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87. Hisashi Nogami, Jun Hasegawa, and Noriko Ikari[*1] : Thiamine Derivatives of Disulfide Type. V.[*2] Kinetic Studies of the Reaction between Thiamine Propyl Disulfide or Homologes and L-Cysteine.[*3]

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The kinetics between thiamine propyl disulfide or its homologes and L-cysteine were studied under the experimental conditions determined in the preceding paper. The effects of alkyl substitution of TPD on kinetic parameter were also studied, using thiamine benzyl disulfide, thiamine allyl disulfide, and thiamine hydroxyethyl disulfide. The following conclusions were drawn from the results presented.

1) From an analysis of the pH-rate profile of the reaction which followed a second order kinetics throughout the pH range of 3.5 to 13.2, the reaction was revealed as the combination of the following elementary reactions.

$$HB_1\text{-}SS\text{-}Pr + NH_3^+\text{-}Cy\text{-}S^- \xrightarrow{k_1} HB_1\text{-}S^- + NH_3^+\text{-}Cy\text{-}SS\text{-}Pr$$

$$B_1\text{-}SS\text{-}Pr + NH_3^+\text{-}Cy\text{-}S^- \xrightarrow{k_2} B_1\text{-}S^- + NH_3^+\text{-}Cy\text{-}SS\text{-}Pr$$

$$B_1\text{-}SS\text{-}Pr + NH_2\text{-}Cy\text{-}S^- \xrightarrow{k_3} B_1\text{-}S^- + NH_2\text{-}Cy\text{-}SS\text{-}Pr$$

where HB_1-SS-Pr is the protonated form of TPD, NH_3^+-Cy-S^- is protonated cysteine anion, and k_1, k_2 and k_3 are specific rate constants of individual elementary reactions. The values determined were 1.82, 0.261 and 1.10 in 10^4 L. mole^{-1} min.$^{-1}$ for k_1, k_2 and k_3, respectively.

2) No detectable effect of ionic strength and buffer concentration on the rate constant was observed at pH 4.0 on Reaction (I).

3) The activation energy obtained from an Arrhenius type plot was 12.3 and 10.9 kcal./mole for Reaction (I) and (II) after correction for ionic dissociation of thiol and amino groups in cysteine.

4) From the kinetic data tabulated on TPD derivatives, the order of the reactivity was TBD>TAD–TOED>TPD. The activation energies of Reaction (I) were about 10 to 12 kcal./mole.

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The ionic scission of the sulfur-sulfur bond has been studied in the reactions between disulfide-thiol,[1~3] alkylthiosulfate-sulfite,[4] alkyldisulfide-sulfite,[5] and disulfide-cyanide.[6] In these reports and the reviews[7] on the scission of sulfur-sulfur bond, the bimolecular reactions following the second order kinetics have been mentioned.

In the preceding paper of this series,[*2] the exchange reaction between thiamine propyl disulfide (TPD) and L-cysteine was examined, and it was proved that the main reaction was represented by Eq. (1) following the second order kinetics and that the expected side reactions were negligible to the kinetic study of the main reaction when both reactants were mixed in the molar ratio of 1:1 as the result of qualitative and

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[*2] Part Ⅳ. This Bulletin, **15**, 685 (1967).

[*3] Presented before the 86th ann. meeting of the Pharmaceutical Society of Japan.

1) L. Eldjarm, A. Pihl: J. Biol. Chem., **225**, 499 (1957).
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quantitative examinations.

$$\text{TPD} + \text{cysteine} \longrightarrow \text{B}_1 + \text{Cy-SS-Pr} \qquad (1)$$

where B_1 is thiamine and Cy-SS-Pr is cysteine propyldisulfide.

In the present paper, the exchange reactions between TPD or its homologes and L-cysteine were examined in the pH-range of 3.5 to 13.2 at 15° as the result of which the elemetary reactions involved were clarified from the analysis of the pH-rate profile and the kinetic parameters for the elementary reactions determined.

Experimental

Materials——TPD and L-Cysteine: reported in the preceding paper. Thiamine benzyl disulfide (TBD): m.p. 152° (decomp.), colorless prisms. Thiamine allyl disulfide (TAD): m.p. 132° (decomp.), colorless crystalline powder. Thiamine β-hydroxyethyl disulfide (TOED): m.p. 148° colorless prisms. The thiamine derivatives were supplied by Takeda Chem. Ind., Ltd.

Kinetic Procedures——Same as the preceding paper.*2

Buffer Solutions——0.1 molar Michaelis' acetate buffer solution of pH 3.5, 4.0, 4.6 and 5.6; 0.1 molar Clark-Lubs' phosphate buffer solution of pH 6.1, 6.8, 8.0 and 8.5; and 0.1 molar Sörensen's borate buffer solution of pH 9.0, 9.5, 10.3, 11.1 and 12.0 were used. 0.1 molar NaOH aq. soln. was used in pH 13.2.

Effect of Ionic Strength——Reactions were examined at pH 4.0 and 15° at varying μ from 0.02 to 0.80 with NaCl.

Effect of Buffer Concentration——Reactions were examined at pH 4.0 and 15° at varying acetate concentration from 0.05 to 0.1 mole/L. and adjusting μ to 0.2

Result and Discussion

The order of the reaction between TPD and L-cysteine in molar ratio 1:1 was calculated from the results obtained in the pH range of 6.1 to 10.3 by the half-life method and is given in Table I, where the second order nature of the reaction was proved as reported in the preceding paper. Since the reaction was very rapid at 15° above pH 10.5, the values of n above this pH are not given in the Table.

TABLE I. Order of Reaction between TPD and L-Cysteine at Various pH (15°)

pH	6.1	7.1	8.0	8.6	9.05	10.3
n	1.87	1.89	2.00	2.10	2.00	1.97

The relation between the rate constant observed and pH is given in Fig. 1.

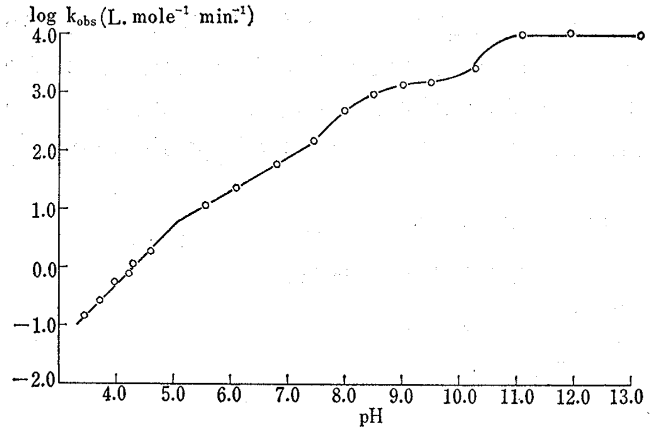


Fig. 1. pH-Rate Profile on the Reaction of TPD and Cysteine at 15°

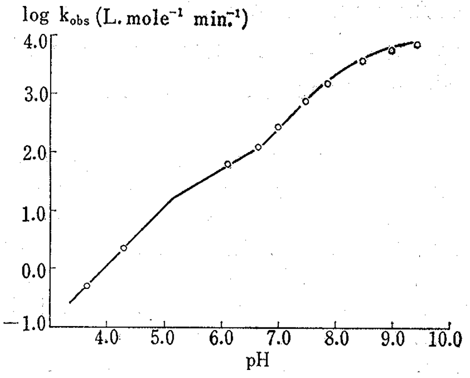


Fig. 2. pH-Rate Profile on the Reaction of TBD and Cysteine at 15°

The tendency for the second order plot of the reaction to deviate from a straight line in the final stages of the run above pH 10.5 was observed, and therefore the rate constant was determined from the initial stages of the reaction. A very complex pH-rate profile was obtained and is shown in Fig. 1. The linear relationship between pH and log k_{obs} with a slope equal to 1.0 was observed within the pH-range of 3.5 to 5.0. The slope decreased to 0.6 between pH 5.0 and pH 7.5. However at the pH-range of 7.5 to 8.5, the slope again returned to 1.0. A constant reaction rate was observed above pH 11.0. The inflection found at pH 5.3 would be caused by the thiamine moiety of TPD, since no inflection was reported by Cecil between pH 5 to 6 on the pH-rate profile for the exchange reaction between cystine and sodium sulfite, and the pK values of the amino-, thiol- and carboxyl-groups, which are the functional groups of cystine or cysteine, do not exist within this pH-range. This assumption is also supported by the following experimental result. The kinetic study between TBD and L-cysteine was carried out under the same experimental condition, and the pH-rate profile is given in Fig. 2 where an inflection was also found at same pH.

Since the pKa value of pyrimidine moiety in TPD was reported as 5.52 at 25°[8] and this moiety is close to the sulfur-sulfur bond stereochemically, it is reasonable to expect that the ionic dissociation of the pyrimidine moiety affects the exchange reaction between disulfide and thiol.

Several ionic species of cysteine may be assumed to depend on the pH value, especially between pH 7 and 12, since the cysteine molecule contains amino- and thiol-groups. The circumstance mentioned above will explain the complex pH-rate profile found between pH 7 to 11.

According to the report by Benesch and Benesch[9] on the pK of cysteine, the ionic dissociation may be as given in Chart 1 where pKa was 8.53, pKb 8.86, pKc 10.36 and pKd 10.03 at 23°, respectively.

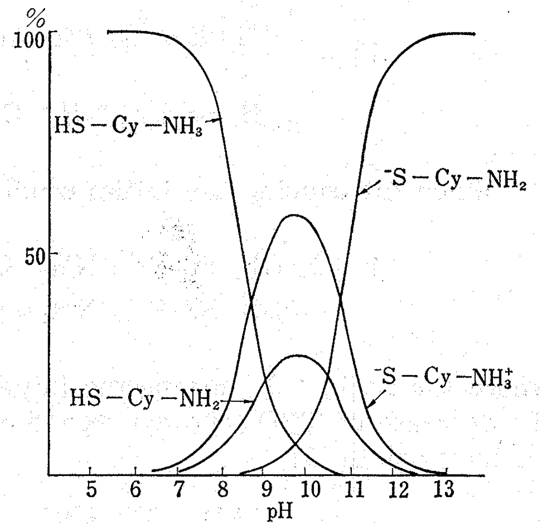
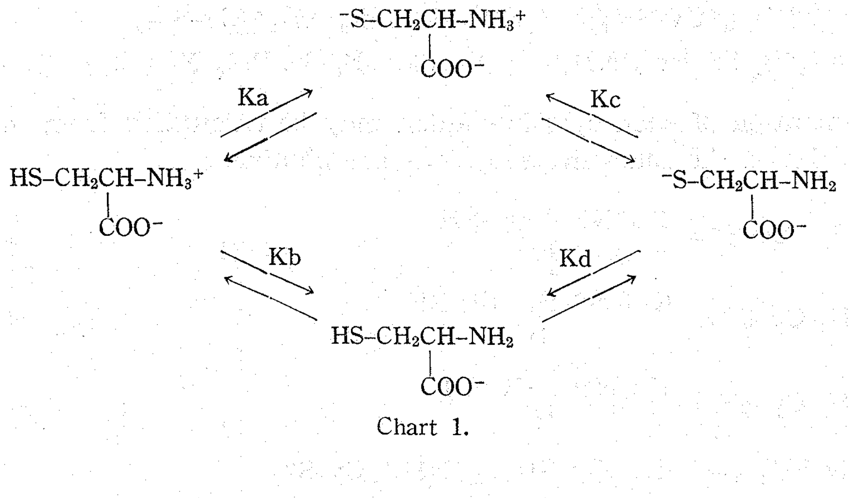


Fig. 3. Concentration of the Different Ionic Forms of Cysteine in Solution as a Function of pH at 23°



$^-S\text{-}CH_2CH(COO^-)\text{-}NH_3^+$, $HS\text{-}CH_2CH(COO^-)\text{-}NH_3^+$, $^-S\text{-}CH_2CH(COO^-)\text{-}NH_2$, $HS\text{-}CH_2CH(COO^-)\text{-}NH_2$

Chart 1.

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[8] T. Matsukawa, S. Yurugi, *et al.*: Ann. Repts. Takeda Research Lab., **12**, 1 (1952).

[9] R. E. Benesch, R. Benesch: J. Am. Chem. Soc., **77**, 5877 (1955).

The treatment of kinetic data obtained is based on the assumption that between the pH-range of 3.5 to 13.2 two cysteine anions, *i.e.* NH_3^+-Cy-S^- and NH_2-Cy-S^-, react with each of two forms of TPD, *i.e.* protonated TPD (HB_1-SS-Pr) and nonprotonated TPD (B_1-SS-Pr), in this pH range. The decrease of TPD or cysteine in the pH range between 3.5 to 13.2 may be shown by the following four equations.

$$V_1=k_1[\mathrm{HB_1\text{-}SS\text{-}Pr}][\mathrm{NH_3^+\text{-}Cy\text{-}S^-}] \qquad (2)$$

$$V_2=k_2[\mathrm{B_1\text{-}SS\text{-}Pr}][\mathrm{NH_3^+\text{-}Cy\text{-}S^-}] \qquad (3)$$

$$V_3=k_3[\mathrm{B_1\text{-}SS\text{-}Pr}][\mathrm{NH_2\text{-}Cy\text{-}S^-}] \qquad (4)$$

$$V_4=k_4[\mathrm{HB_1\text{-}SS\text{-}Pr}][\mathrm{NH_2\text{-}Cy\text{-}S^-}] \qquad (5)$$

where V_4 may be ignored since significant concentration of HB_1-SS-Pr and NH_2-Cy-S^- would not exist simultaneously when the pK value of each is considered. These may be combined into the over-all rate equation,

$$V_4=\frac{-d[\mathrm{TPD}]}{dt}=\frac{-d\,[\mathrm{cysteine}]}{dt}=k_1[\mathrm{HB_1\text{-}SS\text{-}Pr}][\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]$$
$$+k_2[\mathrm{B_1\text{-}SS\text{-}Pr}][\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]+k_3[\mathrm{B_1\text{-}SS\text{-}Pr}][\mathrm{NH_2\text{-}Cy\text{-}S^-}] \qquad (6)$$

When introducing the initial conditions, the equation may be rewritten as Eq. (7).

$$V_0=k_1[\mathrm{HB_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]_0+k_2[\mathrm{B_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]_0$$
$$+k_3[\mathrm{B_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_2\text{-}Cy\text{-}S^-}]_0 \qquad (7)$$

where the suffix "0" means the initial concentration of individual component. Since the decrease of TPD followed second order kinetics as shown in experimental results, we may obtain Eq. (8)

$$V_0=k_{\mathrm{obs}}[\mathrm{TPD_T}]_0[\mathrm{Cy\text{-}SH_T}]_0 \qquad (8)$$

where $[\mathrm{TPD_T}]_0$ and $[\mathrm{Cy\text{-}SH_T}]_0$ mean the total initial concentration of TPD and cysteine, respectively, and k_{obs} is the experimental second order rate constant. Eq. (7) and (8) may then be rewritten as,

$$k_{\mathrm{obs}}[\mathrm{TPD_T}]_0[\mathrm{Cy\text{-}SH_T}]_0=k_1[\mathrm{HB_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]_0$$
$$+k_2[\mathrm{B_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]_0+k_3[\mathrm{B_1\text{-}SS\text{-}Pr}]_0[\mathrm{NH_2\text{-}Cy\text{-}S^-}]_0 \qquad (9)$$

The concentration of each cysteine anion may be calculated from the value of pK given in Chart 1 and the following equations are obtained.

$$\mathrm{NH_3^+\text{-}Cy\text{-}S^-}=\frac{\mathrm{Ka}[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]}{\mathrm{H}} \qquad (10)$$

$$\mathrm{NH_2\text{-}Cy\text{-}S^-}=\frac{\mathrm{KaKc}[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]}{\mathrm{H}^2} \qquad (11)$$

$$\mathrm{NH_2\text{-}Cy\text{-}SH}=\frac{\mathrm{Kb}[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]}{\mathrm{H}} \qquad (12)$$

$$[\mathrm{Cy\text{-}SH_T}]_0=[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]_0+[\mathrm{NH_3^+\text{-}Cy\text{-}S^-}]_0$$
$$+[\mathrm{NH_2\text{-}Cy\text{-}SH}]_0[\mathrm{NH_2\text{-}Cy\text{-}S^-}]_0 \qquad (13)$$

Eq. (14) may be derived by combining Eqs. (10), (11), and (12) with Eq. (13).

$$[\mathrm{Cy\text{-}SH_T}]_0=[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]_0\left(1+\frac{\mathrm{Ka+Kb}}{\mathrm{H}}+\frac{\mathrm{KaKc}}{\mathrm{H}}\right)$$

$$[\mathrm{NH_3^+\text{-}Cy\text{-}SH}]_0=[\mathrm{Cy\text{-}SH_T}]_0\left(\frac{\mathrm{H^2}}{\mathrm{H^2+H(Ka+Kb)+KaKc}}\right) \tag{14}$$

The dissociation constant of pyrimidine moiety in TPD is shown by K_B, then we may get the following relation.

$$\mathrm{HB_1\text{-}SS\text{-}Pr} \overset{K_B}{\rightleftharpoons} \mathrm{B_1\text{-}SS\text{-}Pr+H^+}$$

$$\mathrm{B_1\text{-}SS\text{-}Pr}=\frac{\mathrm{K_B[HB_1\text{-}SS\text{-}Pr]}}{\mathrm{H}} \tag{15}$$

$$[\mathrm{TPD_T}]_0=[\mathrm{HB_1\text{-}SS\text{-}Pr}]_0+[\mathrm{B_1\text{-}SS\text{-}Pr}]_0=[\mathrm{HB_1\text{-}SS\text{-}Pr}]_0\left(1+\frac{\mathrm{K_B}}{\mathrm{H}}\right) \tag{16}$$

$$[\mathrm{HB_1\text{-}SS\text{-}Pr}]_0=[\mathrm{TPD_T}]_0\left(\frac{\mathrm{H}}{\mathrm{H+K_B}}\right) \tag{17}$$

By combining Eqs. (10), (11), (14), (15) and (17) with Eq. (9), Eq. (18) is obtained.

$$k_{\mathrm{obs}}=\frac{\mathrm{KaH^2}\left(k_1+\dfrac{k_2\mathrm{K_B}}{\mathrm{H}}+\dfrac{k_3\mathrm{K_BK_C}}{\mathrm{H^2}}\right)}{\mathrm{(H+K_B)\{H^2+H(Ka+Kb)+KaKc\}}} \tag{18}$$

At $K_B \ll H$, where the effects of K_2 and K_3 may be ignored since B_1-SS-Pr and NH_2-Cy-S^- are negligibile and $K_B \gg Ka$, Eq. (18) may be simplified to Eq. (19)

$$k_{\mathrm{obs}}=\frac{k_1\mathrm{Ka}}{\mathrm{H}} \tag{19}$$

Eq. (19) may be rewritten in logarithm as Eq. (20).

$$\log k_{\mathrm{obs}}=\log k_1\mathrm{Ka}-\log\mathrm{H}=\mathrm{C}+\mathrm{pH} \tag{20}$$

From Eq. (20), the plot of log k_{obs} versus pH can be expected to be linear with a slope equal to 1.0. This relationship was observed between the pH range of 3.5 to 4.5 as above mentioned, and k_1 was calculated from this pH range to be 1.82 × 10^4L. mole^{-1} min^{-1}.

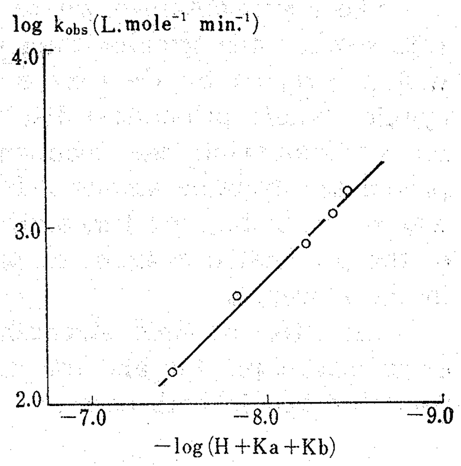


Fig. 4. Plots of log k_{obs} *vs.* −log (H+Ka+Kb) on the Reaction of TPD and Cysteine at 15°

At $K_B \gg H \gg K_C$ and Kd, where the concentration of protonated TPD is negligible as compared with the concentration of nonprotonated TPD, Eq. (18) may be simplified to Eq. (21), which may be rewritten in logarithm as Eq. (22).

$$k_{\mathrm{obs}}=\frac{\mathrm{Ka}k_2}{\mathrm{H+Ka+Kb}} \tag{21}$$

$$\log k_{\mathrm{obs}}=\log k_2\mathrm{Ka}-\log(\mathrm{H+Ka+Kb})=\mathrm{C}-\log(\mathrm{H+Ka+Kb}) \tag{22}$$

From Eq. (22) the plot of log k_{obs} versus − log(H+Ka+Kb) can be expected to be linear. A plot of Eq. (22) is presented at the pH range of 7.5 to 9.0. The value for k_2 calculated from Eq. (22) was 2.61 × 10^3 L. mole^{-1} min^{-1}.

On comparing the rate constant of k_1 and k_2, k_1 was found to be seven times greater than k_2. This would be the reason for the slope decrease observed between the wide pH range of 5.5 to 7.5.

When $H \ll Kc$ and Kd, the concentration of [HB_1-SS-Pr] and [NH_3^+-Cy-S^-] is negligible and the Eq. (18) may be simplified to Eq. (23).

$$k_{obs}=k_3 \tag{23}$$

As expected from Eq. (23), log k_{obs} was constant above pH 11.0, and the value for k_3 was calculated to be 1.10×10^4 L. mole^{-1} min^{-1}.

From the experimental results presented, the main reaction between TPD and cysteine shown by Eq. (1) was interpreted as the mixture of the following elementary reactions between each of the two species of TPD or two active cysteine anion as shown by Eq. (24), (25) and (26), where HB_1-S^- is protonated thiamine and B_1-S^- is thiamine.

$$HB_1\text{-SS-Pr}+NH_3^+\text{-Cy-}S^- \xrightarrow{k_1} HB_1\text{-}S^-+NH_3^+\text{-Cy-SS-Pr} \tag{24}$$

$$B_1\text{-SS-Pr}+NH_3^+\text{-Cy-}S^- \xrightarrow{k_2} B_1\text{-}S^-+NH_3^+\text{-Cy-SS-Pr} \tag{25}$$

$$B_1\text{-SS-Pr}+NH_2\text{-Cy-}S^- \xrightarrow{k_3} B_1\text{-}S^-+NH_2\text{-Cy-SS-Pr} \tag{26}$$

The result obtained where protonated TPD reacted with cysteine anion at a specific rate seven times greater than nonprotonated TPD reacted with cysteine anion agreed with the report by Gawron concerning the reaction between cystine and potassium cyanide where protonated disulfide was reported to react faster than the nonprotonated form. Comparing the reaction between nonprotonated TPD and nonprotonated- or protonated cysteine anions, NH_2-Cy-S^- or NH_3^+-Cy-S^-, the specific rate constant of k_3 was found to be four times greater than k_2. This would indicate that the reactivity of the protonated cysteine anion was weaker due to the closer position of SH to NH_2 in the molecule.

The effect of ionic strength and buffer concentration on the reaction rate were examined at pH 4.0, and the result is given in Fig. 5 and Fig. 6. No detectable effect was observed in both cases.

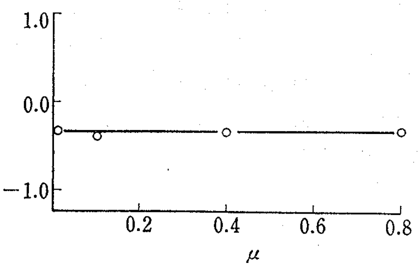


Fig. 5. Plots of log k_{obs} *vs.* μ at pH 4.0

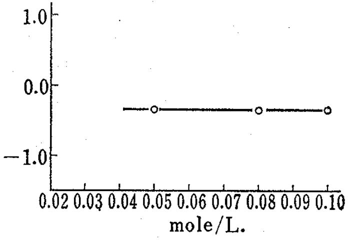


Fig. 6. Plots of log k_{obs} *vs.* Acetate Buffer Concentration at pH 4.0

The temperature dependence was studied at pH 4.0 and 8.0 where only one elementary reaction could be expected. The rate constant was determined at 15°, 20°, 30° and 37°, respectively, in an ionic strength adjusted to 0.1. The result is given in

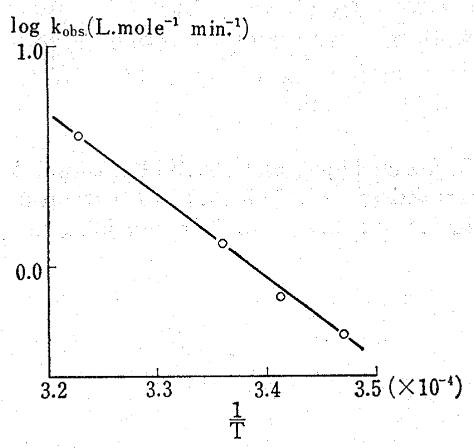


Fig. 7. Arrhenius Type Plots of log k_{obs} *vs.* the Reciprocal of Absolute Temperature on the Reaction of TPD and L-Cysteine at pH 4.0

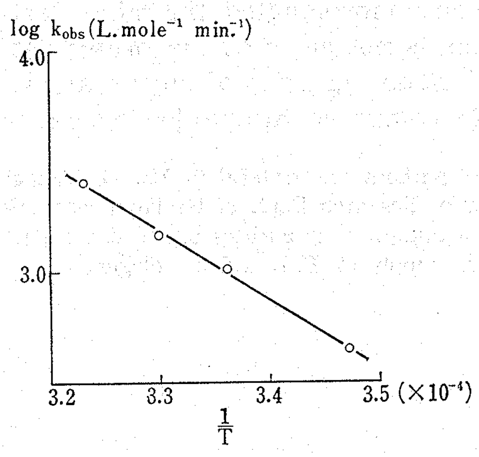
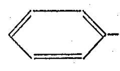


Fig. 8. Arrhenius Type Plots of log k_{obs} *vs.* the Reciprocal of Absolute Temperature on the Reaction of TPD and L-Cysteine at pH 8.0

Fig. 7 and Fig. 8, where an Arrhenius type plot was observed. Assuming the heat of ionization for thiol group and amino group in cysteine to be 6.5[9] and 11.0 kcal.[5] /mole, the activation energy was 12.3 and 10.9 kcal./mole for k_1 and for k_2, respectively.

The specific rate constant and activation energy obtained in the same manner on the reactions between homologes of disulfide type thiamine and cysteine are summarized in Table II. Comparing the order of magnitude of the rate constant, the tendency, TBD>TAD—TOED>TPD, was found. This tendency may be considered to be caused by the electron-withdrawing effect of the substituent, sinse the same tendency was observed on the substitution reaction of the derivatives[10] and these exchange reactions may be understandable as the nucleophilic attack of thiol anion at the sulfur-sulfur bond. The activation energy between protonated thiamine derivatives and NH_3^+-Cy-S^- anion was about 10~13 kcal. /mole, which is the probable magnitude estimated from the value reported.[5] Lower activation energy was found on derivatives where the propyl radical was replaced by more electron-withdrawing radicals such as benzyl, hydroxyethyl, or allyl group, when the exchange reaction was examined

TABLE II. Summarized Parameters on the Reaction of Thiamine Disulfide Derivatives and L-Cysteine

Thiamine disulfide derivatives RSSB$_1$		k at 15° (L. mole^{-1}min^{-1})		Ea (kcal./mole)	Frequency factor A=PZ$_0$(L. mole^{-1}min^{-1})
	R =	k_1 a)	1.82×10^4	12.3	3.64×10^{12}
TPD	CH_3-CH_2-CH_2-	k_2 b)	2.61×10^3	10.9	5.13×10^{11}
TBD	 -CH_2-	k_1	5.11×10^4	12.2	9.55×10^{13}
		k_2	7.90×10^3		
TOED	HO-CH_2CH_2-	k_1	3.05×10^4	8.9	1.66×10^{11}
		k_2	2.87×10^3		
TAD	CH_2=CH-CH_2-	k_1	2.18×10^4	12.5	6.61×10^{13}
		k_2	3.99×10^3		

a) k_1: $RSSB_1H$ + L-NH_3^+-Cy-S^- *b)* k_2: $RSSB_1$ + L-$NH_3^+CyS^-$

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10) M. Murakami, Y. Yukawa: "Kagaku-no-Ryoiki Zōkan 27." (Stereoorganic Chemistry) p. 21, Nankōdō.

between nonprotonated thiamine derivatives and the NH_3^+-Cy-S^- anion. The reason for this is not clear at the present stage. This will be discussed in more detail when the electron densities of sulfur atoms in the sulfur-sulfur bond and the thiol anion will be compared in a following paper of this series.

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