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Thiamine Derivatives of Disulfide Type. III.[1] Enzyme Systems in Rat Intestine contributed for Thiamine Formation from the Disulfide Type Derivatives[2]

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Assuming glutathione (GSH) as the main part of thiol group contributing for thiamine formation in gut wall from disulfide type derivatives, *i.e.*, thiamine propyl disulfide (TPD) and thiamine disulfide (TDS), the reactivity between GSH and TPD or TDS and the characteristics of enzyme system in gut wall was studied. The following conclusions were drawn from the result presented.

1) The rate constant determined at 37° and pH 7.4 was 9.83×10^{2} for TPD and GSH, 3.7×10 for GSH and glutathione propyl disulfide, 1.78×10^{3} for GSH and TDS, and 3.72×10^{3} for GSH and thiamine glutathione disulfide in liter/mole min, respectively.

2) The enzyme preparation was obtained by dialysis of rat gut wall homogenate. The activity was examined under the presence of GSH and NADPH and $K_m=6.25\times10^{-5}$ M/liter and optimal pH=7.4 were same as that of glutathione reductase from rat liver.

3) The mechanism proposed for thiamine formation in gut lumen confirmed as the coupled system shown by Eq. (1), (2), and (3).

In the proceeding papers,[1] the formation of thiamine from thiamine derivatives of disulfide type has been studied by kinetical procedures and concluded that the conversion was caused by the chemical reactions shown in Eq. (1) and (2) coupled with an enzymatic reaction shown in Eq. (3).

$$\text{G(-SH)} + \text{B}_1\text{SSR} \longrightarrow \text{B}_1\text{SH} + \text{G(-SSR)} \quad (1)$$

$$\text{G(-SSR)} + \text{G(-SH)} \longrightarrow \text{G(-SS-)G} + \text{RSH} \quad (2)$$

$$\text{G(-SS-)G} \longrightarrow 2\text{G(-SH)} \quad (3)$$

where G(–SH) is thiol group in gut wall, B_1SSR thiamine alkyl disulfide, G(–SSR) alkyl disulfide derivative in gut wall, B_1SH thiamine, G(–SS–)G disulfide derivative from G(–SH), and RSH alkylthiol. It was estimated that the main part of G(–SH) mentioned above would be glutathione, however, the existence of glutathione reductase and its characteristics in the tissue could not be discussed in detail.

It was, therefore, the purpose of the present paper to study the formation of thiamine from thiamine propyl disulfide (TPD) and thiamine disulfide (TDS) from the standpoint of enzymatic activities estimated from the dialyzed rat intestine homogenate under the presence of glutathione and NADPH. From the results which will be presented, it was confirmed that the enzymatic characteristics were well agreed to the one of glutathione reductase reported and the mechanism mentioned was supported for the conversion of thiamine derivatives during the drug absorption from rat intestine.

1) Part II: H. Nogami, J. Hasegawa, S. Nakatsuka, and K. Noda, *Chem. Pharm. Bull.* (Tokyo), **17**, 228(1969).

2) Presented before the Kanto Branch Meeting of Pharmaceutical Society of Japan, Tokyo, January, 1966

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Experimental

Chemicals——TPD and propyl disulfide were supplied by Takeda Pharm. Ind., Ltd. TDS and thiamine hydrochloride were product of Tanabe Seiyaku Co., Ltd. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Tokyo Kasei Kogyo Ltd. NADPH was obtained from Sigma Chemical Co., Ltd. Wakogel B-5 was purchased from Wako Pure Chemical Co., Ltd.

Kinetic Procedure——Prior to the reaction between glutathione and TPD or TDS in 0.1 M phosphate buffer solution of pH 7.4, nitrogen gas was blown into the buffer solution for 10 min to expel oxygen. Thiamine derivatives and glutathione were weighed and dissolved into the buffer solution separately and the solutions were place in a constant temperature bath. After the maintenance of temperature equilibrium, both solutions were mixed and the sample was withdrawn in suitable intervals for the analysis. The reaction was stopped by addition of hydrochloric acid and thiamine formed was determined. The procedure was carried out under nitrogen atmosphere.

Enzyme Preparation——Small intestine of a freshly slaughtered male albino rat (Donryu stock) was cut into about 1 cm pieces and washed with chilled 0.9% NaCl solution. A portion (14.2 g) of gut wall was placed in a Waring Blender with 100 ml of cold 0.25 M sucrose and blended for 3 min. Suspension was centrifuged at 4000 rpm for 30 min and 25 ml of clear supernatant was dialysed for 24 hr against 3 liter of 0.01 M Tris buffer, pH 7.4. The dialysed solution contained 10.6 mg of protein per ml. This preparation could be stored at 5° for 3 days without any apparent loss of activity.

Analytical Procedure——Thiamine was determined as previously reported.[4] Concentration of protein was determined spectrophotometrically with the method of Kalckar.[5] Reduced glutathione was determined according to the method of Ellman.[6] In the presence of propyl mercaptane, 2 ml of 1N HCl and 2 ml of cyclohexane were added to 2 ml of sample and shaked for 10 min. After centrifugation, 1 ml of aqueous layer was neutralyzed and DTNB solution was added. NADPH was assayed spectrophotometrically at 340 mμ assuming molecular extinction coefficient as 6000.

Assay of Enzyme Activity——Glutathione reductase activity was assayed by measuring the decrease in absorbancy at 340 mμ due to the oxidation of NADPH with Hitachi–Perkin–Elmer 139 spectrophotometer. As a complete system, each cuvette contained 0.5 μmole of substrate, 0.1 ml of enzyme solution, 0.2 μmole of NADPH, 1 μmole of EDTA, and 220 μmole of Tris buffer of pH 7.4 in 3.0 ml of final volume. Enzyme activity was calculated from the linear part of initial period and presented in ΔE_{340} /mg of protein/min.

TLC of Reaction Product——Plates were made with Wako gel B-5 and developed at room temperature by ascending method.

Synthesis of S-Propylmercaptoglutathione (GSSPr)——250 mg of propyl disulfide was dissolved in 5 ml of acetic acid and 0.2 ml of 30% H_2O_2 was added. After standing overnight, 5 ml of water was added, and neutralized with $NaHCO_3$, then 500 mg of glutathione in reduced form was added. After 2 hr, reaction mixture was acidified with hydrochloric acid and extracted with ether. White precipitate was obtained when pH of the aqueous solution adjusted to approximately 3. Recrystallized from 90% ethanol, mp 205° under decomposition. *Anal.* Calcd. for $C_{13}H_{23}O_6N_3S_2$: N, 11.02; S, 16.81. Found: N, 10.84; S, 16.72.

Calculation of Rate Constant by Analog Computer——Hitachi ALM-502T was used.

Results and Discussion

Reaction between TPD and Glutathione

Assuming the main part of thiol group in gut wall is glutathione in reduced form, the reactivity between glutathione and thiamine propyl disulfide was studied. 3.1 mg (10 μmole) of glutathione (GSH) and 3.9 mg (10 μmole) of TPD were dissolved in 1.0 ml of 0.1 M phosphate buffer solution pH 7.4, under the atmosphere of nitrogen, 0.1 ml of reaction mixture was drawn according to time schedule, and the reaction was followed by thin-layer chromatographic procedure after the addition of 1 drop of 5 N hydrochloric acid for the stopping of reaction. The result is given in Fig. 1. The formation of free thiamine was recognized evidently after one minute and the disappearance of TPD and the formation of the product having *Rf* value 0.65—0.7 which showed UV absorption like thiamine and a dark spot under UV light, and reacted to Ninhydrin reagent, were observed in the figure.

4) H. Nogami, J. Hasegawa, and K. Noda, *Chem. Pharm. Bull.*(Tokyo), **17**, 219 (1969).
5) H. Kalckar, *J. Biol. Chem.*, **167**, 461 (1941).
6) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

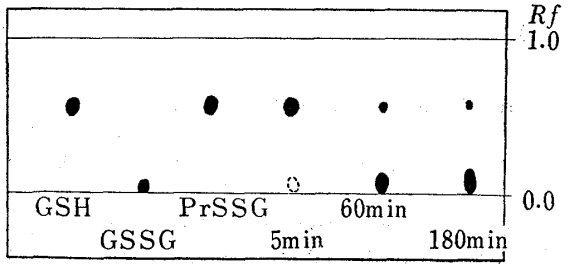

Fig. 1. TLC of the Reaction Product of TPD and GSH

developer: 1% HCl
detective reagent: ninhydrin reagent, dragendorff reagent

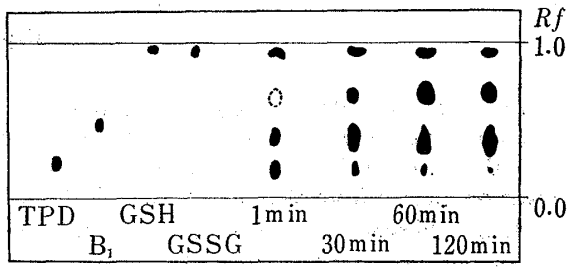

Fig. 2. TLC of Reaction Product of PrSSG and GSH

developer: MeOH-AcOH-acetone (85:10:5)
detective reagent: ninhydrin reagent

From the reports on the formation of S-propyl mercaptocysteine from TPD and cysteine by Yurugi[7] and Ikari, *et al.*,[8] the unknown spot was estimated to be S-propylmercaptoglutathione. The authentic sample obtained from propyl disulfide and GSH showed same *Rf* value as the unknown product. The competitive reaction shown by Eq. (4) and (5) may be considered.

$$B_1SSPr + GSH \xrightarrow{k_1} B_1SH + GSSPr \quad (4)$$

$$B_1SSPr + GSH \xrightarrow{k_1'} PrSH + GSSB_1 \quad (5)$$

The main reaction in experimental condition, however, might be shown by Eq. (4) since no formation of B_1SSG was observed.

The reactivity between GSSPr and GSH was examined by the same procedure mentioned. The result is given in Fig. 2.

The formation of glutathione in oxidized form was proved, therefore, the second step reaction may be presented by Eq. (6).

$$GSSPr + GSH \xrightarrow{k_2} PrSH + GSSG \quad (6)$$

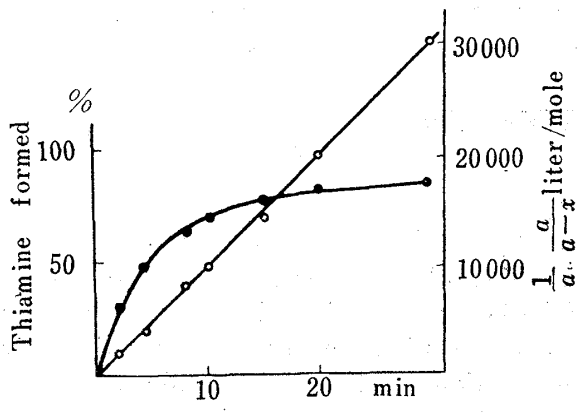

Fig. 3. Second Order Plot for the Reaction of TPD and GSH

●: % of thiamine formed
○: $\frac{1}{a}\frac{x}{a-x}$ liter/mole
initial conc.: TPD 5×10^{-4}M, GSH 5×10^{-4}M
temperature: 37° pH: 7.4

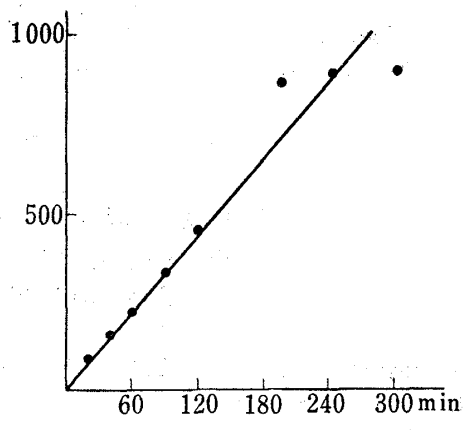

Fig. 4. Second Order Plot for the Reduction of PrSSG and GSH

initial conc.: PrSSG 1×10^{-3} molar
GSH 1×10^{-3} molar
temperature: 370°
pH: 7.4

7) S. Yurugi, *Yakugaku Zasshi*, **74**, 514 (1954).
8) N. Ikari, J. Hasegawa, and H. Nogami, personal communication.

The reverse reaction of Eq. (6) could not be confirmed from TLC of the reaction between propyl mercaptane and GSSG. It was concluded, therefore, that the reaction between GSH and TPD was represented by Eq. (4) and (6).

A kinetic study was made by mixing of equimolar of reactants, 5×10^{-4} mole, at 37° and pH 7.4 as shown in Fig. 3 where a straight line was found in 2nd order plot of the reaction and the rate constant calculated was 9.83×10^{2} liter/mole min.

The reaction between GSSPr and GSH was also examined under same condition as above determining GSH remained. No decrease of GSH was observed standing without GSSPr, therefore, the oxidation of GSH to GSSG, a possible side reaction, may be neglected. The 2nd order plot of the reaction given in Fig. 4 showed a straight line and the value of k_2 calculated was 37 liter/mole min.

Reaction of TDS with GSH

The formation of thiamine from TDS and GSH has been reported by Zima, *et al.*,[9] Matsukawa, *et al.*,[10] Kohno,[11] and Itada.[12] TDS (20 μmole) was reacted with GSH (20 μmole) in 0.1M phosphate buffer solution of pH 7.4 and decrease of TDS and formation of thiamine, GSSG, and a new spot were observed by TLC as shown in Fig. 5.

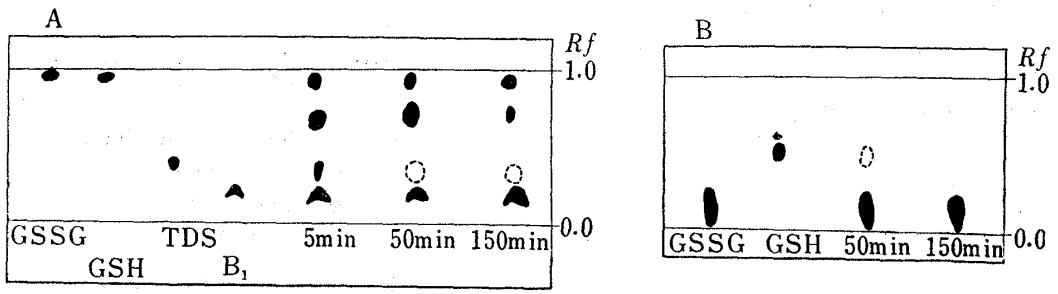

Fig. 5. TLC of Reaction Product of TDS and GSH

developer: A: 0.1N HCl–acetone (8:2) B: MeOH
detective reagent: ninhydrin reagent, dragendorff reagent

Being positive to Ninhydrin and Dragendorff reagents, the new spot was assumed to be the mixed disulfide of GSH and thiamine. The decrease of the spot was recognized after the lapse of reaction when two moles of GSH was added. From the results mentioned, it was assumed that the reaction between TDS and GSH followed two step reaction shown by Eq. (7) and (8).

$$B_1SSB_1 + GSH \xrightarrow{k_3} B_1SSG + B_1SH \qquad (7)$$

$$B_1SSG + GSH \xrightarrow{k_4} GSSG + B_1SH \qquad (8)$$

Although the formation of mixed disulfide from GSSG and thiamine at pH 7.5 has been reported by Matsukawa and Yurugi,[13] no formation was observed by TLC in our experimental condition, *i.e.*, 10 μmole of GSSG was mixed with 20 μmole of thiamine in buffer solution of pH 7.4. The mixed disulfide obtained from TLC was reacted with thiamine in same condition, but the formation of TDS or GSH was not confirmed. It was concluded that the opposite reactions to k_3 and k_4 was negligible small in the expermental condition.

Eq. (9) to (12) may be written from Eq. (7) and (8).

9) O. Zima, K. Pitsert, and Th. Moll, *Z. Physiol. Chem.*, **267**, 210 (1946).
10) T. Matsukawa, S. Yurugi, H. Kawasaki, Y. Aramaki, and J. Suzuoki, *Ann. Rep. Takeda Res. Lab.*, **12**, 1 (1953).
11) K. Kohno, *Vitamins* (Kyoto), **31**, 470 (1965).
12) N. Itada, *J. Vitaminology*, **5**, 72 (1959).

$$\frac{d[B_1SSB_1]}{dt} = -k_3[B_1SSB_1][GSH] \quad (9)$$

$$\frac{d[B_1SH]}{dt} = -\frac{d[GSH]}{dt} = k_3[B_1SSB_1][GSH] + k_4[B_1SSG][GSH] \quad (10)$$

$$\frac{d[B_1SSG]}{dt} = k_3[B_1SSB_1][GSH] - k_4[B_1SSG][GSH] \quad (11)$$

$$\frac{d[GSSG]}{dt} = k_4[B_1SSG][GSH] \quad (12)$$

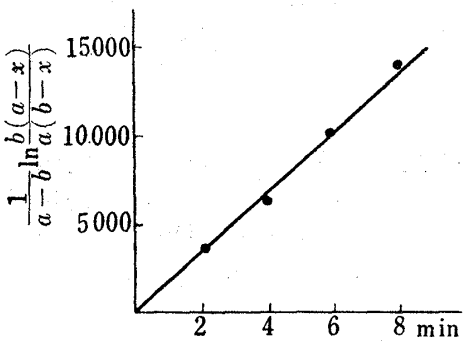

Fig. 6. Second Order Plot for Reaction of TDS and GSH

inital conc.: TDS (a) 14.5×10^{-5}M
GSH (b) 2.98×10^{-5}M
x: formed thiamine
temperature: 37°
pH: 7.4

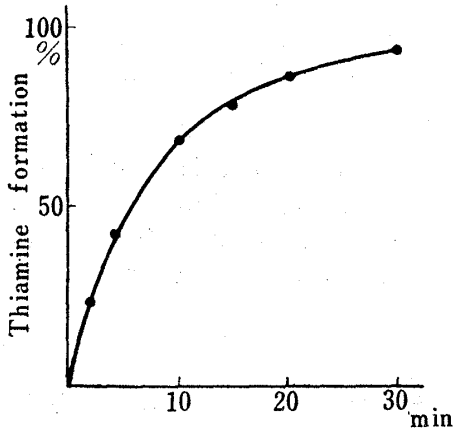

Fig. 7. Calculation of Thiamine Formation by Analog Computer

● : observed value
—: calculated curve

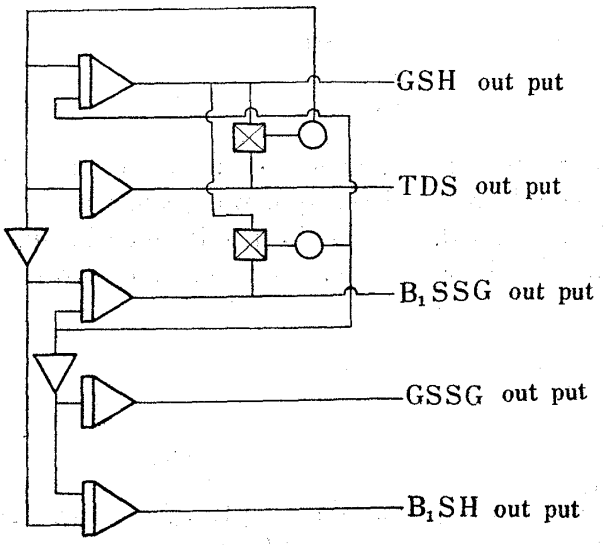

Fig. 8. Circuit Diagram of Analog Computer

It is too complicated to estimate the rate constant of k_3 and k_4 from these differential equations. The procedure which was used in the kinetical study[14] between TDS and cysteine and will be reported in detail as the paper of this series, was applied for the estimation of k_3 and k_4. Using the value of k_3 which was calculated from 2nd order plot given in Fig. 6 where the experimental condition was shown, k_4 was estimated by the curve fitting with the one obtained from analog computer which circuit diagram is given in Fig. 8. The rate constants obtained were 1.78×10^3 and 3.72×10^3 in liter/mole min for k_3 and k_4, respectively.

Glutathione Reductase in Rat Gut Wall

The existence of glutathione reductase in the homogenate of gut wall was examined by the determination of NADPH decreased and thiol group formed when the reaction mixture was stood for 15 min at room temperature. The complete removal of endogenous thiol was not successful by dialysis and the amount of thiol formed was corrected from endogenous thiol previously determined. The result is given in Table I.

13 T. Matsukawa and S. Yurugi, *Yakugaku Zasshi*, **74**, 1373 (1954).
14 K. Hirat, personal communication.

Table I. Stoichiometric Relation of the Enzyme System

GSSG. add. (μmoles)	Oxidized NADPH (μmoles)	Increased SH (μmoles)	NADPH/SH (%)
5	0.165	0.326	50.6
2.5	0.151	0.284	53.2
1.25	0.106	0.192	55.1
0.63	0.053	0.127	41.8
0.32	0.039	0.069	55.5

reaction mixture: 0.62 mg of protein, 0.2 μmoles of NADPH, 1 μmoles of EDTA
220 μmoles of Tris buffer, pH 7.4
total volume 3 ml

As seen in the table, two moles of thiol resulted from one mole of NADPH oxidized, therefore, the reaction may be shown by Eq. (13).

$$NADPH + H^+ + GSSG \longrightarrow 2GSH + NADP \qquad (13)$$

A linear relationship was obtained when decrease of NADPH was plotted against enzyme concentration by Michaelis-Menten kinetics as seen in Fig. 9.

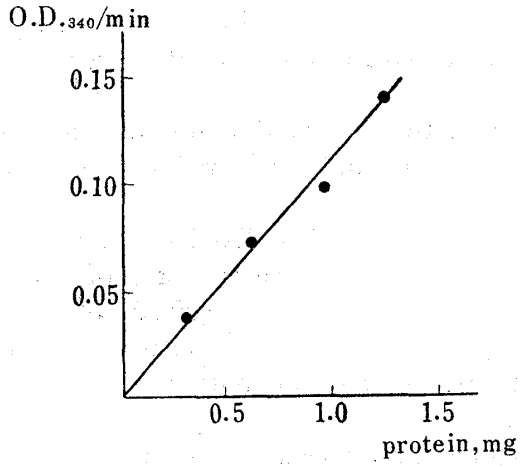

Fig. 9. Decrease of NADPH and Concentration of Protein

reaction mixture
GSSG: 0.5 μmoles NADPH: 0.2 μmoles
EDTA: 1 μmoles Tris: 220 μmoles
enzyme solution: total volume 3 ml

Table II. Substrate Specificity

Substrate	Increased SH (μmoles)
Cystine	0.004
TPD	0.003
GSSPr	0.046

reaction mixture: substrate: 0.5 μmoles
NADPH: 0.2 μmoles
EDTA: 1 μmoles
Tris, pH 7.4: 240 μmoles
protein : 0.62 mg
total volume: 3 ml
incubation time: 15 min
temperature: 30°

The substrate specificity of glutathione reductase in gut wall was examined and the result is given in Table II where slightly increase of thiol was determined for glutathione propyl disulfide but not for cystine and TPD, however, no detective decrease of NADPH was observed for these substrate.

It was reported by Pihl, *et al.*[15] that mixed disulfide was not reduced by rat liver glutathione reductase in the presence of glutathione. It seemed glutathione propyl disulfide might be reduced by the enzyme obtained from rat gut wall eventhough the rate of reduction was only a few per cent of the one for glutathione.

The pH dependent of enzyme activity was examined and shown in Fig. 10. An optimal pH was found at 7.4 which agreed with that obtained from camel gut wall,[16] rat liver,[17] and human erythrocytes.[18] The activity decreased at lower and higher pH region.

15) A. Pihl, L. Eldjarn, and J. Bremer, *J. Biol. Chem.*, **227**, 339 (1957).
16) I.F. Durr and N. Cortas, *Biochem. J.*, **91**, 640 (1964).
17) C.E. Mize and R.G. Langdon, *J. Biol. Chem.*, **237**, 1589 (1962).
18) E.M. Scott, I.W. Duncan, and V. Ekstrand, *J. Biol. Chem.*, **238**, 3928 (1963).

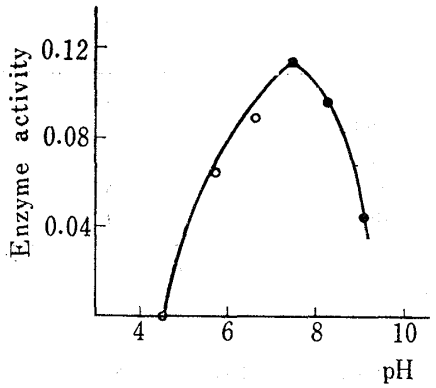

Fig. 10. Effect of pH on Enzyme Activity

enzyme activity: O.D.$_{340}$/mg protein/min
○: Na_2HPO_4–citrate buffer
●: Tris buffer

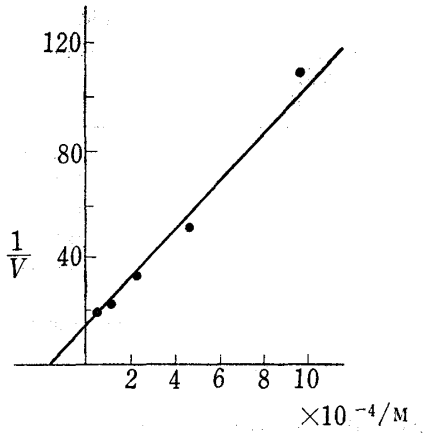

Fig. 11. Lineweaver-Burk Plot for GSSG

V: O.D.$_{340}$/mg protein/min

A lineweaver and Burk type plot of initial rate of reduction is shown in Fig. 11. The Michaelis kinetic constant for GSSG derived from this plot was $K_m = 6.25 \times 10^{-5}$M/liter. It was reported by Mize, *et al.*[17] that K_m for GSSG of rat liver glutathione reductase was 5×10^{-5}M/liter which is similar magnitude to the one mentioned above.

From the results presented in this series of study, the fate of thiamine derivatives in disulfide type will be interpreted as follows when the compounds are administered by oral route.

The disulfide-thiol exchange reaction between the compounds dissolved in intestinal fluid and thiol-derivatives secreted from intestinal tract should be the first step of reaction. Since the ionic nature of the reaction has been reported,[19] the larger magnitude of the rate constant will be assumed. The amount of thiol compounds secreted was neither determined in our study nor reported in rat or human *in vivo* therefore, the contribution of the reaction on drug absorption could not be estimated simply.

The next step would be the interaction of the disulfide derivative with gut wall. However, further examination would be required to prove that whether it occurred at the surface of the mucosal membrane or in the epithelial cell. The free thiamine formed by the exchange reaction of the second step was redistributed in mucosal fluid in our experimental condition. The absorption of the drug *in vivo* would not be quite same as the experiment *in vitro* since some portion of thiamine formed would be carried by blood stream in capillary artery exists at the location where the exchange reaction shown by Eq. (1) and (2) occurs.

The main part of thiol compounds contributing to the exchange reaction would be glutathione from the reasons mentioned previously. The reactivity between the thiamine derivative and glutathione was examined and the rate was large enough as presented and expected. The rate constant between GSSPr and GSH was about one twentieth of that between TPD and GSH. The existence of opposite reaction of k_2 was also assumed, however, the reaction shown by Eq. (6) may proceed more rapidly *in vivo* since propylmercaptane is easily oxidizable[20] and metabolized from report on ethanthiol.[21]

The wide distribution of glutathione reductase in various animal tissues was recognized and the reduction of disulfide derivatives under the existence of glutathione reductase and

19) Chapter 9 by Olav Foss, Organic Sulfur Compounds, ed. by N. Kharasch, Pergamon Press, New York, 1961. The kinetical studies between cysteine and TPD, TTFD, and TDS will be presented in the following papers of this series.
20) J. Xan, E.A. Willson, L.D. Roberts, and N.H. Horton, *J. Am. Chem. Soc.*, 63, 1139 (1941).
21) G.A. Snow, *Biochem. J.*, 65, 77 (1957).

small amount of glutathione has been reported by Pihl, *et al.*,[15] Durr, *et al.*,[16] and Hird.[22] The existence of glutathione reductase in rat gut wall and its characteristics have been proved in this study. The formation of free thiamine from the disulfide type derivatives during drug absorption experiment would be represented by the coupled system of chemical and enzymatic reaction shown by Eq. (1), (2), and (3). The rate of chemical reaction given by Eq. (1) and (2) would be rapid enough in biological system and the catalytic action of glutathione and the first order kinetics of thiamine formation might be understandable. There would be possible, therefore, that the mechanism proposed is valid for the recovery of free thiamine from the disulfide derivatives at other tissues of animal body.

The merits of the compounds, *i.e.*, the higher and longer lasting blood level of thiamine especially exists in erythrocytes and higher availability of the compounds, however, would not be interpreted from the results presented. Further studies would be required for the elucidation of the merits mentioned and will be presented as the series of this study.

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22) F.J.R. Hird, *Biochem. J.*, **85**, 320 (1962).