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86. Hisashi Nogami, Jun Hasegawa, and Noriko Ikari*1 : Thiamine Derivatives of Disulfide Type. IV.*2 The Exchange Reaction between Thiamine Propyl Disulfide and L-Cysteine.

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Experimental conditions for the kinetic study of exchange reaction between thiamine propyl disulfide (TPD) and L-cysteine were examined where the possible side reactions shown in Chart 1 proved by thin-layer chromatography could be safely ignored. The following conclusions were drawn from the results obtained.

1) It was proved that the formation of thiamine was the main product and the formation of propylmercaptan was negligible when the reactants were mixed in a molar ratio of 1:1 and an initial concentration of $1\times10^{-3}\sim5\times10^{-5}$ molar.

2) The exchange reaction followed second order kinetics examined from half-life, the plot of integrated equation, and the result of pseudo first order reaction converted by experimental conditions. The rate constants observed were 1.723 and 444.4 L. mole^{-1} min.$^{-1}$ at 15° and pH 4.6 and 8.0, respectively.

3) The rate constant of the thiamine formed from TPD and propylmercaptan which was the largest side reaction among the various possibilities, was 0.607 L. mole^{-1} min.$^{-1}$ at pH 4.6 and 15° as a second order reaction.

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The merits of thiamine derivatives of disulfide type, *i.e.*, higher and longer lasting blood level and much higher availability estimated from analyses of urinary excretion then the original compound, thiamine, have been reported by many investigators.[1] However, the mechanism as to why the compounds show the favorable effects has not been discussed in detail. Since the formation of free thiamine in the blood stream following the administration of the compounds has been reported, it is reasonable to consider that the scission of the sulfur-sulfur bond in the animal body would be important to the development of the thiamine effect of the compounds. In previous reports of this series,*2 the kinetic study of drug absorption *in vitro* using rat intestine was carried out, the conversion mechanism to thiamine discussed, and the conclusion reached that the exchange reaction between disulfide and thiol was the first step of the conversion. Therefore, the kinetic study between thiamine propyl disulfide (TPD) and L-cysteine, as an example of the most common SH derivative in the body, was planned with the view to understanding the exchange reaction in the animal body.

The following reactions, (A) and (B), may be considered as the possible exchange reaction that takes place between unsymmetrical disulfide and thiol.

$$\text{R-SS-R}' + \text{R}''\text{S}^- \longrightarrow \text{R-SS-R}'' + \text{R}'\text{S}^- \qquad \text{(A)}$$

$$\text{R-SS-R}' + \text{R}''\text{S}^- \longrightarrow \text{R}'\text{-SS-R}'' + \text{RS}^- \qquad \text{(B)}$$

Matsukawa and Yurugi[2] have reported that in terms of synthetic chemistry, the reaction between TPD and L-cysteine may be shown by Eq. (1), which is also used for the determination of thiamine derivatives of the disulfide type.[3] The same reaction

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*2 Part III. This Bulletin, in press.

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1) T. Matsukawa, *et al.* : Ann. Repts. Takeda Research Lab., **12**, 1 (1952).

2) T. Matsukawa, S. Yurugi : Yakugaku Zasshi, **74**, 518 (1954).

3) M. Honda : Vitamin, **15**, 628 (1958).

mentioned above was studied by Utsumi, *et al.*[4], and the reversible reaction shown by Eqs. (2) and (3) was reported

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

$$\text{TPD} + \text{Cy-SH} \rightleftarrows \text{B}_1 + \text{Cy-SS-Pr} \quad (2)$$

$$\text{TPD} + \text{Cy-SH} \rightleftarrows \text{Pr-SH} + \text{B}_1\text{-SS-Cy} \quad (3)$$

where B_1 is thiamine, Cy-SH cysteine, Cy-SS-Pr cysteine propyl disulfide, Pr-SH propylmercaptan and B_1-SS-Cy thiamine cysteine disulfide.

The reaction between TPD and L-cysteine would not be as simple as that shown by the equation above, since the reaction would not be limited by the first and second steps of the exchange reaction. For example, the one between cysteine and Cy-SS-Pr or B_1-SS-Cy, which are formed by Eq. (1) or Eq. (3) and the one between TPD and Pr-SH, which is formed by exchange reaction, should be considered dependent on the initial molar ratio of the reactants or the rate constant of the individual reaction step. Moreover, the oxidation reaction to disulfide on cysteine,[5] thiamine,[6,7] and Pr-SH,[8] the decomposition reaction of disulfide by hydroxyl ion,[9] and the equilibrium state between disulfide[10] have been reported. Therefore, the very complex reaction system shown by Chart 1 might be assumed.

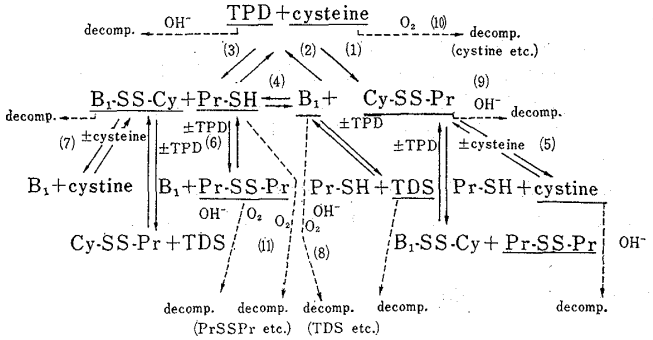

Chart 1.

exchange reaction (——): (-SS- + S-, -SS + -SS-),
decomposition (------).
TDS: thiamine disulfide, PrSSPr: propyldisulfide.

The exchange reaction between disulfide and thiol has been examined by Parker, *et al.*[11] They concluded that the active species was mercaptide ion, and that the rate of reaction was related to the steric circumstance of the compound and to the electronic density of the disulfide. It is considered that the steric hindrance of the thiamine group in TPD is not important in view of the fact that the reaction of thiamine disulfide (TDS) and L-cysteine proceeded quite rapidly as will be reported later in this series of study.[12] Although both sulfur atoms of TPD are electropositive, it would be seen that nucleophilic attack would more likely occur at the sulfur attached to the thiamine which has a lower electronic density.[13]

Moreover Parker demonstrated that the reactions were equilibria, with the positions of equilibria determined by the nucleophilicity toward sulfur of the displaced mercaptide and displacing nucleophiles, and that the cleavage was controlled by the comparative nucleophilicities of the entering group and of the leaving mercaptide group, with the bond-breaking step, rather than the bond-making one, being the critical factor.

4) I. Utsumi, K. Harada, K. Kono: Vitamin, **27**, 86 (1963).
5) E. Chargaff, C. Levine, C. Green: J. Biol. Chem., **175**, 69 (1948).
6) T. Higuch, J. J. Windheuser: J. Pharm. Sci., **51**, 354 (1962).
7) T. Kawasaki, T. Horio: Vitamin, **19**, 48 (1960).
8) J. Xan, E. A. Wilson, L. D. Roberts, N. H. Horton: J. Am. Chem. Soc., **63**, 1139 (1941).
9) A. J. Parker, N. Kharasch: "Chemical Review," **59**, 597,608 (1959).
10) E. R. Bertozzi, F. O. Davis, E. M. Fettes: J. Polymer Sci., **19**, 21 (1956).
11) A. J. Parker, N. Kharasch: J. Am. Chem. Soc., **82**, 3071 (1960).
12) H. Nogami, J. Hasegawa, T. Suzuki, K. Hirata: This Bulletin, in press.
13) Communication from Y. Asahi and E. Mizuta.

The purpose of the present study, therefore, is to investigate the exchange reaction between TPD and L-cysteine based on the assumption that the main reaction is shown by Eq. (1) under the suitable experimental condition where side reactions can be neglected, and to evaluate the magnitude of the reaction shown in Chart 1 and the S-nucleophilicity of thiamine, cysteine and Pr-SH.

Experimental

Materials——TPD : m.p. 128°(decomp.). Thiamine hydrochloride : m.p. 248°. TPD and Thiamine hydrochloride were supplied by the Takeda Chem. Ind., Ltd. Cysteine propyl disulfied : m.p. 205°(decomp.), colorless flakes, synthesized by the method reported by Matsukawa and Yurugi. Propyl disulfied : b.p. 192.5°,[14] colorless oil, propylmercaptan was mixed with equimolar of 30% H_2O_2, allowed to stand for 1 day, extracted by ether, and distilled under reduced pressure after removing the solvent. L-Cysteine : analytical grade, Nihon Rikagaku Co. Cystine : analytical grade, Nihon Rikagaku Co.

Thin-layer Chromatographic Procedure——Qualitative Estimation of the Reactions : The reactants were dissolved in 1 ml. of buffer solutions as shown in Table I, mixed, and allowed to react at 50° for 5 minutes. After adding a few drops 10%-HCl, the reaction mixtures were spotted at the origin of the plate. The plates were prepared with a 250 μ layer of silica gel, Wako Gel B.

Table I. Reactants and Observations upon Reaction with Disulfides and Mercaptants

Reactants	Concentration of reactants		Conditions of reaction mixtures	
	molar ratio	(mg.) : (mg.)	pH 4	pH 8.5
TPD : cysteine	1 : 1	35.6 : 12.1	p.p.t. (white crystals) and smell of mercaptan	p.p.t. (white crystals) and smell of mercaptan
Cy-SS-Pr : B_1HCl	1 : 1	19.5 : 33.7		smell of mercaptan
Cy-SS-Pr : cysteine	1 : 1	19.5 : 12.1	smell of mercaptan	smell of mercaptan
TPD : Pr-SH	1 : 1	35.6 : 7.6	turbidity	turbidity
Pr-SH : cystine	1 : 1	7.6 : 24.0		

Developing Solvents——*N*-HCl aq. sol. : Cysteine and cystine were found closely at Rf 0.9. Water: Cystine and Cy-SS-Pr were found at Rf 0.55. Cysteine and cystine were identified by two dimensional chromatographic procedures using water and *N*-HCl. The Rf value of TPD was lower than thiamine when the development solvent was water, but the reverse was the case when *N*-HCl was used. The spot of propylmercaptan was not recognized by TLC, probably due to the high vapor pressure of the compound.

Detection of Spot——UV-light Detector : (Ōsawa Shigaisen Kogyo Co., UV-LSDP) used for TPD, thiamine, and B_1-SS-Cy. Sodium Dichromate in Sulfuric Acid : (3 g. of $Na_2Cr_2O_7$ in 20 ml. of water and 10 ml. of H_2SO_4) used for TPD, thiamine, cysteine, Cy-SS-Pr and cystine. Iodine Vapor : also used for TPD, thiamine, cysteine, Cy-SS-Pr and cystine. Ninhydrine Reagent : (0.3 g. of ninhydrine was dissolved in 95 ml. MeOH and 5 ml. of collidine was added) used for cysteine, cystine, Cy-SS-Pr and B_1-SS-Cy (in purple) and thiamine (in yellow). Dragendorff Reagent : (Solution A : 17 g. of basic bismuth nitrate and 200 g. of tartaric acid were dissolved in 800 ml. of water. Solution B : 160 g. of KI was dissolved in 400 ml. of water. 50 ml. of equivolume mixture of solution A and solution B, 100 g. of tartaric acid, and 500 ml. of water were mixed) used for TPD, thiamine and B_1-SS-Cy.

Buffer Solution——Acetate buffer solution (pH 4.0) : Dilute mixture of 200 ml. of 0.1*N* Na-acetate, 500 ml. of 0.1*N* AcOH and 4 g. of NaCl with water to make 1 liter. Phosphate buffer solution (pH 7.0): Dissolve 250 ml. of 0.2*M* KH_2PO_4 aq. soln. and 138.2 ml. of 0.2*N* NaOH in water to make 1 liter. Phosphate buffer solution (pH 8.0) : Dilute mixture of 250 ml. of 0.2*M* KH_2PO_4 aq. solu. and 234 ml. of 0.2*N* NaOH aq. soln. with water to make 1 liter. Phosphate-borate buffer solution (pH 8.5) : Mix 68 ml. of 0.2 *M* KH_2PO_4 aq. soln. and 132 ml. of 0.1*M* borax aq. solution.

Determination of Thiamine[15]——Oxidized by 1% BrCN solution and determined O.D. at 368 mμ.

Determination of Cysteine and Propylmercaptan——According to Ellman's procedure,[16] the O.D. of 5-mercapto-2-nitrobenzoic acid at 412 mμ formed by reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)

14) K. Kawahara : Yakugaku Zasshi, **77**, 964 (1957).
15) Y. Kochi, S. Kasahara : Vitamin, **7**, 513 (1953).
16) G.L. Ellman : Arch. Biochem. Biophys., **82**, 70 (1959).

in a phosphate buffer solution (pH 8.0) was determined using a spectrophotometer (Hitach-Parkin Elmer 139). Its log ε was 4.1329 (reported log ε : 4.1335).

Separation of Cysteine and Propylmercaptan——From the test solution acidified by adding 10% HCl, Pr-SH was extracted with a suitable volume of cyclohexane. The extract was caused to react with DTNB phosphate buffer solution by shaking and O.D. of the buffer layer was determined at 412 mμ. The acidified aq. layer was adjusted to pH 8.0, allowed to react with DTNB, and the cysteine amount was determined.

Kinetic Procedure——TPD and L-cysteine were dissolved in buffer solutions which were bubbled with nitrogen to remove oxygen. A suitable aliquot of TPD and of cysteine solutions were mixed at a fixed temperature in nitrogen atmosphere in a concentration varying from 1×10^{-3} to 5×10^{-5} mole/L. Samplings of this mixture were drawn at predetermined intervals, and the quantities of thiamine and cysteine were determined.

Result and Discussion

Qualitative Determination of the Reaction by Thin-Layer Chromatography——The reaction was carried out using TPD and cysteine in equal concentration and the result is given in Fig. 1. The spots of TPD, cysteine, thiamine, Cy-SS-Pr and cystine were detected on thin-layer chromatograms and the smell of mercaptan readily recognized in the reaction mixture of pH 4.0. The formation of B_1-SS-Cy was detected through the forming of a small spot at pH 7.0 and pH 8.5. It may be concluded from the above that the reaction might not be represented solely by Eq. (1), that the course shown by Eq. (3) cannot be ignored even though the existence of a reversible reaction could not be detected from the results.

The existence of side reactions, the ones between a) thiamine and Cy-SS-Pr, b) Cy-SS-Pr and cysteine, c) TPD and Pr-SH, d) Cy-SS-Pr and Pr-SH and e) disulfides formed in the main reaction, were examined by mixing these reactants in a molar ratio

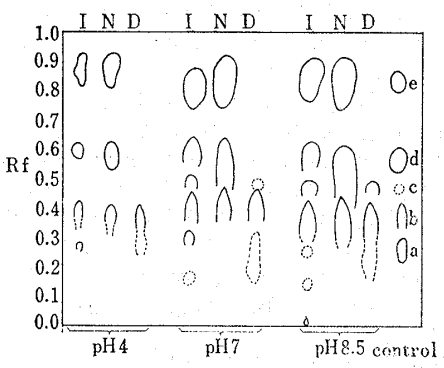

Fig. 1. Composite Representation of Thin-layer Chromatograms on the Reaction Mixture Solution of TPD and Cysteine at pH 4.7 and 8.5

Developing solvent : *N*-HCl aq. soln.
Detecting reagent :
I : iodine vapor;
N : ninhydrine;
D : dragendorff.
Control : a) TPD, b) B_1HCl, c) B_1-SS-Cy, d) Cy-SS-Pr, e) Cysteine, Cystine.

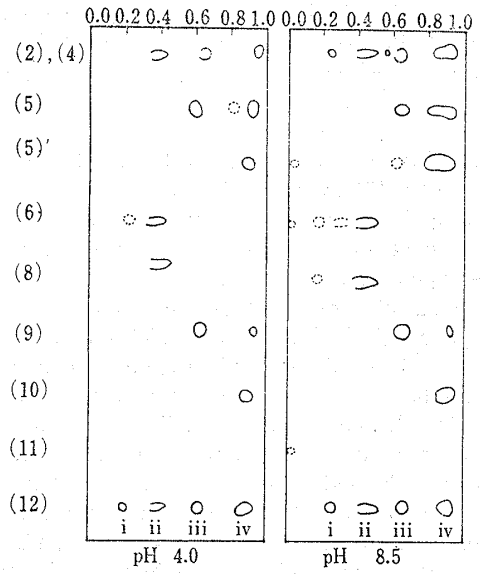

Fig. 2. Composite Representation of Thin-layer Chromatograms on the Reaction Mixture Solutions of Disulfides and Mercaptans at pH 4.0 and 8.5

2), 4) : B_1+Cy-SS-Pr, 5) : Cysteine+CySS-Pr, 5') : Cystine+PrSH, 6): TPD+PrSH, 8) : B_1, 9): Cy-SS-Pr, 10) : Cysteine, 11) : Pr-SH, 12) : Control :
i) TPD, ii) B_1, iii) Cy-SS-Pr, iv) Cysteine, Cystine.
Numbers in the parentheses indicate reactions explained in Chart 1.

of 1:1 at pH 4.0, 7.0, and 8.5. The results are given in the thin-layer chromatogram shown in Fig. 2. The formation of slight quantities of Pr-SH and cysteine from b) and thiamine and propyl disulfide (Pr-SS-Pr) from c) were detected at pH 4.0. Moreover at pH 7.0 and at pH 8.5, the formation of cysteine and Pr-SS-Pr from d) and of a very small amount of Pr-SH, B_1-SS-Cy and cysteine from a) as side reactions were confirmed.

The stability of each reactant and substance formed by the reaction was examined by thin-later chromatography at a pH of 4.0 and of 8.5 with the temperature maintained at 50°. TPD was found to be stable since no increase of thiamine was found after 20 minutes at pH 4.0 and pH 8.5. However, cysteine was found to be not as stable where only one spot was observed within the first 5 minutes but the formation of cysteine was recognized after 20 minutes at pH 8.5 by two dimensional chromatography. Thiamine was not stable since a new small spot was found around Rf 0.15. Cy-SS-Pr was not stable since a new spot was found at Rf 0.9 which reacted positively to ninhydrine reagent. Even where the purity of the Cy-SS-Pr used was supported by melting point and elemental analysis, a sample of the compound obtained from the spot on thin-layerchromatogram developed two spots in subsequent thin-layer chromatogram.

Summarizing the thin-layer chromatographic results, it can be concluded that the main reaction between TPD and L-cysteine was represented by Eq. (1) in Chart 1 but that the contribution of the reactions shown by Eq. (2) to Eq. (11) could not be ignored.

TPD + cysteine	⟸⊖⟹	B_1 + Cy-SS-Pr	(1) (2)
TPD + cysteine	⟶	Pr-SH + B_1-SS-Cy	(3)
Cy-SS-Pr + B_1	⟶	Pr-SH + B_1-SS-Cy	(4)
Cy-SS-Pr + cysteine	⟸⊖⟹	Pr-SH + cystine	(5)
TPD + Pr-SH	⟸⊖	B_1 + Pr-SS-Pr	(6)
B_1-SS-Cy + cysteine	⟸	B_1 + cyseine[12]	(7)
B_1, Cy-SS-Pr, cysteine	⟹	decomp. (2 spots)	(8) (9) (10)
Pr-SH	⟶	decomp. (1 spot)	(11)

Where the arrow sign indicate the magnitude of the reaction rate at pH 8.5, greater reactivities in the order of ⟸, ⟹ and ⟶, and the circles on the arrow show that the same reaction was also observed at pH 4.0.

Quantitative Determination of Main and Side Reactions

The effect of side reactions on the main reaction between TPD and cysteine was examined by observing the quantitative decrease of the each reactant and the corresponding increase of newly found compounds as follows.

At equal TPD and cysteine concentration, the formation of thiamine and the disappearance of cysteine at pH 4.5 and at pH 8.0 were shown in Fig. 3, where the formation of thiamine had the same rate as the disappearance of cysteine. As seen in Fig.4, the second order plot of the thiamine formation from TPD and cysteine formed a straight line up to the point where the reaction was about 90% complete and the rate constant was 444.4 L. $mole^{-1}$ min^{-1} as given in Table II.

Table II. Second Order Rate Constant on the Reaction of TPD and Cysteine at 15°, pH 3.98 and 8.00

pH 3.98	time (min.)	10	15	40	60	100	163	aver.
	k (L. $mole^{-1}$. min^{-1})	0.54	0.57	0.53	0.58	0.53	0.52	0.545
pH 8.00	time (min.)	3	5	7	9	11	16	aver.
	k (L. $mole^{-1}$. min^{-1})	439	448	446	456	444	433	444.4

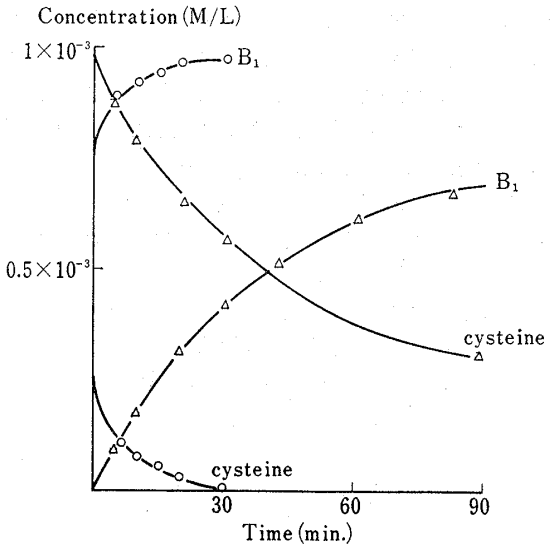

Fig. 3. Reaction of TPD and Cysteine (1:1) at pH 4.5 and 8.0 at 37°
△——△ pH 4.5, ○——○ pH 8.0

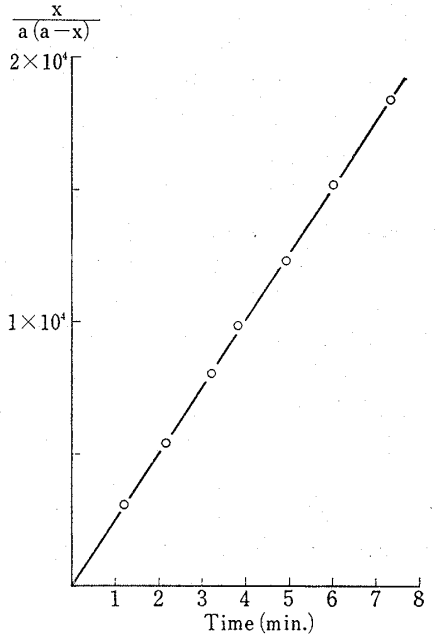

Fig. 4. Second Order Rate Equation Plots on the Reaction of TPD and L-Cysteine (1:1) at 15°, pH 8.0

The second order nature of the reaction was examined in the followign way; *i.e.*, an excess amount of TPD or cysteine was added and the pseudo first order formation of thiamine was observed in the individual experimental condition as given in Table Ⅲ.

Table Ⅲ. Pseudo First Order Rate Constant on the Reaction of TPD and Cysteine at 15°, pH 8.0

$[TPD]_0$ (mole/L.)	$[Cysteine]_0$ (mole/L.)	pH	k (min^{-1})
1×10^{-3}	1×10^{-4}	8.00	0.44
1×10^{-3}	7×10^{-5}	8.05	0.44
1×10^{-3}	5×10^{-5}	8.05	0.46
1×10^{-4}	1×10^{-3}	8.00	0.40
7×10^{-5}	1×10^{-3}	8.00	0.44
5×10^{-5}	1×10^{-3}	7.95	0.45
aver.			0.44

The rate constant was 0.44 min^{-1}, and this was calculated in the second order rate constant, 440 L. $mole^{-1}$ min^{-1}, which agreed with the one obtained for the reaction at equal TPD and cysteine concentration. The order of the reaction was also examined, using the half-life of the reaction according to the Eq. (12)[17]

$$n=1+\frac{\log\{(t_2/t_1)-1\}}{\log\{1/1-y\}} \qquad (12)$$

where n is the order of reaction, and t_1 and t_2 are two successive half-life periods in a single run. The results closely agreed with the second order kinetics.

When TPD and cysteine were allowed to react in a molar ratio of 1:2 at pH 8.0, the recovery of Pr-SH was 16 per cent at the maximum concentration and a gradual

17) A. Frost, R.G. Pearson: "Kinetics and Mechanism," p. 43, New York, London, John Willy & Sons, Inc.

decrease was observed after that. The formation of Pr-SH can be presumed from the Eq. (4) or (5). Since the smaller effect of the side reaction, Eq. (3), on the main reaction was expected, the formation of Pr-SH by Eq. (4) was examined. The cysteine and Cy-SS-Pr were mixed in a molar ratio of 1:1. The recovery of Pr-SH was 13 per cent at 15° and pH 8.0, which was nearly the same as the result mentioned above, and a gradual decrease was also recognized after the peak.

According to the literature on this subject, the rapid oxidation of Pr-SH to disulfide might be expected. As seen in Fig. 5, nearly the same decrease was observed although a deviation greater than the usual kinetic result from a straight line may be seen. This is probably due to the relatively higher vapor pressure of the compound. From the result given in Fig. 5, it may be concluded that the main reaction involved in the decrease in recovery is due to the oxidation of Pr-SH.

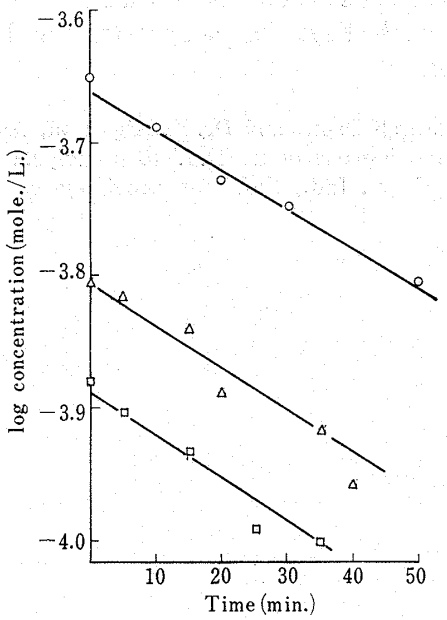

Fig. 5. First Order Decomposition of Propylmercaptan at 37°, pH 8.0

○——○: in phosphate buffer,
△——△: in reaction mixture of TPD and L-cysteine (1:2),
□——□: in reaction mixture of Cy-SS-Pr and L-cysteine (1:1).

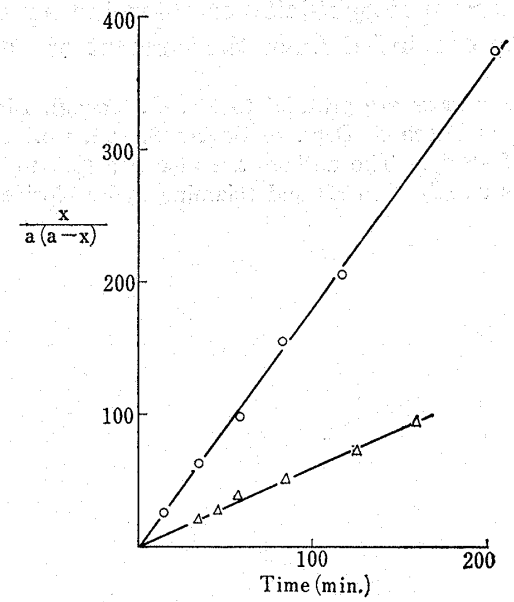

Fig. 6. Second Order Rate Equation Plots on the Reaction of TPD and Mercaptan (1:1) at 15°, pH 4.6

○——○: Cysteine,
△——△: Propylmerceptan.

Since the greatest reactivity among the possible side reactions was expected in the formation of thiamine from Pr-SH and TPD from thin-layer chromatographic results, this reaction was measured to determine the formation of thiamine. The result obtained was a rate constant 0.607 L. mole^{-1} min^{-1} calculated as second order reaction at pH 4.6 and 15° is given in Fig. 6. The thiamine formation from TPD and cysteine, k=1.723 L. mole^{-1} min^{-1} under the same condition, is also given for comparison.

Comparing these rate constants, the reaction between TPD and Pr-SH should not be neglected as a side reaction, assuming that a substantial amount of Pr-SH is formed during the reaction between TPD and cysteine. As mentioned above, however, since a) the formation of thiamine is in close agreement with the second order kinetics throughout the whole process of the reaction, b) the recovery of Pr-SH was 2 per cent at the maximum concentration when reactants were mixed in molar ratio of 1:1, and c) the main reaction in the Pr-SH decrease was thought to be due to the oxidation to

disulfide, it can be concluded that the reaction between TPD and and Pr-SH does not affect the formation of thiamine from TPD and L-cysteine.

The stability of the reactants and the possible compounds formed by the main and side reactions were examined quantitatively under the same conditions as in the qualitative test. The decrease of thiamine and L-cysteine was under 3 percent at pH 4.0 and 8.0. Neither the formation of thiamine from TPD nor the formation of mercaptan from Cy-SS-Pr was observed in appreciable amount.

From the above mentioned results, although some side reactions were observed by thin-layer chromatography, it can be concluded that the effect of side reactions may be ignored as far as the main reaction is concerned where the formation of thiamine from TPD and L-cysteine takes place under the following experimental conditions: *i.e.*, a) in molar ratio 1:1, b) the initial concentration of reactants $1\times10^{-3}\sim5\times10^{-5}$ molar, and c) under the atmosphere of nitrogen. Since the leaving group was thiamine in the exchange reactions between TPD and L-cysteine and between TPD and Pr-SH, the weaker S-nucleophilicity of thiamine as compared with that for L-cysteine or Pr-SH may be concluded from the concept of Parker, *et al.*

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