supplied from Chemical Research Laboratories of this division. Compounds BD 80 and BD 81 were synthesized by Hieda *et al.* (1). Compound BD 82 was synthesized by Oka (2). The other compounds were synthesized by Shimabara and Shirakawa. Chemical studies on some of these thiamine derivatives have been published by them (3).

Bioassay for Thiamine Activity

Vitamin B₁ activity was assayed by a stan-

dard curative test using thiamine deficient rats. Weanling male rats of Sprague-Dawley strain (SD-JCL, Japan CLEA, Inc., Tokyo) were maintained individually in metal cages with screen-bottoms and fed a thiamine-deficient diet (No. T6), of which composition is indicated in Table 2. The rats began to lose their body weights with the development of thiamine deficiency as early as 10 days feeding on the deficient diet (Fig. 1). After a depletion period of 14 to 18 days, the rats were divided into groups of 6 to 7 rats. The rats were then given thiamine or test compound, dissolved in 0.3 ml of water, orally by stomach tube each day or every other days throughout the curative period of two or three weeks. The daily dose of thiamine was 0.01, 0.02 or 0.04 μ moles per rat per day. The dose of test compound was increased, depending on their vitamin activity, so as to give comparative effects as those revealed by the reference thiamine. The compound was classed as "inactive" when no appreciable response was recognized even at the dose of

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TABLE 1
Summary of test compounds and their biological properties

$ \begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_2 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_3 \end{array} \begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_2 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_3 \end{array} \begin{array}{c} \text{CHO} \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_2 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_3 \end{array} \begin{array}{c} \text{S-R} \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_2 \\ \diagup \\ \text{N} \\ \diagdown \\ \text{CH}_3 \end{array} \begin{array}{c} \text{CH}_2\text{CH}_2\text{OH} \end{array} $					
Compound No.	Name	R =	Thiamine activity ^a	Conversion to thiamine by GSH ^b	Urinary excretion of thiamine ^c
BD 108	S-(3-bromo-5-methoxy-2-furanon-4-yl) thiamine		69	54.2	24.0 ± 2.1
BD 99	S-(3-chloro-5-methoxy-2-furanon-4-yl) thiamine		48	37.0	21.1 ± 5.3
BD 110	S-(3-chloro-5-acetoxy-2-furanon-4-yl) thiamine		22	-	-
BD 109	S-(3-chloro-5-benzyloxy-2-furanon-4-yl) thiamine		22	-	-
BD 120	S-(5-ethoxy-5-phenyl-2-furanon-4-yl) thiamine		25	-	-
BD 98	S-(4-chloro-5-methoxy-2-furanon-3-yl) thiamine		33	9.3	2.8 ± 2.0
BD 101	S-(4-chloro-3-pyridazon-5-yl) thiamine		0	1.0	0.04 ± 0.02
BD 112	S-(1-methyl-4-nitroimidazol-5-yl) thiamine		45	54.2	19.9 ± 10.4
BD 97	S-(α -carboxy- β -chloro- β -formylvinyl) thiamine		35	25.0	10.5 ± 1.5
BD 142	S-cis-(β -methoxycarbonylvinyl) thiamine		0	2.3	0.4 ± 0.16
BD 143	S-trans-(β -methoxycarbonylvinyl) thiamine		16	4.9	8.3 ± 0.12
BD 138	S-cis-(β -chloro- β -methoxycarbonylvinyl) thiamine		32	5.0	18.0 ± 3.6
BD 102	S-trans-(β -chloro- β -methoxycarbonylvinyl) thiamine		34	11.2	11.8 ± 0.9
BD 133	S-(β -chloro- β -benzenesulfonylvinyl) thiamine		12	-	-
BD 185	S-(β -bromo- β -methoxycarbonylvinyl) thiamine		27	-	-
BD 82	S-(2-cyanovinyl) thiamine		30	-	-
BD 81	S-cis-benzoylvinyl thiamine		70	-	-
BD 80	S-trans-benzoylvinyl thiamine		90	-	-

^a Rat growth assay (Thiamine · HCl=100%).^b Per cent conversion (See Table 4).^c Per cent of dose/24 hr (See Table 3).

TABLE 2
Composition of thiamine-deficient diet, No. T6

Vitamin free casein	18.0 g
Sucrose	67.6
Cottonseed oil*	8.0 ml
Salt mixture (15)**	4.0 g
Powdered cellulose	1.5
DL-Methionine	0.3
Choline chloride	0.1
Vitamin mixture***	0.5
* Cottonseed oil supplement (in 8 ml)	
Vitamin A palmitate	2000 IU
Vitamin D ₂	200 IU
DL- α -Tocopheryl acetate	10 mg
** Salt mixture (4 g)	
CaCO ₃	1.26 g
K ₂ HPO ₄	1.36
CaHPO ₄ ·2H ₂ O	0.32
MgSO ₄	0.21
NaCl	0.71
Ferric citrate·6H ₂ O	0.12
KI	0.0034
MnSO ₄	0.016
ZnCl ₂	0.001
CuSO ₄ ·5H ₂ O	0.0012
*** Vitamin mixture (0.5 g)	
Riboflavin	0.8 mg
Pyridoxine·HCl	0.5
Niacin	5
Ca pantothenate	4
Biotin	0.04
Folic acid	0.2
Vitamin B ₁₂	0.003
p-Aminobenzoic acid	10
Inositol	10
Ascorbic acid	20
Menadione	0.5
Glucose was added to the mixture to make	0.5 g.

FIG. 1 Body weight curves in curative assay using thiamine-deficient rats

Cross indicates death due to thiamine deficiency. Experimental conditions are described in the text.

FIG. 2 Dose-response curves in curative assay using thiamine-deficient rats

Data are the same as those described in Fig. 1.

0.16 μ moles per day per rat. Weekly weight gains obtained during the curative period were plotted against logarithm of dose (Fig. 2). This semi-logarithmic plotting usually gave two parallel lines of both standard thiamine and test compound, permitting calculation of the vitamin potency as described by György (4).

In Vivo Conversion of Thiamine Derivatives to Thiamine

Male Sprague-Dawley rats weighing about 200 g were maintained on a usual semi-synthetic complete diet, No. T6, supplemented with 5 mg/kg of thiamine hydrochloride. The rats were administered 20 μ moles of thiamine (6.74 mg as thiamine hydrochloride) or thiamine derivatives by intraperitoneal injection and then placed in individual metabolism cages. The rats were fed on a thiamine-deficient diet, No. T6, in order to avoid contamination of dietary thiamine to urine. The urine was collected for three intervals of 0-4, 4-8, 8-24 hours. Urinary thiamine was determined by the thiochrome method (5).

In Vitro Conversion of Thiamine Derivatives to Thiamine

Male Sprague-Dawley rats weighing about 230 g were anesthetized by ether inhalation. Blood was collected by heart puncture into heparinized injection syringe. Blood was then separated into plasma and blood cells by centrifugation. Plasma was added with 4 volumes of saline. Blood cells, after washed by saline, were hemolyzed by addition of 4 volumes of water.

Liver, kidney and upper part of small intestine were excised and homogenized with 20 volumes of 0.1 M phosphate buffer, pH 6.5 by Teflon homogenizer of Potter-Elvehjem type.

The reaction mixture consisted of 0.2 ml of 1 mM thiamine derivative solution, 0.2 ml of tissue homogenates or blood components and 1.6 ml of 0.1 M phosphate buffer, pH 6.5. In some experiments, 0.2 ml of 0.1 M glutathione (GSH) was added in place of tissue homogenates or blood components. The reaction mixture was incubated at 37°. Three 0.5 ml aliquots of the reaction mixture were pipetted out at appropriate intervals and added to 0.2 ml of 10% perchloric acid solution. After centrifugation, 0.5 ml aliquots of the supernatants were subjected to thiamine determination by the thiochrome method.

RESULTS

Thiamine Activity in Rat Growth Assay

Figure 1 shows one of typical results obtained in rat growth assay with a S-(α -ketovinyl) thiamine derivative, BD 108. From semi-logarithmic plot of dose and response (body weight gain), thiamine activity of BD 108 was calculated to be 69% of thiamine hydrochloride (Fig. 2). Table 1 summarizes the results obtained on eighteen thiamine derivatives. Of these, BD 108, BD 80 and BD 81 possessed the most active potency, while BD 101 and BD 142 were quite inactive.

In Vivo Conversion of Thiamine Derivatives to Thiamine

Thiamine derivatives of thiol type have no vitamin activity unless they are converted to thiamine. We have therefore examined the conversion both *in vivo* and *in vitro*. Table 3 shows the urinary excretion of thiamine after oral or intraperitoneal administration of thiamine or derivatives to rats. Control rats excreted only 3.4 ± 0.7 μ g of thiamine into daily urine under the present experimental condition. Rats receiving 20 μ moles (6.74 mg) of thiamine hydrochloride by injection excreted 14.8 μ moles of thiamine into the 24-hr urine. This amounted 73.9% of the given dose. Thiamine tetrahydrofurfuryl disulfide hydrochloride (TTFD) is one

TABLE 3
Urinary excretion of thiamine in rats after administration of thiamine, TTFD and thioether type thiamines

Compound*	Urinary thiamine (% of dose)**			
	0-4	4-8	8-24	0-24 hr
1) Intraperitoneal injection				
Thiamine·HCl	64.3	5.7	3.9	73.9 $\pm$ 9.4
TTFD·HCl	38.5	11.5	14.6	64.6 $\pm$ 3.8
BD 97	6.9	1.7	1.9	10.5 $\pm$ 1.5
BD 98	1.9	0.5	0.5	2.8 $\pm$ 2.0
BD 99	13.0	3.6	4.5	21.1 $\pm$ 5.3
BD 101	—	—	—	0.04 $\pm$ 0.02
BD 102	7.0	2.2	2.7	11.8 $\pm$ 0.9
BD 108	12.1	5.9	6.0	24.0 $\pm$ 2.1
BD 112	15.3	2.2	2.4	19.9 $\pm$ 10.4
BD 138	9.3	5.5	3.3	18.0 $\pm$ 3.6
BD 142	—	—	—	0.40 $\pm$ 0.16
BD 143	4.5	2.0	1.8	8.3 $\pm$ 0.12
2) Oral administration				
Thiamine·HCl	2.4	2.1	4.7	9.2 $\pm$ 1.1
TTFD·HCl	13.9	8.9	4.3	27.1 $\pm$ 3.7
BD 108	1.5	2.3	2.7	6.6 $\pm$ 0.4

* Dose, 20 μ moles (6.74 mg as thiamine·HCl).

** Each group consists of 3 rats. Data are expressed as mean $\pm$ S.D.

of thiol type thiamines, quickly and quantitatively transformed into thiamine in the animals and exerts vitamin B₁ activity as equal as that of thiamine itself (6). TTFD was therefore used as a positive control of thiol type thiamines. After intraperitoneal injection, 64.6% of the dosed TTFD was excreted as thiamine into the 24-hr urine. Following oral administration, only 9.2 $\pm$ 1.1% of the ingested thiamine was eliminated into the urine, whereas much more thiamine (27.1 $\pm$ 3.7%) was excreted from oral TTFD. These facts, as already pointed out by many investigators (7,8), reflect limited intestinal absorption of thiamine as well as better bioavailability of TTFD. Among the tested compounds, BD 108 which showed the most active vitamin activity, caused the most remarkable increase in urinary thiamine excretion. This result clearly indicated that considerable part of BD 108 was converted into thiamine in rats. In contrast, BD 101 and BD 142, which have little or no thiamine activity, showed no appreciable increase in urinary excretion of thiamine. This demonstrated failure of these compounds to be biotransformed into thiamine. As shown in Fig. 3, a correlation was recognized between thiamine activities and amounts of urinary thiamine among the tested compounds with an

FIG. 3 Relation between thiamine activity and urinary excretion of thiamine

Urinary thiamine excreted 24 hr after intraperitoneal injection of test compound (20 μ moles) to rats is expressed in percent of dose.

exception of a S-(α -ketovinyl) thiamine, BD 98. It was therefore concluded that the vitamin activity depended upon degree of liberation of thiamine from these derivatives in the animal.

In Vitro Conversion of Thiamine Derivatives to Thiamine

As mentioned above, some of thioether type thiamines were transformed into thiamine when they were administered to rats. We then examine whether the reaction is enzymic. Shimabara and Shirakawa (3) reported that some of these compounds were transformed into thiamine by heating with excess cysteine in 50% ethanol

TABLE 4
In vitro conversion into thiamine by GSH

Test compounds	Supplement	Percent conversion into thiamine		
		Incubation time		
		5 min	15 min	30 min
BD 97	GSH	12.7	18.2	25.0
BD 98	GSH	—	9.1	9.3
BD 99	GSH	7.5	20.5	37.0
BD 101	GSH	0.3	0.3	1.0
BD 102	GSH	1.8	5.3	11.2
BD 108	GSH	12.4	31.6	54.2
BD 108	—	0	0	0
BD 112	GSH	10.8	30.3	54.2
BD 138	GSH	—	4.1	5.0
BD 142	GSH	—	2.4	2.3
BD 143	GSH	0.9	3.2	4.9
TTFD·HCl	GSH	100	100	100
TTFD·HCl	—	0	0	0

Final concentrations in the reaction mixture (pH 6.5) are 0.1 mM for test compounds and 10 mM for GSH.

TABLE 5
In vitro conversion of BD 108 into thiamine by tissue homogenates or blood components

Supplement	Percent conversion into thiamine		
	Incubation time		
	5 min	15 min	30 min
—	0	0	0
GSH	12.4	31.6	54.2
Liver	0	1.6	2.7
Kidney	0	0.6	2.4
Intestine	0	0	0.6
Plasma	0	0	0
Hemolysates	2.8	7.9	15.7

Final concentrations in the reaction mixture (pH 6.5) are 0.1 mM for BD 108 and 10 mM for GSH.

FIG. 4 Relation between thiamine activity and in vitro conversion into thiamine by glutathione

Experimental conditions are described in the text.

solution (pH 5, 80°, 60 min). We examined the conversion in presence of glutathione (GSH) or tissue homogenates under physiological conditions. As shown in Table 4, BD 108 was most readily converted but the conversion was not complete after 30-min incubation. BD 101 and BD 142 with little or no thiamine activity were hardly transformed by GSH. Under the same condition, a disulfide type thiamine, TTFD, was quantitatively reduced to thiamine in 5 minutes. As shown in Fig. 4, thiamine activities of the derivatives were rather correlated to susceptibilities to GSH but the relation was not of a straight line but seemed to consist of two lines.

DISCUSSION

A number of thioether type thiamines were synthesized by Matsukawa and Iwatsu (9) and Shirakawa (10) but they had little or no biolo-

gical activity in animal bioassay. Later, Fusco and Tenconi (11) reported that several S-(β -acylvinyl) thiamines were endowed with considerable vitamin activity. S-(β -Benzoylvinyl) thiamine was completely transformed into thiamine *in vitro* by tissue homogenates (Rindi and de Giuseppe (12)). Oka *et al.* (13) synthesized S-acyloxyalkyl thiamines, of which some derivative such as S-benzoyloxymethyl thiamine possessed a full vitamin activity, as compared with that of thiamine itself (14). In the present studies we examined a series of S-acylvinyl thiamines with respect to thiamine activity as well as thiamine liberation both *in vitro* and *in vivo*. The results showed that some of them were inactive but some were of considerable biological activity. These compounds would exert no vitamin activity unless they were converted to thiamine, because the activity depended upon largely degree in thiamine liberation from the derivatives. The present studies also clarified that the conversion in the animal was non-enzymic, because the reaction proceeded with GSH *in vitro* whereas practically no conversion occurred with tissue homogenates or plasma. Among the tested living materials, only blood cell hemolysates could convert BD 108 into thiamine in appreciable amount. The reaction would be driven by GSH, because it is well-known that red blood cells contain GSH abundantly with powerful GSH regenerating system catalyzed by glutathione reductase and glucose-6-phosphate dehydrogenase. Thiamine activities were highly correlated to amounts in urinary excretion of thiamine, as shown by an approximate linear relationship between both parameters (Fig. 3). A correlation was also recognized between thiamine activity and thiamine liberation by GSH. In this respect, BD 98, S-(α -ketovinyl) thiamine, seemed an exception (Fig. 3). Of the intraperitoneally injected BD 98, only 2.8% was excreted as thiamine into the 24-hr urine, nevertheless the compound showed 33% of thiamine activity. A possibility might arise that orally administered BD 98 would be more readily converted to thiamine than when inject-

ed. Considering chemical properties of BD 98, the compound is decomposed possibly via BD 97 as an intermediate during biological modification and the latter exert a considerable thiamine activity. Potency as a vitamin is an overall reflexion of bio-availability of compound, that is, integration of many factors, such as rate of intestinal absorption, tissue distribution pattern, nature and velocity of biotransformation and renal excretion rate.

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