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Thiamine Derivatives of Disulfide Type. VII.[1] Kinetics between Thiamine Tetrahydrofurfuryl Disulfide and L-Cysteine or Its Derivatives[2]

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The kinetics in aqueous solution on the exchange reaction between thiamine tetrahydrofurfuryl disulfide and thiols, *i. e.*, L-cysteine, N-acetyl-L-cysteine, and glutathione, was conducted at the experimental condition of 15° to 37° and pH 3.5 to 8.5 where it was proved that the main reaction could be followed from the formation of thiamine neglecting the possible side reactions examined by paper partition chromatographic procedure.

The following conclusions were drawn from the result presented.

1) The formation of thiamine followed the second order reaction concluded as S_N2 and was converted to a first order kinetics by the addition of excess amount of reactant.

2) From the analysis of pH-rate profile of the reaction, the following two elementary reactions were postulated, *i. e.*, between the molecular or ionized form of thiamine derivative and thiol anion, which well explained the result obtained.

3) No detectable effect on rate was proved for the ionic strength and concentration of buffer solution used.

4) The kinetical parameters for each elementary reaction, *i. e.*, specific rate constant, activation energy, frequency factor, and activation entropy, were tabulated.

5) The activation energies were around 10 kcal/mole. Larger negative activation entropy was found in reactions between molecular form of thiamine derivative and thiol anions than ones between ions. The reactivity of thiols was $^{-}$NACyS^{-}>$^{-}$GS^{-}>CyS^{-}.

In the proceeding papers[4],[5] of this series, the conversion mechanism of thiamine derivatives of disulfide type to thiamine at rat intestine *in vitro* had been studied for the understanding of the merits of the compounds and the kinetics on the reaction between thiamine propyl disulfide or its homologes and L-cysteine was conducted to examine the reactivity of the compound since the exchange reaction between sulfur-sulfur bond and thiol had been proved the initial step of the metabolic fate of the compounds.

The present study was planned for further examination of the exchange reaction. Thiamine tetrahydrofurfuryl disulfide (TTFD), as the thiamine derivative, and L-cysteine or its derivatives, *i.e.*, glutathione, and N-acetyl-L-cysteine, were selected to examine the ionic displacement nature of the exchange reaction.

Experimental

Material——Thiamine tetrahydrofurfuryl disulfide (TTFD): mp 125°, white crystalline powder. Tetrahydrofurfuryl bromide: bp 60—65°, colorless liquid. The materials mentioned were supplied from Takeda

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[4] H. Nogami, J. Hasegawa, and K. Noda, *Chem. Pharm. Bull.* (Tokyo), in press Part I, II, III.
[5] H. Nogami, J. Hasegawa, and N. Ikari, *Chem. Pharm. Bull.* (Tokyo), **15**, 693 (1967); *Chem. Pharm. Bull.* (Tokyo), **15**, 685 (1967).

Chem. Ind. Tetrahydrofurfuryl mercaptan (FSH): synthesized from tetrahydrofurfuryl bromide by the report of Bary,[6] bp 117—120°, colorless liquid. L–Cysteine tetrahydrofurfuryl disulfide (FSSCy).[7] synthesized by Meiwa–Kogyo Co., mp 185—190° white crystalline powder. Glutathione (GSH): mp 195° white crystalline powder supplied from Yamanouchi Pharm. Co. N–Acetyl–L–cysteine (NACySH): mp 110° white crystal supplied from Senju–Seiyaku Co. Other materials: same as the proceeding report.

Buffer Solutions——The solution used for kinetics was prepared by dilution from the stock solution concentrated in five times. The ionic strength of the stock solution was adjusted to 0.5 by the addition of sodium chloride and one in reaction mixture was 0.1. The water used for buffer solution was deionized and bubbled with nitrogen gas for removing of oxygen or carbon dioxide dissolved. 0.1M Michaelis' acetate buffer solution of pH 3.5—5.6 and 0.1M Michaelis' phosphate buffer solution of pH 6.2—8.2 were used.

Procedure of Paper Partition Chromatography (PPC)——0.1M of TTFD and 0.1M of L–cysteine (CySH) were reacted in buffer solution of pH 4.9 and 8.0 for 50 min at 37°, acidified under pH 1.0 by the addition of hydrochloric acid for the stopping of reaction, and extracted with ether. The both aqueous and etherial layer were concentrated to ten and fifty times by evaporation, respectively, and used for the sample of PPC. The filter paper, No. 51A from Tōyō–Roshi Co., was used and developed by the mixed solvent of butanol—acetic acid—water in volume ratio of 40—10—50 using ascending method. The following detection procedures were used: Ultraviolet (UV) (2500 Å) irradiation in dark room, ninhydrine and Dragendorff's reagent, and iodine vapor. *Rf* values are given in table I.

Determination of Possible Side Reaction——Formation of FSH or FSSF was examined as follows. 6.66×10^{-3}M of TTFD and CySH were reacted at 25° for 3 min at pH 4.9 or 10 sec at pH 8.0, acidified to under pH 1.0 by addition of hydrochloric acid, and extracted with ether and the etherial layer was washed with 0.7% hydrochloric acid to remove TTFD contaminated in the etherial solution and concentrated by 9 times and then the optical density at 240 and 260 mμ was determined. The molar extinction determined was 325 and 345 for FSSF and 63.3 and 6.33 for FSH at 240 mμ and 260 mμ respectively.

TABLE I. *Rf* Values and Detection Procedures of PPC

Substance	*Rf* value	Detection procedure UV	Nin.	Drag.	I_2
CySSCy	0.05—0.088	+	+	−	+
VB_1	0.15—0.24	+	+	+	+
CySH	0.32—0.35	+	+	−	+
FSSCy	0.50—0.60	+	+	+	+
TTFD	0.60—0.66	+	−	+	+
FSH	0.18—0.23	+	−	±	+
FSSF	0.92—0.96	+	−	±	+

Procedure of Kinetics——The pre–incubated solutions of TTFD and thiols under nitrogen atmosphere in a constant temperature bath were mixed, 5 ml of aliquots were drawn according to the time table, mixed with 0.2 ml of 10% hydrochloric acid for stopping of the reaction, and amount of thiamine formed was determined following the procedure reported by Kōchi and Kasahara.[8] Thiamine chydrochloride standard solution prepared by Takeda Chem. Ind., Ltd. was used for standardization. The optical density at 368 mμ of thiochrome was determined by a spectrophotometer, Hitachi–Perkin–Elmer 139. The second order rate constant of the reaction was determined reacting equimolar of reactants to simplify the calculation and the initial concentration was between 10^{-3} and 10^{-5}M. The value of pH in reaction mixture was determined by a pH–meter with glass electrode, Horiba–Hitachi Model–P and Tōa–Demppa HM–5A.

The Reverse Reaction of the Main Reaction——The reverse reaction was examined reacting 3.59×10^{-4}M of FSSCy and thiamine in buffer solutions of pH 4.9 and 8.2 at 25°.

Stability of TTFD——The stability of TTFD was examined from the formation of thiamine at 37° in buffer solutions of pH 4.1 and 7.3.

Determination of p*K* of Carboxyl Groups——Determined by the analysis of neutralization curve using Tōa–Demppa HM–5A.

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6) Vincent C. Barry and Joan E. McCornik, *Proc. Roy. Irish Acad.*, *Sect. B*, **59**, 350 (1958).

7) The identification of FSSCy was carried out as follows. The *Rf* value of this substance at paper partition chromatography and thin–layer chromatography agreed with that of the precipitation obtained by the reaction between TTFD and L–cysteine in a sufficiently concentrated aqueous solution. Further more L–cysteine, FSH, and FSSF were identified by the reaction between this substance and L–cysteine.

8) Y. Kōchi and S. Kasahara, *Bitamin*, **7**, 513 (1954).

Result and Discussion

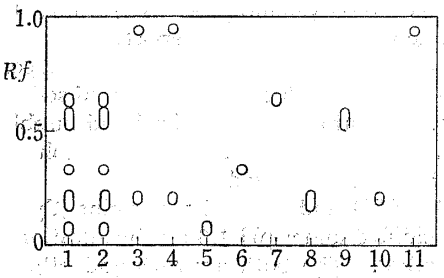

Fig. 1. The Paper Partition Chromatogram of the Reaction Solutions and Authentic Substances

1: aqueous layer of the reaction at pH 4.9
2: aqueous layer of the reaction at pH 8.0
3: eherial layer of the reaction at pH 4.9
4: etherial layer of the reaction at pH 8.0
5: CySSCy 6: CySH 7: TTFD 8: B_1
9: FSSCy 10: FSH 11: FSSF

The analysis of reaction mixture at pH 4.9 and 8.0 was carried out by PPC procedure. The result is given in Fig. 1 where not only the formation of thiamine (B_1) and FSSCy from the main reaction but also the formation of L-cystine (CySSCy), tetrahydrofurfuryl mercaptan (FSH), and its disulfide (FSSF) from side reactions were recognized.

The main reaction between TTFD and L-cysteine (CySH) may be written as Eq. (1), however, several side reactions may be postulated from the result shown in Fig. 1.

$$\text{TTFD}+\text{CySH} \longrightarrow B_1+\text{FSSCy} \qquad (1)$$

The main and possible side reactions are given in Chart 1 neglecting some slower side reactions.

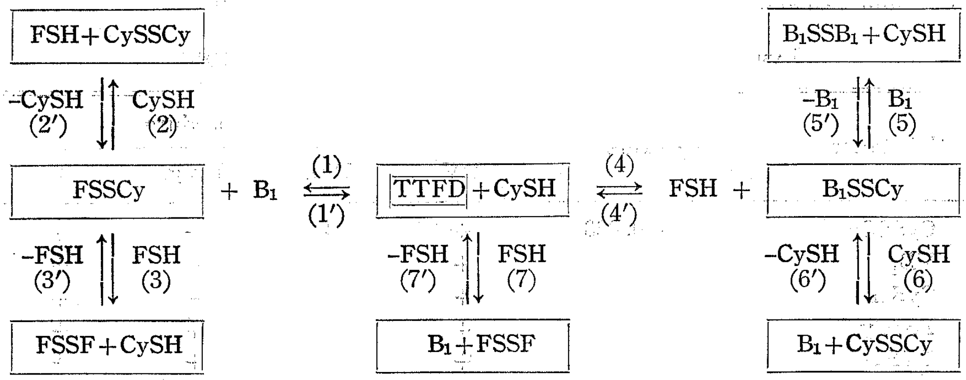

Chart 1. Main and Possible Side Reactions

The magnitude of side reactions may be estimated from the ratio of thiamine formed to FSH or its disulfide. The formation of FSH or its disulfide was examined as described in experimental but no detectable formation of FSH or its disulfide was found at initial stage of the reaction.

The reverse reaction of the main reaction was examined and a constant thiamine concentration was proved as seen in Table II. The stability of TTFD was examined and the results are given in Table III where no detectable thiamine formation was concluded.

Table II. Reaction of FSSCy and Thiamine

Reaction time / pH	0 min	10 min	20 min	30 min
4.94	0.587	0.583	0.583	0.576
8.16	0.580	0.600	0.583	0.587

The table shows the optical density at 368 mμ of thiochrome.

From these results presented, it may be concluded that the kinetics on the main reaction shown by Eq. (1) is conducted when the formation of thiamine is followed at the initial stage

Table III. Stability of TTFD from the Formation of Thiamine

pH	Initial concentration of TTFD	Reaction time and O.D$_{368}$ of thiochrome		
		2 min	10 min	30 min
4.05	1.86×10^{-3}M	0.092	0.072	0.077
7.33	8.00×10^{-5}M	0.027	0.008	0.014

at 37° in buffer solutions of pH 4.1 and 7.3

of the reaction.[9] However, the pH range of the reaction mixture was limited between 8.2 and 3.5 since the reaction proceeded so rapidly at higher pH region and the molecular form of carboxylic group in L-cysteine molecule can be neglected in this pH region.

As reported in the proceeding paper, the reaction shown by Eq. (1) should follow second order kinetics and the rate depends on the concentration of both reactants, TTFD and CySH. The assumption mentioned was supported by the following experimental results. The second order nature of the reaction should be converted to first order character when the excess amount of one component, TTFD or CySH, is added. The results are shown in Fig. 2 where the linear logarithmic decrease of reactant to time was observed. The rate constant of a pseud first order reaction was 2.06×10^{-2} min^{-1} determined at given experimental condition. The mean value of pseud first order rate constant obtained from eight run was $(2.14\pm0.08)\times10^{-2}$ min^{-1} at 25° and pH 4.90 which well agreed with 12.6 ± 0.3 liter mole^{-1} min^{-1} as a second order rate constant.

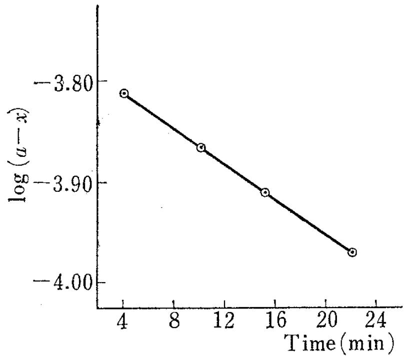

Fig. 2. Pseudo-First Order Reaction at 25°, pH 4.90 where a Represents the Initial Concentration of TTFD and CySH and x the Concentration of Produced B_1

$[\mathrm{CySH}]_0=1.69\times10^{-3}$ M
$[\mathrm{TTFD}]_0=1.69\times10^{-4}$ M$=a$

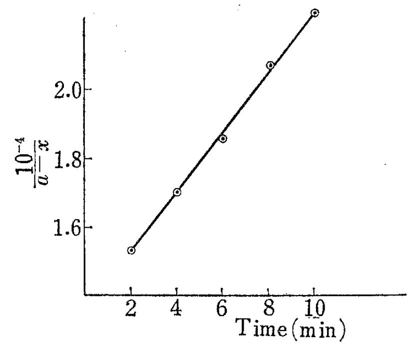

Fig. 3. Second Order Plot of the Reaction at 25°, pH 7.51 where $a=[\mathrm{TTFD}]_0=[\mathrm{CySH}]_0=7.58\times10^{-5}$ M and x is the Concentration of B_1

The second order plot of the reaction was a straight line as seen in Fig. 3 where the equimolar of disulfide and thiol were reacted at pH 7.51.

The second order rate constant of reaction (1) was determined at 37°, 25°, and 15° within pH-range of 3.5 to 8.2. The result is given in Fig. 4 where the relation between log k_{obs} and pH is seen. The pH-rate profile of the reaction may be revealed as follows.

Two species of TTFD, ionic and molecular form, may be postulated within the pH-range mentioned as shown in Eq. (2)

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[9] Side reactions were recognized on PPC at completion of the reaction and the second order rate constants determined at the latter stage of reacion were not always constant, probably depending on the side reactions, (2), (3), or (7) in chart 1.

$$\mathrm{HB_1SSF} \rightleftarrows \mathrm{B_1SSF} + \mathrm{H^+} \qquad (2)$$

where B_1SSF is molecular form of TTFD and HB_1SSF its protonated form. The dissociation constant, K_F, may be given in Eq. (3).

$$K_F = \frac{[\mathrm{B_1SSF}][\mathrm{H^+}]}{[\mathrm{HB_1SSF}]} \qquad (3)$$

The value of pK_F has been reported as 5.50 at 25°.[10] Eq. (4) may be written since the total concentration of TTFD is sum of molecular and monoprotonated form at experimental condition.

$$[\mathrm{TTFD}]_{\mathrm{total}} = [\mathrm{B_1SSF}] + [\mathrm{HB_1SSF}] \qquad (4)$$

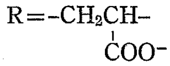
From these relationships, each species may be represented as Eqs. (5) and (6).

$$[\mathrm{HB_1SSF}] = [\mathrm{TTFD}]_{\mathrm{total}} \frac{[\mathrm{B_1SSF}][\mathrm{H^+}]}{K_F([\mathrm{HB_1SSF}]+[\mathrm{B_1SSF}])} = \frac{[\mathrm{TTFD}]_{\mathrm{total}}}{1+\dfrac{K_F}{[\mathrm{H^+}]}} \qquad (5)$$

$$[\mathrm{B_1SSF}] = [\mathrm{TTFD}]_{\mathrm{total}} - [\mathrm{HB_1SSF}] = \frac{[\mathrm{TTFD}]_{\mathrm{total}}}{1+\dfrac{[\mathrm{H^+}]}{K_F}} \qquad (6)$$

The ionic species of L-cysteine has been reported as Eq. (7)

$$\begin{tikzcd} \mathrm{HSRN^+H_3} \arrow[r, shift left, "pK_a=8.53"] \arrow[d, shift left, "pK_b=8.86"'] & \mathrm{^-SRN^+H_3} \arrow[l, shift left] \arrow[d, shift left, "pK_c=10.36"] \\ \mathrm{HSRNH_2} \arrow[r, shift left] \arrow[u, shift left] & \mathrm{^-SRNH_2} \arrow[l, shift left, "pK_d=10.03"] \arrow[u, shift left] \end{tikzcd} \qquad (7)$$



$$\mathrm{R} = -\mathrm{CH_2}\underset{\mathrm{COO^-}}{\mathrm{CH}}-$$

The value of pK of carboxylic group in L-cysteine was reported as $pK=1.86$[11] and only ionized form of acid group, exists at the pH-range over 3.5. From the relation shown in Eq. (7), Eq. (8) was given by Benesch.[12]

$$\frac{[\mathrm{^-SRN^+H_3}]+[\mathrm{^-SRNH_2}]}{[\mathrm{CySH}]_{\mathrm{total}}} = \frac{K_a/K_b + K_d/[\mathrm{H^+}]}{[\mathrm{H^+}]/K_b + K_a/K_b + K_d/[\mathrm{H^+}]+1} \qquad (8)$$

The reaction between the disulfide and $^-SRNH_2$ may be negligible at pH 3.5—8.2 because the ratio of the concentration of $^-SRNH_2$ to $^-SRN^+H_3$ is calculated from pK_c (=10.36) to be about 1/100 at pH 8.2 and the ratio of the reactivity of $^-SRNH_2$ with thiamine derivatives to that of $^-SRN^+H_3$ was about 4 as reported in the proceeding paper.[5] More over, K_d/H^+ may be negligible small at pH 3.5—8.2. From these two reasons, Eq. (8) may be simplified to Eq. (9).

$$\frac{[\mathrm{CySH}]_{\mathrm{total}}}{[\mathrm{^-SRN^+H_3}]} = \frac{[\mathrm{H^+}]/K_b + K_a/K_b + 1}{K_a/K_b} \qquad (9)$$

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[10] T. Matsukawa, L. Yurugi, and H. Kawasaki, *Takeda Kenkyusho Nenpo*, **12**, 1 (1952).
[11] R.K. Cannan, *Biochem. J.*, 21, 1384 (1927); cited from "Daiyuki-Kagaku," Asakura Shoten, 1963.
[12] R.E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, 77, 5877 (1955).

Eq. (9) may be simplified further to Eq. (10) since $[H^+]/K_b \gg K_a/K_b > 1$, at pH 3.5—7.5.

$$\frac{[^-SRN^+H_3]}{[CySH]_{total}} = \frac{K_a}{[H_3]} \tag{10}$$

The following elementary reactions shown by Eqs. (11) and (12) can be assumed from the relations mentioned for the reaction between CySH and TTFD at given experimental condition,

$$HB_1SSF + {}^-SRN^+H_3 \xrightarrow{k_1} FSSRN^+H_3 + B_1{}^{13)} \tag{11}$$

$$B_1SSF + {}^-SRN^+H_3 \xrightarrow{k_2} FSSRN^+H_3 + B_1{}^{13)} \tag{12}$$

The formation of thiamine may be shown by Eq. (13)

$$\frac{d[B_1]}{dt} = k_{obs}[TTFD]_{total}[CySH]_{total} \tag{13}$$

and the equation can be rewritten as Eq. (14).

$$\frac{d[B_1]}{dt} = [TTFD]_{total}\left(\frac{k_1}{1+K_F/[H^+]} + \frac{k_2}{1+[H^+]/K_F}\right) \times [CySH]_{total}\frac{K_a/K_b}{[H^+]/K_b + K_a/K_b + 1} \tag{14}$$

Eq. (15) may be obtained from Eq. (14).

$$k_{obs} = \left(\frac{k_1}{1+K_F/[H^+]} + \frac{k_2}{1+[H^+]/K_F}\right)\frac{K_a/K_b}{[H^+]/K_b + K_a/K_b + 1} \tag{15}$$

Eq. (15) can be simplified to Eq. (16) from the relation between pH and pK_F (=5.50).

$$\frac{k_1}{1+K_F/[H^+]} + \frac{k_2}{1+[H^+]/K_F} = k_1 (3.5 \leq \text{pH} \leq 4.5) \text{ or } k_2 (7.5 \leq \text{pH}) \tag{16}$$

from Eqs. (15), (16), (9), and (10), the following simplified equations may be obtained.
pH 3.5—4.5

$$k_{obs} = k_1\frac{K_a}{[H^+]} \qquad \log k_{obs} = \log k_1 \cdot K_a + \text{pH} \tag{17}$$

pH 7.5

$$k_{obs} = k_2\frac{K_a}{[H^+]} \qquad \log k_{obs} = \log k_2 \cdot K_a + \text{pH} \tag{18}$$

The linear relationship of slope 1.0 between pH and $\log k_{obs}$ given in Fig. 4 may be revealed from the Eqs. (17) and (18) by which the specific rate constants were calculated as

$k_1 = 6.56 \times 10^4$ and $k_2 = 9.05 \times 10^3$

in liter·mole⁻¹ min⁻¹ at 25°, respectively.

The curve ① in Fig. 4 is calculated from Eq.

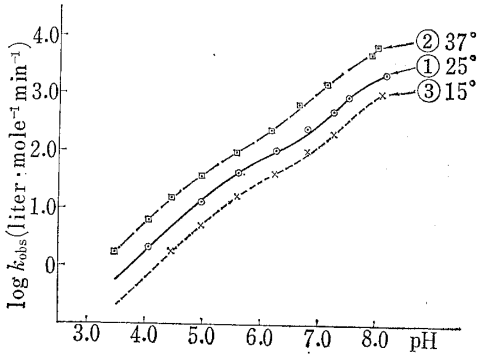

Fig. 4. pH–Rate Profile of the Reaction between TTFD and CySH in Buffer Solutions

The curve, 1, was calculated from Eq.(15) where $k_1 = 6.56 \times 10^4$ and $k_2 = 9.05 \times 10^3$ in liter·mole⁻¹ min⁻¹ and plots ⊙, ⊡, ×, were observed.

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13) B_1 is used as the generic symbol of thiamine involving several species and electric charge — or + are omitted.

(15) using these values. The well agreement between the experimental and calculated values is observed in the figure and it would be one of the evidence that the interpretation of the reaction mentioned is reasonable.

The effect of ionic strength and buffer concentration on the reaction rate was studied at given experimental condition and the result is shown in Fig. 5 and Fig. 6 where no detectable effect was confirmed.

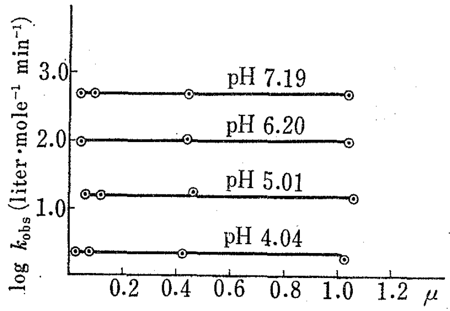

Fig. 5. Effect of Ionic Strength on the Logarithm of the Observed Rate Constants

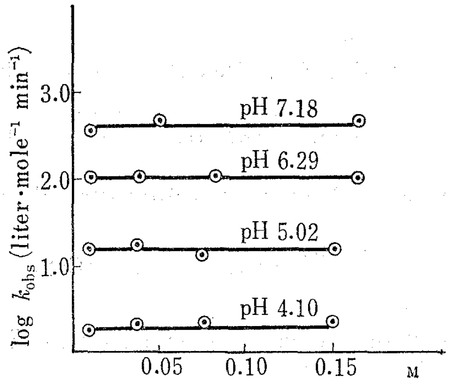

Fig. 6. Effect of the Buffer Concentration on the Logarithm of the Observed Rate Constants

The chemical kinetics between TTFD and glutathion (GSH) or N-acetyl-L-cysteine (NACySH) was studied by the same procedure to L-cysteine since the same reaction mechanism was assumed for these compounds and k_{obs} may be represented by Eq. (19).

$$k_{obs}=\left(\frac{k_1}{1+K_F/[H^+]}+\frac{k_2}{1+[H^+]/K_F}\right)\times\frac{[RS^-]}{[RSH]_{total}} \qquad (19)$$

The value of pK for the thiol group of glutathione has been reported by Benesch[12] as follows.

$$\log\frac{[GS^-]^{14)}}{[GSH]}=pH-pK \qquad pK=9.2 \text{ at } 23° \qquad (20)$$

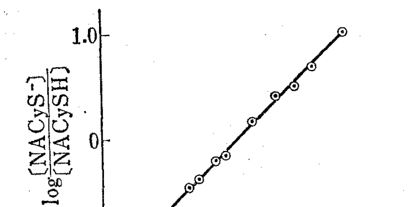

Fig. 7. Determination of pK Value of Thiol Radical of NACySH following the Method of R.E. Benesch[12]

The relation was examined on NACySH and the result is given in Fig. 7 where the value of pK was calculated as 10.0 at 23°.

$$\log\frac{[NACyS^-]}{[NACySH]}=pH-pK \qquad pK=10.0 \text{ at } 23° \qquad (21)$$

The log k_{obs} at given temperature and pH is summerized in Table IV and the specific rate constant for GSH and NACySH is given in Table V. A pK of the weaker carboxyl group of GSH was found to be 3.65 at 25° and the pK of the carboxyl group of NACySH was found to be 3.15 at 25°. From these pK values, it may be concluded that the species of GSH at pH 4.5—8.2 would be mainly $^-$GSH and $^-$GS$^-$ and that of NACySH at pH 4.0—8.2 $^-$NACyS$^-$ and $^-$NACySH where $^-$GS$^-$, $^-$NACyS$^-$ represent the species shown in Fig. 9. Then the two specific rate constants

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[14] Here [GS$^-$] and [GSH] represent concentration of thiol anion and nonionized thiol species of GSH, respectively.

Table IV. The log k observed in the Reaction of TTFD and Glutathione or N-Acetyl-L-cysteine at given Temperature and pH

React. temp. (°C)	RSH	pH	log k_{obs} (liter·mole^{-1}min^{-1})	React. temp. (°C)	RSH	pH	log k_{obs} (liter·mole^{-1}min^{-1})
15	GSH	3.92	−0.351	25	GSH	6.40	1.99
15	GSH	4.97	0.597	25	GSH	7.48	2.66
15	GSH	5.57	1.09	25	GSH	8.00	3.22
15	GSH	6.23	1.52	25	NACySH	3.94	−0.19
15	GSH	6.80	1.85	25	NACySH	6.01	1.37
15	GSH	7.51	2.28	25	NACySH	6.26	1.48
15	NACySH	3.90	−0.602	25	NACySH	6.41	1.59
15	NACySH	4.97	0.301	25	NACySH	7.48	2.25
15	NACySH	6.23	1.16	25	NACySH	7.99	2.80
15	NACySH	6.80	1.45	37	GSH	4.01	0.640
15	NACySH	7.51	1.85	37	GSH	7.32	3.03
25	GSH	3.94	0.25	37	NACySH	4.01	0.386
25	GSH	6.00	1.80	37	NACySH	7.38	2.66
25	GSH	6.26	1.91				

Table V. The Specific Rate Constants at 25°

$$\mathrm{HB_1SSF + RS^-} \xrightarrow{k_1} \mathrm{FSSR + B_1}$$

$$\mathrm{B_1SSF + RS^-} \xrightarrow{k_2} \mathrm{FSSR + B_1}$$

RSH	k_1 (liter·mole^{-1}min^{-1})	k_2 (liter·mole^{-1}min^{-1})
CySH	6.56×10^4	9.05×10^3
GSH	3.28×10^5	2.70×10^4
NACySH	7.55×10^5	6.36×10^4

of $^-$GS$^-$ were calculated from k_{obs} over pH 6.00 and for $^-$NACyS$^-$ from k_{obs} over pH 3.94. The agreement between experimental and calculated value may be seen in Fig. 8. As reported in the proceeding paper,$^{5)}$ the larger, about 10 times or less, specific rate on the reaction between ionized form of thiamine derivative and thiol anion was found than the one between molecular form and thiol anion. The relative specific reaction rate between the ionized or molecular form of the thiamine derivative and the thiol anions are given in Fig. 9. The reactivity of thiols increased in the order, CyS$^-$<$^-$GS$^-$<$^-$NACyS$^-$.

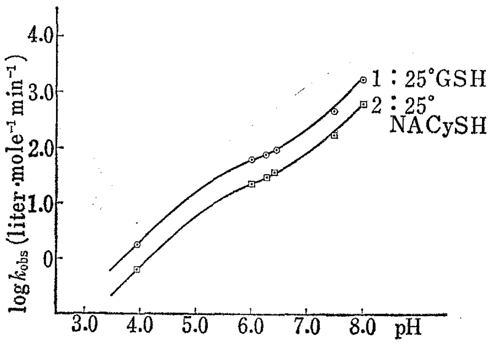

Fig. 8. pH-Rate Profile of the Reaction between TTFD and Glutathione or N-Acetyl-L-cysteine in Buffer Solutions

The curves, 1 and 2, were calculated from Eqs. (19), (20) and (21) and plots ⊙, ⊡, were observed.

In the reaction with ionized form of TTFD, it probably due to the net charge of thiol anion which related directly to the electrostatic attractive force of reacting species. In the case of uncharged molecular form of TTFD, the cause of the order of the reactivity is not clear. The difference between $^-$GS$^-$ and $^-$NACyS$^-$ in reactivity might be due to the steric effect or relative distance between two negative charges in thiol ions.

The kinetical parameters were examined on both elementary reactions. The results were given in Fig. 10, 11 where an Arrhenius' type plot was observed. The parameters cal-

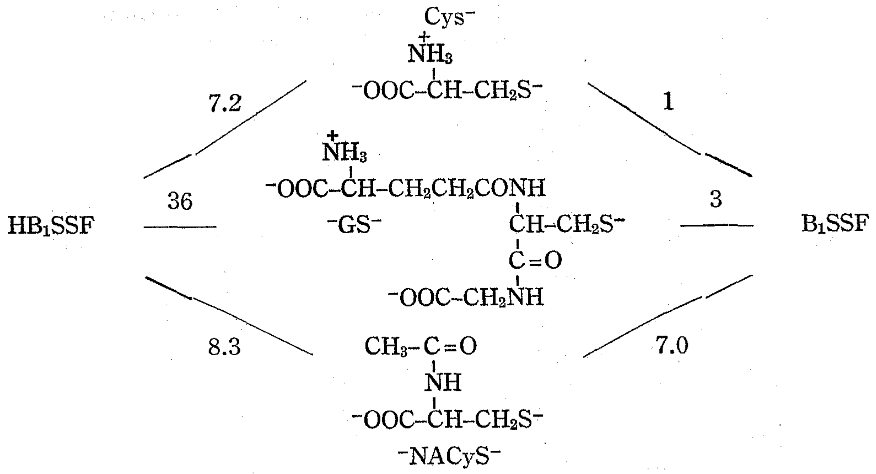

Fig. 9. Comparison of the Specific Rate Constants

culated are summerized in Table VI where Ea is the mean value after correction of the heat of dissociation of thiol group[15] determined at 18° and 31° by Benesch[12] and from which the frequency factor and entropy for activation were calculated. The activation energy of the reaction was around 10 kcal/mole, not depending on the reaction species, however, the activation entropy was different depending on reacting species.

The similar relationship were reported in the reactions between bisulfite anion and disulfides reported by Cecil, *et al.*[16] They studied the kinetics of the reaction,

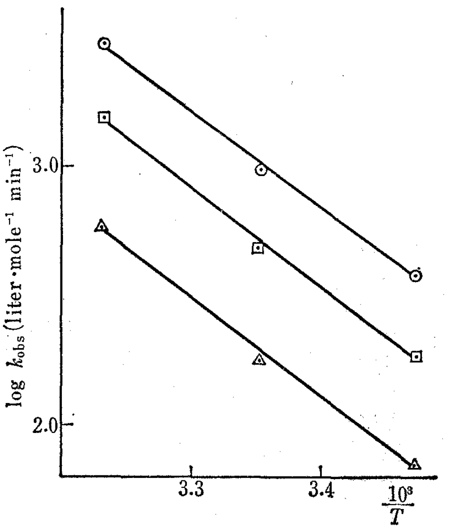

Fig. 10. The Arrhenius' Type Plot of the Reactions of TTFD and the Thiols, CySH, GSH, and NACySH, represented by ⊙, ⊡ and △, Respectively at pH 7.5

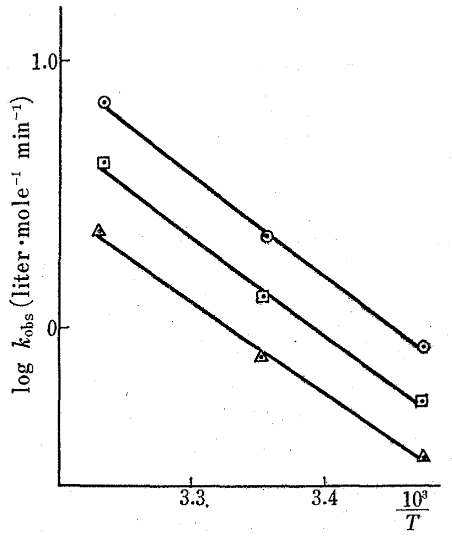

Fig. 11. The Arrhenius' Type Plot of the Reactions of TTFD and the Thiols, CySH, GSH, and NACySH, represented by ⊙, ⊡ and △, Respectively at pH 4.0

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15) The heat of dissociation of the thiol group was found by Benesch using thioglycolic acid as the thiol compound. The value, $\Delta H = 6.4$ kcal/mole which is the average of 5.8 and 7.0 kcal/mole was used in this study.

Method	Heat of dissociation
Spectrophotometric	$\Delta H_{31^\circ} = 6.9$ cal/mole
Electrometric	$\Delta H_{18^\circ} = 5.8$
	$\Delta H_{31^\circ} = 7.0$

16) R. Cecil and J.R. Mcphee, *Biochem, J.*, **60**, 496 (1955).

TABLE VI. The summary of the Kinetical Parameters

RSH	Specific rate consts. at 25° (liter·mole^{-1} sec^{-1})	E_a (kcal · mole^{-1})	A (liter·mole^{-1} sec^{-1})	ΔS (cal · deg^{-1} mole^{-1})
CySH	$k_1=1.09\times10^3$	10.7	6.31×10^{10}	−11.1
	$k_2=1.51\times10^2$	9.9	3.16×10^9	−17.5
GSH	$k_1=5.47\times10^3$	10.4	2.24×10^{11}	− 8.6
	$k_2=4.50\times10^2$	10.8	3.63×10^{10}	−12.5
NACySH	$k_1=1.27\times10^4$	9.9	2.24×10^{11}	− 8.6
	$k_2=1.06\times10^3$	10.9	1.02×10^{11}	−10.1

$$RSSR+SO_3^{2-} \xrightarrow{k} RS^- + RSSO_3^-$$

Where RSSR represents three species of cystine or three species of oxydized glutathione, which are different in net charge. The activation energy reported was from 10 to 13 kcal/mole and the activation entropy was from −10 to −29 cal·mole^{-1} deg^{-1}. The activation entropy obtained was negative although the positive value was reported by Kōno, *et al.*[17] on the reaction between the disulfide type thiamine derivatives and thiol group in protein molecule.

Since the relations mentioned above are not simple and the kinetical parameters will be discussed more in detail in the following paper of this series.

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17) K. Kōno, *Bitamin*, 31, 470 (1965).