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Thiamine Derivatives of Disulfide Type. VI.[1] Kinetic Studies of the Thiol-Exchange Reaction between Thiamine Disulfide and L-Cysteine[2]

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The reaction between thiamine disulfide and L-cysteine was studied in the pH range of 3.45 to 8.77 at 15.0°. The reaction was found to be represented by two consecutive irreversible bimolecular reactions. The first bimolecular rate constants were obtained from the initial second-order plots of thiamine concentration, while the second bimolecular rate constants were obtained by an analog computer on which the differential equations of the reaction mechanism were programmed. The elementary reactions involved were clarified from the analysis of the pH-rate profile, and the specific rate constants were determined. The bimolecular rate constants of thiamine disulfide with L-cysteine at 15.0° were greater than the corresponding rate constants of thiamine propyl disulfide.

Since the isolation of thiamine disulfide (TDS) by Zima and Williams[5,6] in 1940, several workers have studied the reactions[6–13] between TDS and thiols and between TDS derivatives and proteins. The reactivity of cysteine or glutathione in reduced form toward TDS (B_1SSB_1) was investigated by Matsukawa and Yurugi[7,8] using partition paper chromatography, and the following two-reversible sequence in which unsymmetrical disulfides (B_1SSR) were the intermediates was reported.[14]

$$B_1SSB_1 + R\text{-}SH \rightleftarrows B_1SSR + B_1SH \quad (1)$$

$$B_1SSR + R\text{-}SH \rightleftarrows RSSR + B_1SH \quad (2)$$

where, R-SH is cysteine or glutathione in reduced form, B_1SSR is thiamine-cysteine (or thiamine-glutathione) disulfide, B_1SH is thiamine, and RSSR is cystine or glutathione in oxidized form. It has been reported that thiol-disulfide exchange reactions are of S_{N2}-type, and thiol ions are reactive species.[15–17] Numerous thiol-exchange reactions follow second-

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5) O. Zima and Williams, *Ber.*, **73**, 941 (1940).
6) O. Zima, K. Ritsert, and T. Moll, *Z. Physiol. Chem.*, **267**, 210 (1941).
7) T. Matsukawa and S. Yurugi, *Science*, **118**, 109 (1953).
8) T. Matsukawa and S. Yurugi, *Yakugaku Zasshi*, **74**, 1373 (1954).
9) P. Sykes and A.R. Todd, *J. Chem. Soc.*, **1951**, 534.
10) G.E. Bonvicino and D.J. Hennessy, *J. Org. Chem.*, **24**, 451 (1959).
11) I. Utsumi, K. Harada, Y. Kondo, and H. Hirano, *Vitamins* (Kyoto), **25**, 74 (1962).
12) I. Utsumi, K. Harada, K. Kohno, and H. Hirano, *Vitamins* (Kyoto), **26**, 134 (1962).
13) K. Kohno, *Vitamins* (Kyoto), **31**, 470 (1965).
14) Thiamine transits reversibly to the thiol type by alkali and to thiazolium salt type by acid in aqueous solution. Both of the types were represented by B_1SH for convenience in this report.
15) A.J. Parker and N. Kharasch, *Chem. Rev.*, **59**, 583 (1959).
16) A. Fava, A. Iliceto, and E. Camera, *J. Am. Chem. Soc.*, **79**, 833 (1957).
17) A.J. Parker and N. Kharasch, *J. Am. Chem. Soc.*, **82**, 3071 (1960).

order kinetics which shows ionic scission of sulfur–sulfur bonds.[15] According to the reports[18,19] on reactivities of nucleophiles toward disulfides, the rate constant was influenced by an electrostatic effect and steric factor around the disulfide bond. For example, the rate constant between glutathione in oxidized form and sulfite ions[18] or between cystine and cyanide ions[19] was dependent upon the number of ionized amino groups in the disulfide molecules as shown by the following order.

$$(H_3N^+)RSSR(N^+H_3) > (H_3N^+)RSSR(NH_2) > (H_2N)RSSR(NH_2)$$

The effect of the position of the charge also was examined and reported that the closer positive charge around S–S bond caused the reaction rate to be greater than the separated charge in glutathione (in oxidized form) molecule.

The thiol–disulfide exchange reactions have been studied on thiamine derivatives of disulfide type in the preceding papers of this series.[20,21] The present study was planned to extend further these investigations. Kohno[13] reported that several thiamine disulfide derivatives including TDS were useful as the reagent for determination of thiol group, and it was shown that TDS at the concentration of 4—10 times that of cysteine was able to liberate thiamine quantitatively. It would be interesting to determaine to what extent thiamine would be formed in a given time period from the exchange reaction between TDS and cysteine as an example of the most common thiol derivative in the body. It will be related to the rate constants of the forward and reverse reactions in the two reversible reactions as shown by Eqs. (1) and (2), if the reactions proceed as reported.[7,8]

The kinetic model of the reactions was one of the reasons to initiate the present investigation. A special consideration would be required for the kinetic study of the present exchange reactions, since it is only possible to determine the common reactant (cysteine) and reacted product (thiamine) to the two consecutive second-order reactions.

Results

Thin-layer Chromatographic Analysis

The progress of the reaction between TDS and cysteine at pH 3.60 and 15.0° was followed by thin-layer chromatography (TLC) and the result is given in Fig. 1, where the spots were detected by iodine vapor. Intensity of a spot with an *Rf* value (0.43) corresponding to thiamine disulfide (TDS) decreased with time, while a faint spot with an *Rf* value (0.30) corresponding to thiamine in the 5 min sample intensified with time. The other faint spot with an *Rf* value (0.68) in the 5 min sample intensified with time and reached a maximum in the 20 min sample. Thereafter, this spot decreased in intensity with time and no corresponding spot was in the final reacted sample. The spots of TDS, thiamine and the unknown intermediate were detectable by UV-light and Dragendorff reagent. The spots corresponding to cysteine (CySH) and cystine (CySSCy) assumed to be one of the final products were not differentiated with the developing solvent, 0.1 N hydrochloric acid–acetone (4:1), and the intensity of the spot was almost same throughout the reaction. The spots corresponding to cysteine, cystine, and the intermediate were detectable by Ninhydrin reagent. The portion corresponding to the spot of the intermediate on the plate was scraped off and extracted by 0.1 N hydrochloric acid. The absorption maximum of the extracted solution was found at

18) R. Cecil and J.R. Mcphee, *Biochem. J.*, **60**, 496 (1955).
19) O. Gawron, S. Mahboob, and J. Fernando, *J. Am. Chem. Soc.*, **86**, 2283 (1964).
20) H. Nogami, J. Hasegawa, and N. Ikari, *Chem. Pharm. Bull.* (Tokyo), **15**, 685 (1967).
21) H. Nogami, J. Hasegawa, and N. Ikari, *Chem. Pharm. Bull.* (Tokyo), **15**, 693 (1967).

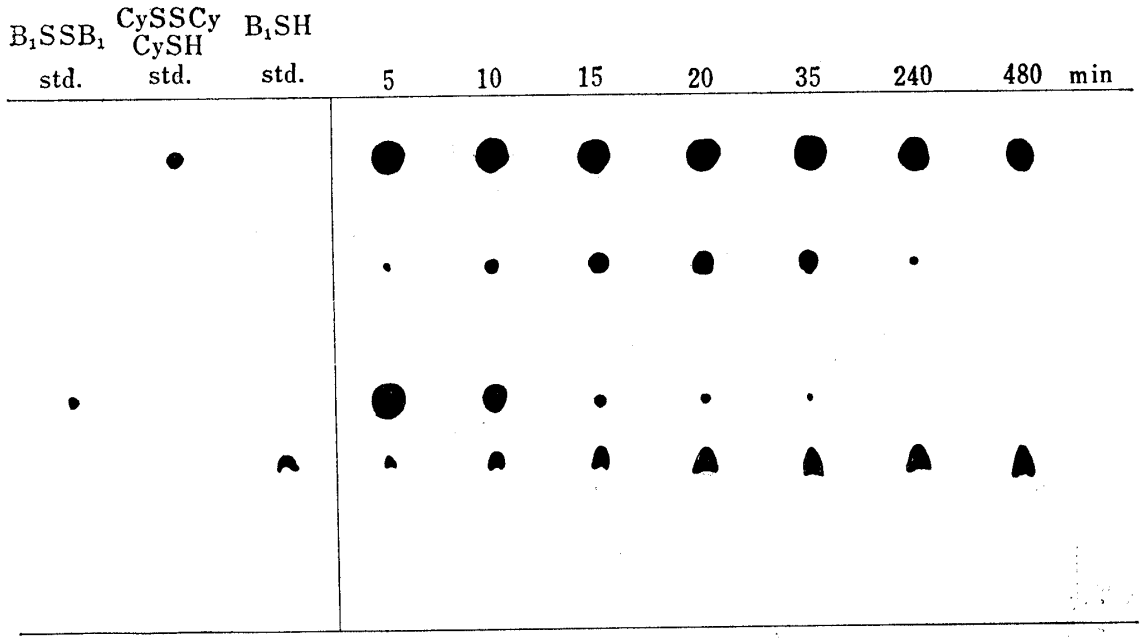


Fig. 1. Thin-layer Chromatogram of the Reaction between 1×10^{-2}M Thiamine Disulfide and 2×10^{-2}M Cysteine in Acetate Buffer at pH 3.60 and 15.0°

The reaction mixture was spotted on a silica gel plate, and developed in 0.1N HCl–acetone (4:1), and detected by iodine vapor.

246 mμ assigned to the absorption of pyridine ring. From the UV-spectral characteristics and the color reaction on the plate, the compound found at *Rf* 0.68 may be identified as thiamine–cysteine disulfide.

The reactivity of thiamine with cystine was also examined at pH 3.60, 6.90 and 10.64 and at 15.0°. No detectable formation of substances except the initial materials was found after 12 hr at pH 3.60, and after 2 hr at pH 6.90 and 10.64. The reaction between thiamine–cysteine disulfide and thiamine was followed at pH 7.00 and 37.0° for 2 hr by TLC, and the formation of TDS also was not detected. Exchange reactions between thiamine disulfide and protein disulfide were reported.[22] However, the reaction between TDS and cystine was examined at pH 6.90 and 37.0° for 2 hr, and no spot except TDS and cystine was found on the plate. The solvolytic degradation of thiamine–cysteine disulfide extracted from the TLC plate was studied at pH 4.00, 5.55 and 8.00 and at 15.0° for 2 hr. The stability of the compound in these conditions was satisfactory, since no degraded compound was found on the TLC plate.

Quantitative Examination of the Reactions

The stoichiometric relation of the reaction between TDS and cysteine was examined from the yield of thiamine after an equilibrium state, if any, was attained. The formation of thiamine was followed at 37.0° for initial molar concentrations of $[TDS]_0=18.7\times10^{-5}$ M and $[CySH]_0=40.0\times10^{-5}$ M, and for $[TDS]_0=18.7\times10^{-5}$ M and $[CySH]_0=20.0\times10^{-5}$ M at pH 5.55 in phosphate buffer and at pH 8.24, 9.18 and 10.64 in ammonium buffer. The thiamine concentrations in the final reacted solution were 98.1—101.7% of 2 times the initial TDS concentration for $[CySH]_0=40.0\times10^{-5}$ M or of the initial cysteine concentration for $[CySH]_0=20.0\times10^{-5}$ M. The change of thiamine and cysteine concentration with time at pH 5.55 and 37.0° is given in Fig. 2. The decreased molarity of cysteine was equal to the increased molarity of thiamine at any time. The amount of thiamine agreed with that calculated from Eq. (3).

22) I. Utsumi, K. Harada, and K. Kohno, *Vitamins* (Kyoto), **31**, 487 (1965).

$$B_1SSB_1 + 2CySH = 2B_1SH + CySSCy \qquad (3)$$

The reaction between cystine and thiamine at pH 5.55, 9.18 and 10.64 and at 37.0° was examined for 1 hr, and no significant decrease of the thiamine concentration was observed.

The contribution of the reverse reactions of Eqs. (1) and (2) was negligible small under the conditions mentioned in our study from these experimental results and the TLC analysis. The reaction between TDS and cysteine may be represented by two consecutive irreversible bimolecular reactions as shown by Eqs. (4) and (5).

$$B_1SSB_1 + CySH \xrightarrow{k_1} B_1SSCy + B_1SH \qquad (4)$$

$$B_1SSCy + CySH \xrightarrow{k_2} CySSCy + B_1SH \qquad (5)$$

where, B_1SSCy is thiamine–cysteine disulfide.

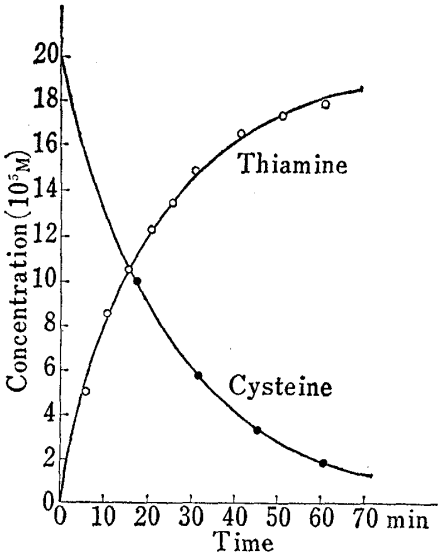


Fig. 2. Disappearance of Cysteine and Formation of Thiamine in the Reaction between Thiamine Disulfide and Cysteine at pH 5.55 in Phosphate Buffer and at 37.0°

initial thiamine disulfide concentration; 18.7×10^{-5}M
initial cysteine concentration; 20.0×10^{-5}M

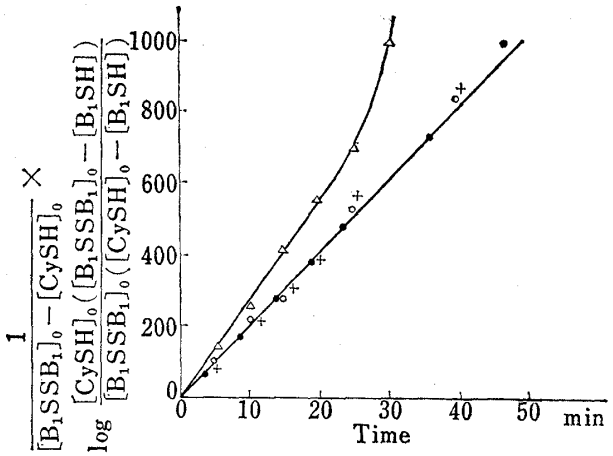


Fig. 3. Second-Order Plots for the Reaction between Thiamine Disulfide and Cysteine at pH 5.55 and 15.0°

initial molar concentrations of thiamine disulfide (B_1SSB_1) and cysteine (CySH)
—△— B_1SSB_1; 16.2×10^{-5}M + CySH; 37.5×10^{-5}M
—○— B_1SSB_1; 17.5×10^{-5}M + CySH; 18.9×10^{-5}M
—+— B_1SSB_1; 27.0×10^{-5}M + CySH; 8.96×10^{-5}M
—●— B_1SSB_1; 35.9×10^{-5}M + CySH; 8.59×10^{-5}M

Determination of Rate Constant (k_1) of the First Reaction and Rate Constant (k_2) of the Second Reaction

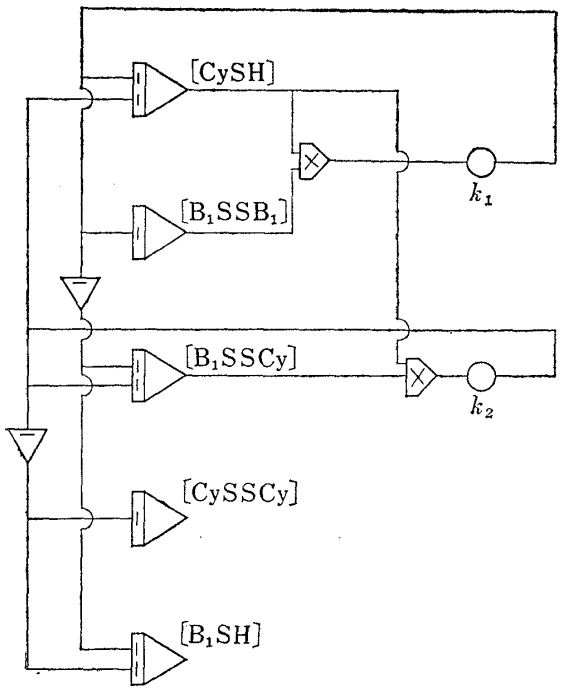
Thiamine is simultaneously formed from the two second-order consecutive reactions. The second-order plots of the thiamine concentration, on the assumption that the thiamine formation was based on the first second-order reaction alone, in the various initial molar ratios of TDS to cysteine at pH 5.55 and 15.0° may be represented by a straight line in the early stage of the reaction as seen in Fig. 3. The rate constants against the initial molar concentration ratios of TDS to cysteine are shown in Table I. It was considered that the formation of thiamine from TDS and cysteine at the early stage of the reaction may be represented by the first irreversible second-order reaction, as far as judged from the consistency of the rate constants obtained at the presence of TDS in excess. An initial molar concentration

Table I. Rate Dependency on Initial Concentrations of Thiamine Disulfide and Cysteine at pH 5.55 and 15.0°

Initial concentration		Molar ratio	Rate constant
TDS(10^5M)	CySH(10^5M)	TDS/CySH	(liter mole^{-1} min^{-1})
16.2	37.5	0.43	64.5
17.5	18.9	0.93	48.0
27.0	8.96	3.0	45.7
35.9	8.59	4.2	47.4

ratios, TDS to cysteine, over 1 were used in order to determine the first second-order rate constant. This may be a favorable condition to reduce the interfering effect of the reaction between cysteine and thiamine-cysteine disulfide on determining the first second-order rate constant.

The next step of the study is the determination of the rate constant for the second reaction by means of the analog computer. The rate constants of the competitive, consecutive second-order reaction as shown in Eqs. (4) and (5) were generally obtained from the table calculated in advance which shows relation between a ratio of k_1 to k_2 and ratios of periods of time necessary for specified percent consumptions of a common reactant in the two reactions.[23] However, the ratios of k_1 to k_2 appeared in our study are not found in this reference, and the calculations are very time-consumming.



CySH : Cysteine
B_1SSB_1 : Thiamine Disulfide
B_1SSCy : Thiamine–Cysteine Disulfide
CySSCy : Cystine
B_1SH : Thiamine

Fig. 4. Analog Computer Program for the Consecutive Reactions of Thiamine Disulfide with Cysteine, based on Equations (6)—(9)

The curve of the concentration of thiamine formed against time is determined from giving specified initial concentrations of TDS and cysteine, k_1 and k_2. The following differential equations are obtained from Eqs. (4) and (5).

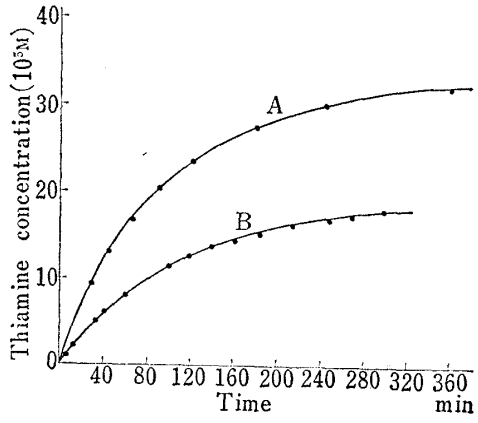


Fig. 5. Typical Analog Computer Fit of Thiamine Concentration formed from the Reaction of Thiamine Disulfide with Cysteine in Phosphate Buffer at pH 5.55 and 15.0°

initial concentration
A: B_1SSB_1; 18.7×10^{-5}M
CySH; 40.0×10^{-5}M
B: B_1SSB_1; 18.7×10^{-5}M
CySH; 20.0×10^{-5}M

23) A.A. Frost and W.C. Schwemer, *J. Am. Chem. Soc.*, **74**, 1268 (1952).

$$d[B_1SSB_1]/dt = -k_1[B_1SSB_1][CySH] \quad (6)$$

$$-d[CySH]/dt = d[B_1SH]/dt = k_1[B_1SSB_1][CySH] + k_2[B_1SSCy][CySH] \quad (7)$$

$$d[B_1SSCy]/dt = k_1[B_1SSB_1][CySH] - k_2[B_1SSCy][CySH] \quad (8)$$

$$d[CySSCy]/dt = k_2[B_1SSCy][CySH] \quad (9)$$

The second-order sequence of Eqs. (6)—(9) was programmed on the analog computer (Fig. 4). The k_2 value which best fits the experimental thiamine concentration against time on a chart paper of the analog computer was obtained. The k_2 values should be determined in the experimental conditions, under which the interference of the first reaction is small and the change of formation-curve of thiamine by varying of k_2 is comparatively large. The k_2 values may be accurately estimated from the formation of thiamine at the later phase of the reaction process. A typical result for determining k_2 at the initial concentrations of TDS (18.7×10^{-5} M) and cysteine (40.0×10^{-5} M) is shown in Fig. 5.

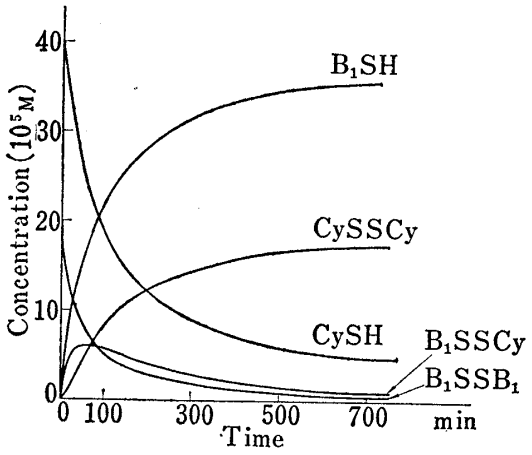


Fig. 6. Curves computed by Aanlog Computer, showing the Concentrations of Thiamine Disulfide (B_1SSB_1), Thiamine-Cysteine Disulfide (B_1SSCy), Cystine (CySSCy), Cysteine (CySH) and Thiamine (B_1SH) as a Function of Time at pH 5.55 and 15.0° Initial Concentrations of B_1SSB_1 (18.7×10^{-5}M) and CySH (40.0×10^{-5}M)

The concentration curve of thiamine formed from the reaction of TDS with cysteine started at other initial concentrations (18.7×10^{-5} M TDS and 20.0×10^{-5} M cysteine) in same pH and temperature was also able to be fitted satisfactorily by setting the potentiometer to the initial concentrations and the values of k_1 (47.0 liter mole^{-1} min^{-1}) and k_2 (60.0 liter mole^{-1}min^{-1}) obtained from the previous computation (Fig. 5). This fact shows that the curve fitting is a satisfactory method to determine k_2. The concentation-time curves of B_1SSB_1, B_1SSCy, CySSCy, CySH and B_1SH computed by the analog computer for the reaction between TDS and cysteine at the initial concentrations of TDS (18.7×10^{-5} M) and cysteine (40.0×10^{-5} M), using the values of k_1 and k_2 determined above, were shown in Fig. 6.

Rate(k_1)-Dependency on pH

The values of k_1 and k_2 determined at given pH were shown in Table II. The log rate (k_1 and k_2)-pH profiles for the thiol-exchange reaction between TDS and cysteine at 15.0° are given in Fig. 7. According to the preceding report,[21] it may be reasonable to be assumed that the two active species[24] of cysteine in the reactions are the dissociated forms on carboxyl group and thiol group, $(S^-)Cy(N^+H_3)(COO^-)$ and $(S^-)Cy(NH_2)(COO^-)$, and the three species of TDS (diprotonated TDS, $(H_3N^+)B_1SSB_1(N^+H_3)$, monoprotonated TDS, $(H_3N^+)B_1SSB_1(NH_2)$, and nonprotonated TDS, $(H_2N)B_1SSB_1(NH_2)$) contribute to the exchange reaction at

24) The undissociated form of carboxylic group (pK_a 1.86)[25] of cysteine at 30° can be neglected throughout this study. According to Benesch, *et al.*[26] the dissociation constants of cysteine at 23.0° are shown as follows:

$$K_A=[(S^-)Cy(N^+H_3)][H^+]/[(HS)Cy(N^+H_3)]=2.95\times10^{-9}$$
$$K_B=[(HS)Cy(NH_2)][H^+]/[(HS)Cy(N^+H_3)]=1.38\times10^{-9}$$
$$K_C=[(S^-)Cy(NH_2)][H^+]/[(S^-)Cy(N^+H_3)]=4.37\times10^{-11}$$
$$K_D=[(S^-)Cy(NH_2)][H^+]/[(HS)Cy(NH_2)]=9.33\times10^{-11}$$

25) R.K. Cannan and B.C.J.G. Knight, *Biochem. J.*, **21**, 1384 (1927).

26) R.E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **77**, 5877 (1955).

Table II. Observed Consecutive Second-Order Rate Constants for the Reactions of Thiamine Disulfide and Thiamine–Cysteine Disulfide with Cysteine at $\mu=0.2$ and 15.0°

pH	Buffer composition (10^2M)		k_1 (liter mole^{-1} min^{-1})	k_2 (liter mole^{-1} min^{-1})
3.45	HCl	18.8	0.746	0.500
	CH_3COONa	20.0		
3.72	CH_3COOH	18.0	1.42	0.700
	CH_3COONa	2.00		
4.54	CH_3COOH	11.0	11.4[a,b]	4.00[a,b]
	CH_3COONa	9.00		
5.55	Na_2HPO_4	0.33	47.0	60.0
	KH_2PO_4	6.33		
6.17	Na_2HPO_4	1.33	77.5	83.5
	KH_2PO_4	5.33		
7.00	Na_2HPO_4	4.00	192	240
	KH_2PO_4	2.67		
7.43	Na_2HPO_4	5.33	413	320
	KH_2PO_4	1.33		
8.15	NH_4OH	0.39	2430	800
	NH_4Cl	9.62		
8.77	NH_4OH	1.43	4400	2000
	NH_4Cl	8.57		

a) for $\mu=0.3$ (pH 4.49) and $\mu=0.6$ (pH 4.42), $k_1=9.00$, $k_2=4.87$ ($k_1=8.10$, $k_2=4.34$)[27] in liter mole^{-1}min^{-1} and $k_1=5.97$, $k_2=3.67$ ($k_1=6.90$, $k_2=3.53$)[27] in liter mole^{-1}min^{-1}, respectively.

b) $k_1=8.00$, $k_2=4.83$ ($k_1=8.10$, $k_2=4.34$)[27] in liter mole^{-1}min^{-1} and $k_1=7.00$, $k_2=4.00$ ($k_1=7.56$, $k_2=3.97$)[27] in liter mole^{-1}min^{-1} for 2 times ($\mu=0.4$, pH 4.49) and 4 times ($\mu=0.8$, pH 4.47) the buffer solution ($\mu=0.2$, pH 4.54), respectively.

the pH range studied (pH 3.4—8.8).

Therefore, the overall rate constant (k_1) of TDS disappearance can be given by Eq. 10.[28]

$$-d[B_1SSB_1]/dt=k_1[B_1SSB_1][CySH]$$
$$=k_{1-2}[(H_3N^+)B_1SSB_1(N^+H_3)][(S^-)Cy(N^+H_3)]$$
$$+k_{1-1}[(H_3N^+)B_1SSB_1(NH_2)][(S^-)Cy(N^+H_3)]$$
$$+k_{1-0}[(H_2N)B_1SSB_1(NH_2)][(S^-)Cy(N^+H_3)] \quad (10)$$

where, k_{1-2}, k_{1-1} and k_{1-0} are the specific rate constants of the elemental reactions of (S^-)-$Cy(N^+H_3)$ with diprotonated TDS, monoprotonated TDS and nonprotonated TDS, respectively. The total concentration of TDS may be written as Eq. (11) and the ionic dissociation constants of TDS may be represented as Eqs. (12) and (13).

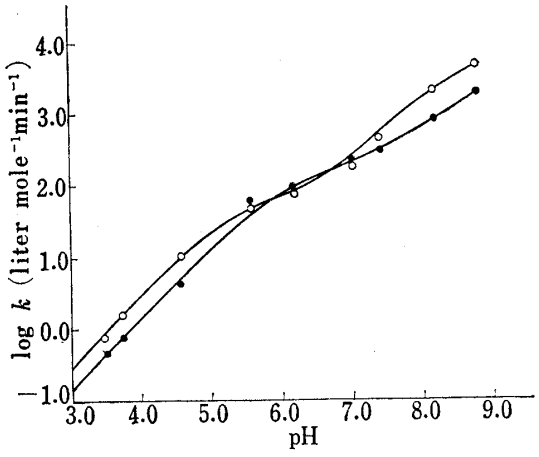


Fig. 7. Log k–pH Profiles for the Reactions of Thiamine Disulfide (k_1) and Thiamine–Cysteine Disulfide (k_2) with Cysteine at 15.0°

○: thiamine disulfide
●: thiamine–cysteine disulfide

27) Interpolated values ($\mu=0.2$) at pH values changed in Fig. 7.

28) The contribution of the reaction between $(S^-)Cy(NH_2)$ and nonprotonated TDS was neglected, since the concentration of $(S^-)Cy(NH_2)$ is negligible small at the pH range of this study, and the specific rate constant of the reaction between $(S^-)Cy(NH_2)$ and nonprotonated TDS might be nearly same in magnitude as that between $(S^-)Cy(N^+H_3)$ and nonprotonated TDS, as judged from the result reported in the paper of this series, Part V.[21]

$$[B_1SSB_1]=[(H_3N^+)B_1SSB_1(N^+H_3)]+[(H_3N^+)B_1SSB_1(NH_2)]+[(H_2N)B_1SSB_1(NH_2)] \quad (11)$$

$$K_{a1}=[(H_3N^+)B_1SSB_1(NH_2)][H^+]/[(H_3N^+)B_1SSB_1(N^+H_3)] \quad (12)$$

$$K_{a2}=[(H_2N)B_1SSB_1(NH_2)][H^+]/[(H_3N^+)B_1SSB_1(NH_2)] \quad (13)$$

where, K_{a1} and K_{a2} are the first and second dissociation constants of TDS. The values of pK_{a1} and pK_{a2} were determined to be 4.94 and 5.81 at 15.0° by potentiometric titration, respectively. The concentration of each species of TDS may be shown as Eqs. (14), (15) and (16).

$$[(H_3N^+)B_1SSB_1(N^+H_3)]=[H^+]^2[B_1SSB_1]/([H^+]^2+K_{a1}[H^+]+K_{a1}K_{a2}) \quad (14)$$

$$[(H_3N^+)B_1SSB_1(NH_2)]=K_{a1}[H^+][B_1SSB_1]/([H^+]^2+K_{a1}[H^+]+K_{a1}K_{a2}) \quad (15)$$

$$[(H_2N)B_1SSB_1(NH_2)]=K_{a1}K_{a2}[B_1SSB_1]/([H^+]^2+K_{a1}[H^+]+K_{a1}K_{a2}) \quad (16)$$

The concentration of thiol anion of cysteine, $(S^-)Cy(N^+H_3)$, at given pH may be written as Eq. (17).[21] The overall second-order rate constant (k_1) of the first reaction may be rewritten

$$[(S^-)Cy(N^+H_3)]=K_A[H^+][CySH]/(K_A[H^+]+K_B[H^+]+K_AKc+[H^+]^2) \quad (17)$$

as Eq. (18), substituting Eqs. (14)—(17) into Eq. (10).

$$k_1=(k_{1-2}/K_{a1}K_{a2}[H^+]+k_{1-1}/K_{a2}[H^+]^2+k_{1-0}/[H^+]^3)/(1/K_{a1}K_{a2} +1/K_{a2}[H^+]+1/[H^+]^2)(1/[H^+]+K_B/K_A[H^+]+K_C/[H^+]^2+1/K_A) \quad (18)$$

Eq. (18) may be simplified at the following conditions.

Case 1. $pH \ll pK_{a1}$.

The concentrations of $(H_3N^+)B_1SSB_1(NH_2)$ and $(H_2N)B_1SSB_1(NH_2)$ and the contribution of the reaction corresponding to k_{1-1} and k_{1-0} to the whole reaction rate are negligible small at lower pH region. The equation can be simplified as shown by Eqs. (19) and (19a), since $1/[H^+] \ll 1/K_{a1}$. The dissociation constants of cysteine at 15.0° were calculated to be 8.66 for pK_A from ΔH (6.5 kcal/mole)[26] and 9.09 for pK_B from ΔH (11.0 kcal/mole).[18]

$$k_1=k_{1-2}K_A/[H^+] \quad (19)$$

$$\log k_1=pH+\log k_{1-2}-pK_A \quad (19a)$$

The linear portion with slope 1.0 at lower pH (3.4—4.5) in Fig. 7 may be revealed as Eq. (19a). The specific rate constant (k_{1-2}) was calculated to be 1.22×10^5 in liter mole^{-1} min^{-1} from the intercept of the linear portion on the log k axis at pH 0.

Case 2. $pH \gg pK_{a2}$.

Eq. (18) may be simplified as Eq. (20), since $1/[H^+] \gg 1/K_{a2}$.

$$k_1=(k_{1-0}/[H^+])/(1/[H^+]+K_B/K_A[H^+]+K_C/[H^+]^2+1/K_A) \quad (20)$$

As mentioned above, $pK_C=10.36$ at 23.0° was reported and $K_C/[H^+]^2$ may be neglected at pH 8.0 to 8.8. Thus, Eq. (20) was simplified further as Eq. (20a) in logarithmic expression.

$$\log k_1=\log k_{1-0}+pH-\log(1/[H^+]+K_B/K_A[H^+]+1/K_A) \quad (20a)$$

The value of k_{1-0} was calculated to be 1.13×10^4 and 9.60×10^3 in liter mole^{-1} min^{-1} from the data at pH 8.15 and 8.77 at 15.0°, respectively. The agreement of the values probably reflects that the contribution of term of $K_C/[H^+]^2$ to k_1 was negligible small.

Case 3. $pH \simeq pK_{a1}$ and pK_{a2}.

The term of $K_c/[H^+]^2$ in Eq. (18) may be ignored at these pH region. Substituting the values of k_{1-2} and k_{1-0} to Eq. (18), k_{1-1} was calculated to be 1.01×10^5 liter mole^{-1} min^{-1} from the rate constant, 47.0 liter mole^{-1} min^{-1}, at pH 5.55. Using these specific rate constants (k_{1-2}, k_{1-1} and k_{1-0}), the calculated log k_1 values against pH were shown with a smooth line in Fig. 7, which was reasonably in agreement with experimental values.

Rate (k_2) -Dependency on pH

Since thiamine–cysteine disulfide (B_1SSCy) has not been isolated, the following assumptions were made.

a) The carboxylic group in cysteine moiety of B_1SSCy would dissociate completely at the pH range over pH 3.45.

b) The amino group in cysteine moiety would exist as protonated form at pH range of 3.45 to 8.77.

c) The value of pK_a in thiamine moiety of the compound would be nearly equal to that of thiamine propyl disulfide.[21]

From these assumptions, two species of B_1SSCy, $(H_3N^+)B_1SSCy(N^+H_3)$ and $(H_2N)B_1SSCy(N^+H_3)$, are responsible for the thiol–exchange reaction between B_1SSCy and cysteine under the conditions of this study. Thus, the overall appearance rate of cystine may be written as Eq. (21).

$$\begin{aligned} d[CySSCy]/dt &= k_2[B_1SSCy][CySH] \\ &= k_{2-1}[(H_3N^+)B_1SSCy(N^+H_3)][(S^-)Cy(N^+H_3)] \\ &\quad + k_{2-0}[(H_2N)B_1SSCy(N^+H_3)][(S^-)Cy(N^+H_3)] \end{aligned} \tag{21}$$

where, k_{2-1} and k_{2-0} are the specific rate constants of the two elementary reactions, respectively. When the dissociation constant, K_{a3}, of B_1SSCy is represented by Eq. (22), the overall rate constant (k_2) may be represented by Eq. (23).

$$K_{a3} = [H^+][(H_2N)B_1SSCy(N^+H_3)]/[(H_3N^+)B_1SSCy(N^+H_3)] \tag{22}$$

$$k_2 = (k_{2-1}/K_{a3}[H^+] + k_{2-0}/[H^+]^2)/(1/K_{a3} + 1/[H^+])(1/[H^+] + K_B/K_A[H^+] + 1/K_A) \tag{23}$$

Case 1. $pH \ll pK_{a3}$.

As mentioned previously, Eq. (23) is simplified to Eq. (24).

$$\log k_2 = pH + \log k_{2-1} - pK_A \tag{24}$$

From Eq. (24), the linear relationship with slope 1.0 in the log rate–pH profile may be expected at lower pH region, and the value of k_{2-1} may be calculated from the intercept of the log rate–pH profile at pH 0. The expectation was realized in Fig. 7, where the value of k_{2-1} obtained was 6.12×10^4 in liter mole^{-1} min.$^{-1}$

Case 2. $pH \gg pK_{a3}$.

Eq. (23) may be simplified to Eq. (25).

$$\log k_2 = \log k_{2-0} + pH - \log(1/[H^+] + K_B/K_A[H^+] + 1/K_A) \tag{25}$$

The value of k_{2-0} was calculated to be 3.73×10^3 and 4.28×10^3 in liter mole^{-1} min^{-1} for pH 8.15 and 8.77 at 15.0°, respectively.

Case 3. $pH \simeq pK_{a3}$

The value of pK_{a3} was calculated to be 5.96 (pH 6.17), 6.17 (pH 7.00) and 5.96 (pH 7.43), using k_{2-1}, k_{2-0} and the experimental values of k_2 obtained. The calculated curve (as

$k_{2-1}=6.12\times10^4$, $k_{2-0}=4.01\times10^3$ in liter mole^{-1} min^{-1} and p$K_{a3}=5.96$) is shown as a smooth curve in Fig. 7, which is in good agreement with the observed experimental results, and it was evident that the assumptions mentioned were reasonable.

Rate Dependency on Ionic Strength, General Acid and Base

The effect of ionic strength was examined at pH 4.54 in acetate buffer where no detectable effect based on ionic strength, 0.2—0.6, was found (Table II). Since the effect of acetate ions was not found as examined in four times concentrated buffer solution, it may be concluded that the general acid-base catalytic reaction on the exchange reaction between thiamine disulfide and cysteine does not exist as reported previously.[21]

Discussion

A mercaptide ion (RS^-) displaces another mercaptide ion ($R'S^-$) from sulfur, when the attacking mercaptide ion has a greater affinity (S-nucleophicity[15]) for sulfur than the displaced mercaptide ion. It was reported that thiamine displaced propyl mercaptan from cysteine propyl disulfide,[20] and that thiamine displaced cysteine from cysteine propyl disulfide with formation of thiamine propyl disulfide.[29] The reverse reactions for Eqs. (4) and (5), however, were not observed under the conditions in this investigation, as shown in the chromatographic analysis and the quantitative analysis mentioned above. Hence, it may be considered that CyS^- has a greater affinity for sulfur than B_1S^-. Table III shows the kinetic constants

Table III. Summarized Rate Constants (liter mole^{-1} min^{-1}) of the Reaction of the Disulfide and Cysteine

Thiamine disulfide		Thiamine–Cysteine disulfide		Thiamine propyl disulfide[21]
k_{1-2}	1.22×10^5			
k_{1-1}	1.01×10^5	k_{2-1}	6.12×10^4	1.82×10^4
k_{1-0}	1.05×10^4	k_{2-0}	4.01×10^3	2.61×10^3

obtained at 15.0° in our study, and also the corresponding rate constants for the reaction of cysteine with two species of thiamine propyl disulfide. From Table III, it is noted that the rates increase as the net charge on the thiamine disulfide becomes more positive. This increase in rate would be attributable to an increase electrostatic attraction for the attacking anion, as shown in the reaction of cystine with cyanide.[19]

The difference of the affinity for sulfur between a displacing mercaptide and a displaced mercaptide is dependent upon the anionic stabilities, the charges and the steric factors of these mercaptide groups.[15,19] It is of interest to note that the bimolecular rate constants for the reaction with thiamine disulfide (symmetric disulfide) are greater than the corresponding rate constants of thiamine propyl disulfide (unsymmetric disulfide) for cysteine anion attack.[30]

Experimental

Materials——Thiamine disulfide (TDS) was supplied from Tanabe Seiyaku Co., Ltd. (mp 168—170°. *Anal.* Calcd. for $C_{24}H_{34}O_4N_8S_2$: C, 51.23; H, 6.09; N, 19.91. Found: C, 51.89; H, 6.00; N, 20.00). The

29) I. Utsumi, K. Harada, and K. Kohno, *Vitamins* (Kyoto), **27**, 299 (1963).
30) After completion of this study, it was found that Utsumi, *et al*, *Vitamins* (Kyoto), **35**, 340, 508 (1967), reported that the second-order rate constants of thiamine formation from symmetric disulfide such as benzylthiamine disulfide and unsymmetric disulfide such as thiamine propyl disulfide with glutathione in reduced form at pH 7.3 and 37° were $2—6\times10^3$ in liter mole^{-1} min^{-1}.

thiamine content of the compound was determined to be 99.4% by reducing at pH 8.0 and 37.0° for 1 hr with cysteine to thiamine. L-cysteine and L-cystine were of analytical grade (Nippon Rikagaku Co., Tokyo). A standard solution of thiamine hydrochloride for assay supplied from Takeda Chem. Ind., Ltd. was used.

Assay Procedures——Thiamine[31]: A sample solution was diluted to make 5.0 ml and pH 1 to 2 by an appropriate quantity of 0.5 N HCl. Three ml of saturated solution of cyanogen bromide was added, and allowed to stand for 5 min at the room temperature. Two ml of 10% NaOH solution was added and total volume was made 10.0 ml. The absorbance at 368 mμ was determined by a spectrophotometer (Hitachi-Perkin-Elmer Model 139). The presence of TDS, cysteine, cystine, or buffer ingredients did not interfere the determination of thiamine.

L-cysteine[32]: 39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) was dissolved in 100 ml of phosphate buffer solution (pH 7.0). Two ml of the sample solution and 0.5 ml of the solution mentioned above were mixed with a suitable volume of phosphate buffer solution (pH 8.0) in order to keep pH 8.0 during the reaction, and the final volume was adjusted to 10.0 ml. The concentration of cysteine in the sample solution was determined from the absorbance of 5-mercapto-2-nitrobenzoic acid formed at 412 mμ.

Thin-layer Chromatographic Procedure——The reaction of TDS with cysteine at pH 3.60 in acetate buffer was examined by thin-layer chromatography (TLC). The TLC plates were prepared with 0.4 mm layer of silica gel (Wakogel B-5, Wako Pure Chem. Ind., Ltd. Osaka). One-hundredth M TDS and 0.02 M cysteine were reacted at 15.0°. The sample solutions were drawn at intervals, and made pH 1 to 2 by addition of 0.5 N HCl to stop the reaction. A constant volume of sample solution was spotted at the origin of the plate, and the developing mixture solvent of 0.1 N HCl and acetone (4:1) was used for about 10 cm travel. The spots were detected with UV-light (2537Å), iodine vapor, Dragendorff reagent, J.P., and Ninhydrin reagent, J.P. The TLC procedure was also used to extract thiamine-cysteine disulfide. One-hundredth M TDS and 0.02 M cysteine were reacted at pH 3.60 and 15.0° in acetate buffer for 20 min. The acidified solution was streaked across a plate (0.7 mm in thickness). The portion detected by UV-light, corresponding to thiamine-cysteine disulfide, was scraped off and extracted with acetate buffer at pH 4.00 and phosphate buffers at pH 5.55, 7.00 and 8.00 for the examination of reaction between thiamine-cysteine disulfide and thiamine and of the stability of thiamine-cysteine disulfide. The reaction between saturated cystine and 0.01 M thiamine was examined at 15.0° and at pH 3.60 (acetate buffer), pH 6.90 (phosphate buffer) and pH 10.64 (ammonium buffer). The reaction between saturated cystine and 0.001 M TDS was examined at 37.0° and pH 6.90 (phosphate buffer).

Determination of pK_a of TDS——Fourty to 50 mg of TDS was weighed accurately and dissolved in 25 ml of water. Twenty ml of the solution was taken and titrated with 0.1 N HCl using Radiometer TTTlc equipped with a recording devise (SBR2c) and jacketed electrode vessel around which water from a thermostted water bath (15.0°) was circulated. The titration curve obtained was analyzed by the method reported by Noyes.[33]

Kinetic Procedure——Acetate, phosphate and ammonium buffer solutions were used, and the compositions were given in Table II. The buffer solutions were prepared using deionized and nitrogen-purged water to avoid the oxidation of thiol to disulfide. The ionic strength of reaction mixture was adjusted to 0.2 by addition of NaCl. The both reactants were separately dissolved in same buffer solutions, and kept in a constant temperature bath maintained to 15.0° within ±0.1°, and the reactants dissolved in the buffer solutions were mixed after the temperature was equilibrated. The value of pH in the reaction mixture was determined at the experimental temperature using a glass electrode pH meter (Beckman Model G). The shift of pH value after the reaction was not detectable throughout this study. The samples were drawn at intervals, and the formation of thiamine was determined as described above after the acidification of the sample solution to stop the reaction. Initial concentrations of TDS and cysteine were selected appropriately so that the rate constants were determined within 8 hr.

The effect of change in the ionic strength on k_1 and k_2 was studied at 15.0° by adjusting the ionic strength of the reaction mixtures with NaCl to 0.3 and 0.6 in acetate buffer at pH 4.54. The change of rate was studied in 2- and 4-fold ranges of acetate buffer concentrations to evaluate the general acid-base catalysis. The rate constants in the different ionic strengths and buffer concentrations were compared with the rate constants obtained by setting pH values changed in Eqs. (19a) and (24).

Procedure for Determination of k_1——Initial rate of thiamine formation from an excess amount of TDS added to cysteine approximates to the rate of thiamine formation from the first irreversible second-order reaction shown as Eq. (4). A second-order plot of concentration of thiamine formed from the reaction between TDS and cysteine based on Eq. (4) exhibited a straight line relationship as shown in Fig. 3 in the early stage of the reaction. The second-order rate constants of the reactions between cysteine and TDS at increasing initial concentrations in pH 5.55 and 15.0° were shown in Table I. An initial molar ratios of TDS

31) Y. Kochi and S. Kasahara, *Vitamins* (Kyoto), **7**, 513 (1954).
32) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
33) A. Albert and E.P. Serjeant, "Ionization Constants of Acids and Bases," Methuen & Co., Ltd., 1962.

to cysteine over 1 were postulated to be sufficient to determine the value of k_1 at pH 5.55 and 15.0°, and were used to determine k_1 at any other pH.

Procedure for Determination of k_2——The second-order sequence based on Eqs. (4) and (5) and the value of k_1 calculated by the preceding procedure were programmed on the analog computer (Hitachi ALM-502T, Tokyo) as shown in Fig. 4. The outputs of each reactant and product were adjusted within 50 volt at the maximum concentration. The concentrations of thiamine with time were plotted on a recording paper used by Unicorder Model U-100M (Nippon Denkikikai Co., Ltd. Tokyo). The rates (k_2) of the second-order reaction (Eq. (5)) were determined by setting the potentiometer representing k_2 so that the best curve fitting by the analog computation to the experimental [B_1SH] *vs.* time plots was given. The accuracy of the curve fitting is dependent upon the extent of shift of [B_1SH] *vs.* time curves resulted from variation of value of k_2 at given k_1 and initial concentrations of TDS and cysteine. A favorable condition, under which k_2 is determined accurately by curve-fitting by the analog computation, is to keep the concentration of thiamine-cysteine disulfide higher and also the concentration of cysteine higher to lay stress on the second reaction. A preliminary test conducted at different initial molar ratios of TDS and cysteine showed that the favorable conditions of the initial concentration ratios were between 1:2 and 1:3.