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Thiamine Derivatives of Disulfide Type. I. Formation of Thiamine from Thiamine Propyl Disulfide in Rat Intestine *in Vitro*[1]

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The conversion of thiamine from thiamine propyl disulfide was studied *in vitro* using rat intestine set at a modified Wiseman and Smyth's apparatus. The following conclusions were drawn from the experimental results obtained.

1) It was found that thiamine propyl disulfide was converted to free thiamine in mucosal solution and that propyl disulfide appeared in intestinal tissues during the conversion.

2) The formation of thiamine in mucosal and serosal solutions followed the first order kinetics apparently and were same magnitude.

3) The conversion may be considered as the coupled reactions between chemical and enzymic systems and the participation of glutathione reductase to this conversion was expected.

Since the discovery of thiamine allyl disulfide (allithiamine) by Fujiwara and Watanabe[3] in 1952, many thiamine derivatives of disulfide type have been synthesized and developed their clinical applications in our country from their merits, *i.e.*, the rapid absorption from intestinal tract, the higher availability estimated by free thiamine excreted in urine, and the longer lasting blood level, *etc.* When the derivatives were given by oral route, although this was the most usual way to administer the compounds, several investigators found free thiamine in blood stream, therefore, it may be considered the reduction of disulfides to thiamine has to be studied for the understanding of the merits mentioned above.

Honda[4] reported that thiamine allyl disulfide (TAD) was converted to thiamine by cysteine, sodium thiosulfite, sodium hydrosulfite, or potassium cyanide. The reduction

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3) M. Fujiwara and H. Watanabe, *Proc. Japan Acad.*, **28**, 156 (1952).

4) M. Honda, *Vitamins* (Kyoto), **15**, 628 (1958).

of TAD in animal tissues was recognized by several workers; *i.e.*, the reduction in liver and kidney,[5-8] in blood,[8-14] in cerebral fluid[15] has been reported.

The reduction of TAD by homogenate of animal stomach or intestine was reported by Itoh, *et al.*[16] and the recovery of free thiamine in intestinal fluid was recognized by several investigators[17-20] in the absorption experiment *in vivo*. The mechanism of the reduction in animal tissues was estimated by Zima, *et al.*[21] that the thiamine disulfide (TDS) was reduced by glutathione in the tissues. The reduction was also studied by Matsukawa, *et al.*[22] who proposed the following mechanisms; the reduction by a) the thiol compounds of small molecular weight, b) thiol group in protein molecule, and c) the oxidation–reduction systems coupled with dehydrogenase.

When the non–specific reduction of the drugs to thiamine in animal tissues is not neglected at the oral administration of thiamine derivatives of disulfide type, the investigation of mechanism of reaction may be one of the most interesting topic of the drug absorption from intestinal tract since the relation between the chemical change and drug absorption has not been studied in detail.

It was reported by Suda, *et al.*[23] that the methionine sulfoxide was found in intestinal wall at the absorption study of the methionine and the original form was recovered in the portal circulation. The hydrolysis of chloramphenicol stearate was also reported as the previous stage of absorption of the drug.[24]

It was, therefore, our purpose to study the rate, mechanism, and site of the conversion of thiamine derivatives to thiamine at drug absorption experiment *in vitro*. The rat intestine was selected using the modified Wiseman and Smyth's apparatus and the kinetic studies conducted in mucosal and serosal solution for the elucidation of the reactions.

Experimental

Chemicals——Thiamine propyl disufide (mp 128° (decomp.)) (TPD) and propyl disulfide (bp 75°/14 mmHg) were supplied by Takeda Pharm. Ind., Ltd. TDS (mp 171° (decomp.)) and thiamine·HCl (mp 248° (decomp.)) were products of Tanabe Seiyaku Co., Ltd. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Tokyo Kasei Kogyo Ltd. *p*-Chloromercurybenzoic acid was purchased from Katayama Kagaku Kogyo Ltd. Wakogel B-5 was the product of Wako Pure Chemical Ind., Ltd.

Animals——Male albino rat (Donryu) weighing 200—250 g was used. The animal was fasted for a whole night before experiments and water only was given freely. The animal was anesthesized by the intraperitoneal injection of pentobarbital sodium (6 mg/100 g body weight) and intestine segment was removed. About 45 cm of segment was used for experiment.

5) T. Matsukawa and J. Suzuoki, *Vitamins* (Kyoto), **6**, 285 (1953).
6) H. Watanabe, *Vitamins* (Kyoto), **6**, 121 (1953).
7) S. Uchino, *Vitamins* (Kyoto), **6**, 629 (1953).
8) H. Nanjo, *Vitamins* (Kyoto), **9**, 290 (1955).
9) J. Suzuoki and T. Suzuoki, *J. Biochem.*, **40**, 11 (1953).
10) T. Tamaki, *Vitamins* (Kyoto), **7**, 352 (1954).
11) S. Sasagawa and K. Ikeda, *Vitamins* (Kyoto), **28**, 81 (1963).
12) K. Takenouchi, K. Aso, S. Shimizu, and T. Kobayashi, *Vitamins* (Kyoto), **26**, 257 (1962).
13) Y. Itokawa, *Vitamins* (Kyoto), **28**, 568 (1963).
14) E. Hamamoto, *Vitamins* (Kyoto), **27**, 229 (1963).
15) I. Tsuchimoto, *Vitamins* (Kyoto), **14**, 601 (1958).
16) S. Itoh and S. Miyazaki, *Vitamins* (Kyoto), **7**, 104 (1954).
17) T. Shimizu, H. Teraoka, and Y. Takeuchi, *Vitamins* (Kyoto), **6**, 691 (1953).
18) K. Matsubara, *Vitamins* (Kyoto), **12**, 80 (1957).
19) K. Takenouchi, K. Aso, S. Shimizu, and T. Kobayashi, *Vitamins* (Kyoto), **26**, 261 (1962).
20) Y. Itokawa, *Vitamins* (Kyoto), **28**, 574 (1963).
21) O. Zima, K. Ritsert, and Th. Moll, *Z. Physiol. Chem.*, **267**, 210 (1941).
22) T. Matsukawa, S. Yurugi, H. Kawasaki, Y. Aramaki, and J. Suzuoki, *Ann. Rep. Takeda Res. Lab.*, **12**, 1 (1953).
23) M. Suda, T. Sugawa, and H. Akedo, *J. Biochem.*, **47**, 131 (1960).
24) A.J. Glazko, W. Dill, A. Kazenko, L.M. Wolf, and H.E. Carnes, *Antibiot. Chemotherapy*, **8**, 516 (1958).

Circulation Apparatus——Modified Wiseman and Smyth's circulation apparatus reported by Nogami and Matsuzawa[25] was used.

Thin-Layer Chromatography—Plates were made with Wakogel B-5 and developed at room temperature with ascending method.

Determination of Free Thiamine, TPD, Thiol Group, and Mercuric Ion——Free thiamine was determined according to the method reported by Kochi and Kasahara[26], *i.e.*, thiamine was oxidized to thiochrome with cyanogen bromide and sodium hydroxide and optical density at 368 $m\mu$ was determined with Hitachi-Perkin-Elmer 139 Spectrophotometer. In the case of low concentration, fluorometrical determination was employed. TPD was reduced to free thiamine with 5 mg of cysteine for 60 min at 37° in pH 8.5 buffer solution and determined photometrically. Thiol group was determined with the method of Ellman[27] using DTNB. Mercuric ion was determined with dithizone method described below. Five ml of sample were mixed with 5 ml of dil. H_2SO_4 (1:17), 2 ml of 6 N AcOH, and 10 ml of dithizone solution (10 mg/1000 ml of $CHCl_3$), then shaked for 5 min and optical density of organic layer was measured at 500 $m\mu$.

Results and Discussion

Eighty ml of 1×10^{-3} molar solution of TPD in Tyrode solution and 80 ml of Tyrode solution were perfused for two hours at mucosal and serosal side of rat intestine attached to a modified Wiseman and Smyth's apparatus. The formation of thiamine from TPD in the gut lumen was examined with thin-layer chromatography and the conversion was proved as shown in Fig. 1. The enzyme inhibitor was dissolved in Tyrode solution. Sixty ml of enzyme inhibitor solution was perfused for 10 minutes at mucosal side and 20 ml of 4×10^{-3} molar TPD solution was added to the inhibitor solution. After the perfusion of two hours, the concentration of thiamine and TPD in mucosal solution and the drugs moved to serosal Tyrode solution was determined. The result is given in Table I, where the formation of thiamine was reduced by the addition of the enzyme inhibitors reacting to SH group. The main portion of the drug diffused to serosal side was found as free thiamine.

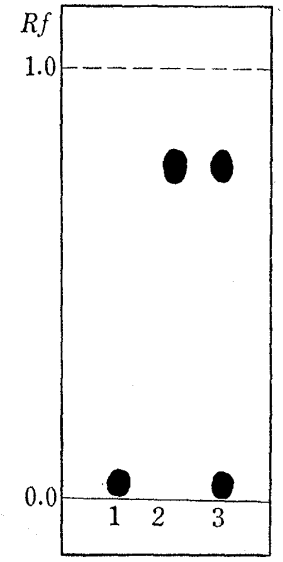

Fig. 1. TLC of TPD Solution circulated in Rat Gut Lumen

1: thiamine
2: TPD
3: circulated solution
developer: MeOH
detecting reagent: $KMnO_4$ soln

When 80 ml of 1×10^{-3} molar TPD in Tyrode solution and 80 ml of Tyrode solution were perfused for 2 hours at serosal and mucosal side, respectively, about 34% of free thiamine calculated from TPD was found in serosal solution. Since the slightly difference was observed on the formation of thiamine from disulfide derivative at the both sides of intestine, the time course of the thiamine formation was conducted adding 0.8×10^{-4} mole of TPD to both side of intestine to diminish the accumulation of drug in the gut wall from mucosal solution. Since the recovery of total thiamine was over 95% in the experiment mentioned above, it may be reasonable to assume that the accumulation of drug in gut wall, the movement of TPD across gut wall, and the conversion of thiamine to cocarboxylase are negligible. The result is given in Fig. 2 where a straight line was observed.

It was concluded that the formation of thiamine was first order reaction at the initial stage of the experiment. The rate constants determined at various initial concentrations of TPD are given in Table II.

The pH dependence of thiamine formation was examined as follows. Each 80 ml of the isotonic buffer solution dissolved TPD in 1×10^{-3} molar and Tyrode solution were used

25) H. Nogami and T. Matsuzawa, *Chem. Pharm. Bull.* (Tokyo), **9**, 523 (1961).
26) Y. Kochi and S. Kasahara, *Vitamins* (Kyoto), **7**, 513 (1954).
27) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

Table I. Effect of Enzyme Inhibitors against the Formation of Thiamine

Inhibitor	Conc. (M)	Mucosal side Free B_1	Serosal side Total B_1	Free B_1/Total B_1
Control		36.6[a] (100)[b]	6.23[a] (100)[b]	99[c]
M.I.A.	5×10^{-2}	0 (0)	2.65 (42)	0
M.I.A.	1×10^{-2}	6.2 (17)	9.34 (149)	31
M.I.A.	1×10^{-3}	8.5 (23)	5.00 (80)	67
P.C.M.B.	1×10^{-3}	6.0 (16)	5.79 (93)	55
$K_3Fe(CN)_6$	1×10^{-3}	25.9 (71)	3.98 (64)	99
Phlorizine	1×10^{-3}	36.4 (99)	7.18 (115)	100

a) in per cent, calculated from the initial amount of TPD
b) relative value, compared to the control
c) in per cent
M.I.A.: monoiodoacetic acid P.C.M.B.: *p*-chloromercury benzoic acid

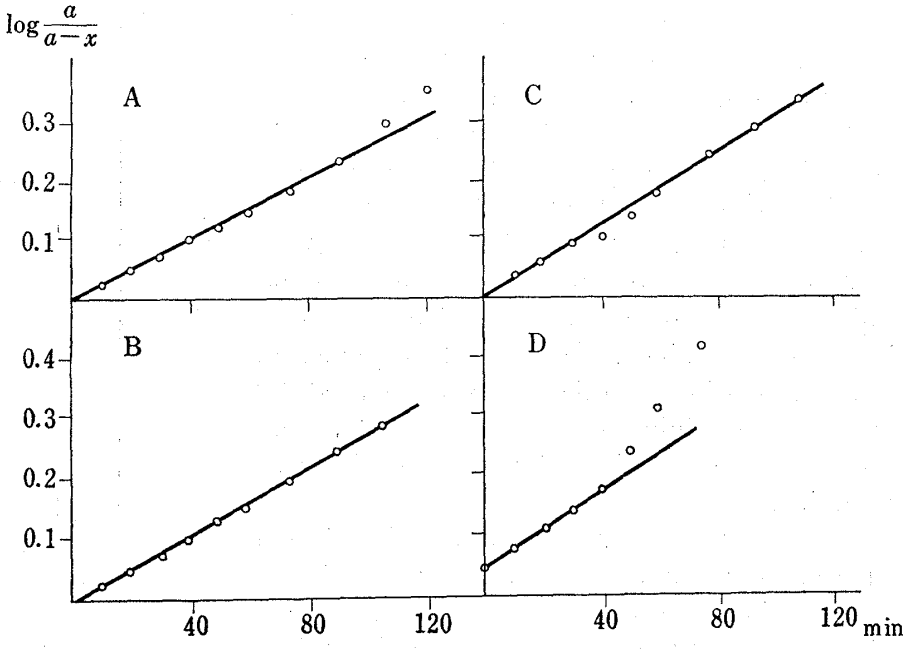

Fig. 2. First Order Plot for Thiamine Formation at Different Initial Concentration

A : 1×10^{-3}M TPD B : 5×10^{-4}M TPD
C : 1×10^{-4}M TPD D : 1×10^{-5}M TPD

Table II. Apparent Rate Constant and Half-Life of Thiamine Formation

Conc. (M)	Rate constant (hr^{-1})	Half-Life (hr)
5×10^{-3}	0.34	2.1
5×10^{-4}	0.35	2.0
1×10^{-4}	0.33	2.1
1×10^{-5}	0.55	1.3

for mucosal and serosal solution and the amount of thiamine formed in mucosal solution was plotted against time for 60 minutes. The pH value was determined after every experiment, the buffer solutions of which initial pH values are over 9.0 showed the change of pH value to neutral region, however, the change was not observed in other buffer solutions. The

result is given in Fig. 3 where the rate constant was calculated from the initial linear portion. The rate of thiamine formation was plotted against pH as seen in Fig. 4, where an apparent pKa value was found around 7.

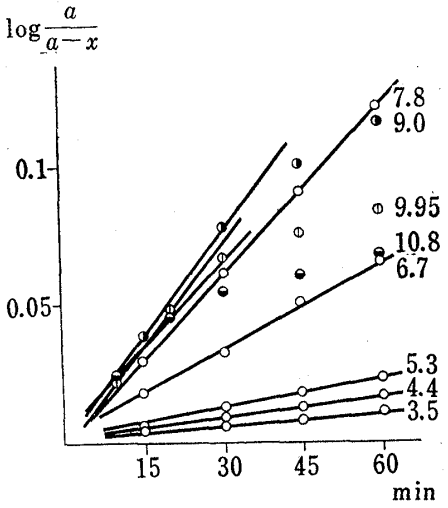

Fig. 3. First Order Plot for Thiamine Formation at Various pH

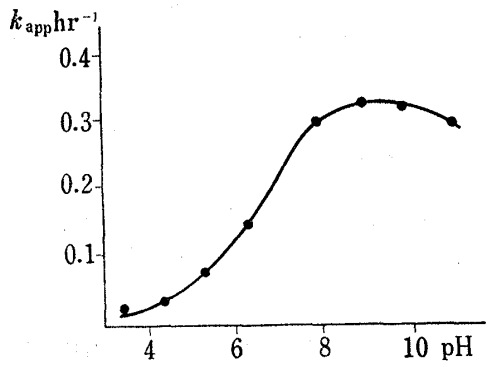

Fig. 4. pH Profile of Apparent Rate Constant (k_{aPP})

As mentioned previously, the formation of thiamine in mucosal solution was about 40% after two hours perfusion and the conversion was restrained by the addition of monoiodoacetic acid or *p*-chloromercurybenzoic acid, which meant the contribution of thiol group for the thiamine formation. The formation of thiamine and S-propylmercapto cysteine from TPD and cysteine was reported by Yurugi.[28] Utsumi, *et al.*[29] found that the reaction between TDS and thiol group in protein molecule was shown by Eq. (1).

$$\text{P-SH} + \text{B}_1\text{SSB}_1 \longrightarrow \text{P-SSB}_1 + \text{B}_1\text{SH} \qquad \text{Eq. (1)}$$

where P-SH is protein thiol group.

It may be considered, therefore, that the main reaction of thiamine formation *in vitro* experiment mentioned before is an exchange reaction as shown in Eq. (2) between TPD and thiol groups in intestinal tissues,

$$\text{G(-SH)} + \text{B}_1\text{SSPr} \longrightarrow \text{G(-SSPr)} + \text{B}_1\text{SH} \qquad \text{Eq. (2)}$$

where G(–SH) is assumed to be thiol groups in intestinal tissues or in the secretion from intestine in mucosal solution. For the examination of the possibilities, the determination of thiol group secreted in Tyrode mucosal solution was carried out. About 2.9×10^{-5} mole per liter of thiol group was found after circulation of 2 hr and the value was negligibly small for the amount of TPD in the experiment. It was concluded, therefore, that the thiol group contributed to the reaction shown in Eq. (2) existed in the gut wall.

It is reasonable to assume from the lipoid theory by Brodie, *et al.*[30] that only the molecular form of TPD penetrates into intestinal wall. Representing K_1 as the dissociation constant of pyrimidine moiety in TPD, (H^+) as the concentration of hydrogen ion, the amount of TPD penetrated per unit time, ds/dt, may be shown by Eq. (3), where k_1 is a constant for proportion and C the concentration of TPD.

28) S. Yurugi, *Yakugaku Zasshi*, **74**, 514 (1954).
29) I. Utsumi, K. Harada, K. Kohno, and H. Hirao, *Vitamins* (Kyoto), **26**, 134 (1962).
30) B.B. Brodie, P.A. Shore, and C.A. Hogben, *J. Pharmacol. Exper. Therap.*, **119**, 361 (1957).

$$\frac{dS}{dt}=k_1\cdot C\cdot\frac{1}{1+\frac{(H^+)}{K_1}} \qquad \text{Eq. (3)}$$

The formation of thiamine depends on ds/dt and G(–SH) from Eq. (2). Put the initial amount of thiol group in gut wall is B_o mole and the residuum is (B_o-X) mole after the reaction of X mole of thiol group. Since the exchange reaction between thiol and disulfide may be considred as an ionic reaction with thiol ion, the amount of thiol ion for the reaction may be represented by Eq. (4), where K_2 is the dissociation constant of thiol moiety in G(–SH).

$$G(S^-)=(B_0-X)\cdot\left(\frac{1}{1+\frac{(H^+)}{K_2}}\right) \qquad \text{Eq. (4)}$$

When the change of volume is neglected, the initial amount of TPD A_0 mole, and thiamine formed X mole, then, Eq. (5) may be written, where k_2 is a constant.

$$\frac{dX}{dt}=k_1\cdot k_2\cdot(A_0-X)\cdot\left(\frac{1}{1+\frac{(H^+)}{K_1}}\right)(B_0-X)\cdot\left(\frac{1}{1+\frac{(H^+)}{K_2}}\right) \qquad \text{Eq. (5)}$$

When the reaction mentioned may occur at the close portion to mucosal fluid, it is reasonable that thiamine exists in mucosal solution from its partition coefficient. Eq. (5) is converted to Eq. (6) when pH is constant.

$$\frac{dX}{dt}=k_3\cdot(A_0-X)\cdot(B_0-X) \qquad \text{Eq. (6)}$$

Eq. (5) is written as Eq. (7) when $B_o-X\fallingdotseq B_o$

$$\frac{dX}{dt}=k_4\cdot(A_0-X)\cdot\left(\frac{K_1K_2}{K_1K_2+K_1(H^+)+K_2(H^+)+(H^+)^2}\right) \qquad \text{Eq. (7)}$$

Further, Eq. (7) is simplified as Eq. (8) when pH is fixed.

$$\frac{dX}{dt}=k_5\cdot(A_0-X) \qquad \text{Eq. (8)}$$

k_3,k_4, and k_5 described above are constants for proportion.

When the assumptions mentioned agree to the experiment, the first order formation of thiamine obtained *in vitro* experiment may be understood from Eq. (8). In this case, the disagreement between $B_o-X\fallingdotseq B_o$ and the amount of thiol group determined by mercuric ion decrease at mucosal side after circulation of mercuric chloride solution for 2 hours, 24.4 μmole at 37°, may be explained as follows.

When the TPD solution was circulated in mucosal side, the formation of thiamine followed the first order kinetics for 90 minutes and the reaction did not saturate after that.

The total amount of thiamine formed within 90 minutes from 0.8×10^{-4} mole of TPD was about 32 μmole which exceeded the thiol group determined in gut wall by mercuric ion. The formation of propyl mercapto derivatives in intestinal tissue and the excretion of propyl mercapto moiety in urine was reported by Takenouchi[12] and Itokawa[20] in their intestinal absorption experiment of TPD labeled on sulphur atom attached to propyl group. From the result, it may be considered that the experimental system includes not only the reaction shown by Eq. (2) but also the one shown by Eq. (9).

$$G(\text{-SSPr}) + G(\text{-SH}) \longrightarrow G(\text{-SS-})G + PrSH \qquad \text{Eq. (9)}$$

$$G(\text{-SS-})G \longrightarrow 2G(\text{-SH}) \qquad \text{Eq. (10)}$$

When the reaction of Eq. (9) and (10) are rapid enough, then we may assume $Bo-X \rightleftharpoons Bo$ and Eq. (8) is realized.

Glutathione may be considered as G(–SH) instead of the thiol group in gut tissue *in vitro* experiment, then, the probable existence of the relation shown by Eq. (8) is understood since the wide distribution of glutathione reductase is recognized generally. The following results would be the support of the hypothesis proposed. The same rate constants were obtained at different initial concentrations of TPD as shown in Fig. 2. Nonlinear tendency observed at higher pH region seen in Fig. 3 may be caused by the pH dependence of glutathione reductase. The formation of thiamine in Tyrode solution was nearly constant within the initial concentration range of TPD from 1×10^{-3} to 1×10^{-4} molar at neutral pH region as seen in Fig. 5. The formation, however, depended on the initial concentration of TPD in isotonic buffer solution of pH 10.8 as shown in Fig. 5, where the second order reaction represented by Eq. (6) was observed.

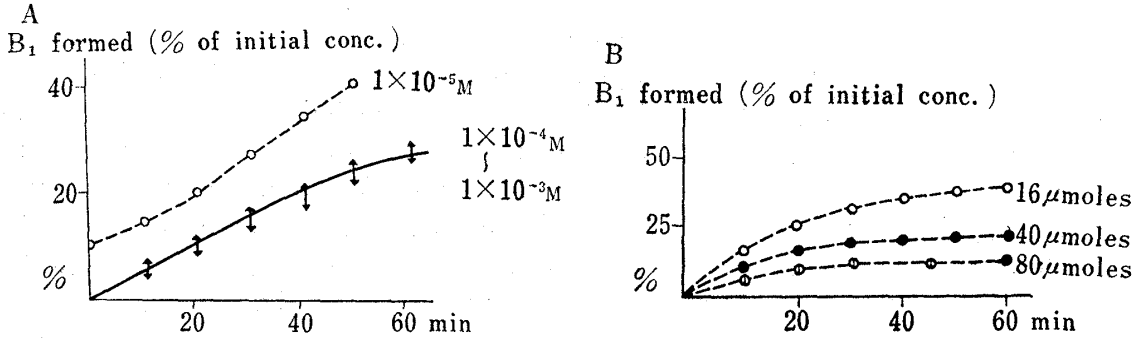

Fig. 5. Time Course of Thiamine Formation in Various Initial Concentration of TPD

A) in Tyrode solution[a] B) in pH 10.8 buffer solution

a) It was assumed that the apparent reduction of TPD at initial time in the case of low concentration was due to the small amount of thiol compounds secreted into gut lumen, but in high concentration, the reduction with these secreted thiols was negligibly small compared with the total amount of TPD.

Assumming 24.4 μmole as *Bo*, the second order plot was made as seen in Fig. 6 and the rate constant calculated was given in Table III where nearly consistency of the value was observed when we may assume the wide variation of the value of *Bo* in experimental condition.

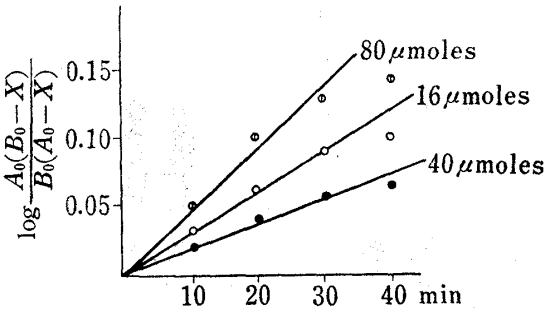

Fig. 6. Second Order Plots of Thiamine Formation in Isotonic Buffer Solution of pH 10.8

A_0: initial amount of TPD (80—16 μmoles)
B_0: initial amount of thiol group (24.4 μmoles)
X: formed thiamine

Table III. Apparent Rate Constant of Thiamine Formation calculated from Equation (6)

Initial conc. of TPD (M)	Rate constant
1×10^{-3}	1.5×10^{-2}
5×10^{-4}	6.9×10^{-2}
2×10^{-4}	5.2×10^{-2}

rate constant: system/μmole, hr

Considering the series of the reaction shown by Eq. (2), (9), and (10), the pH dependence of thiamine formation should follow Eq. (7) and the apparent pK_a should agree to the value of glutathione, however, the result differed from 9.2.[31] The thin-layer chromatogram of

31) R.E. Benesh and R. Benesh, *J. Am. Chem. Soc.*, 77, 5877 (1958).

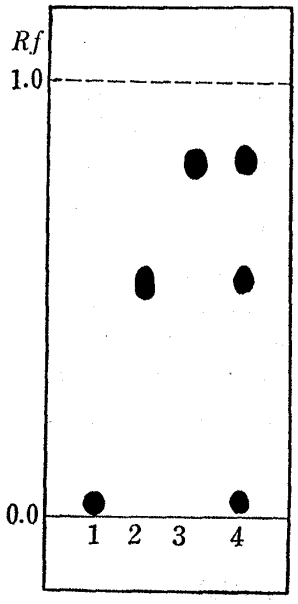

Fig. 7. TLC of TPD Solution, pH 10.8, circulated in Gut Lumen

1: thiamine 2: thiamine disulfide
3: TPD 4: circulated solution
developer: MeOH
detective reagent: $KMnO_4$ soln

the mucosal buffer solution of pH 10.8 after th circulation of TPD is shown in Fig. 7, where the presence of TDS was observed. It may be one of the reason why the value of pK_a observed was different from the one of glutathione since TDS was formed from thiamine[32] converted from TPD and the system could be more complicated.

When Eq. (2), (9), and (10) are assumed for the formation of thiamine, the presence of propyl mercapto derivatives should be proved in other side. The mucosal solution acidified after perfusion was examined by the method of Ellman[27] for propyl mercaptane and by thin-layer chromatography or UV absorption for propyl disulfide, but, the results were negative. It would be reasonable since propyl mercaptane is relatively unstable and oxidized to propyl disulfide[33] which is much lipoid soluble than thaimine. The rat intestine was washed with diluted hydrochloric acid after perfusion, homogenized and extracted with cyclohexane. The presence of propyl disulfide was proved by UV absorption of the organic solvent extracted and thin layer chromatography. The result is given in Fig. 8 and 9.

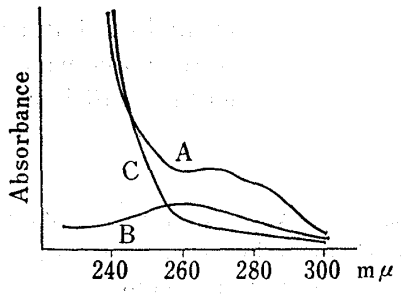

Fig. 8. UV Spectra of Gut Homogenate extracted by Organic Solvent

A) cyclohexane extract of gut homogenate after circulation of TPD
B) cyclohexane extract of propyl disulfide aqueous solution
C) etherial extract of gut homogenate

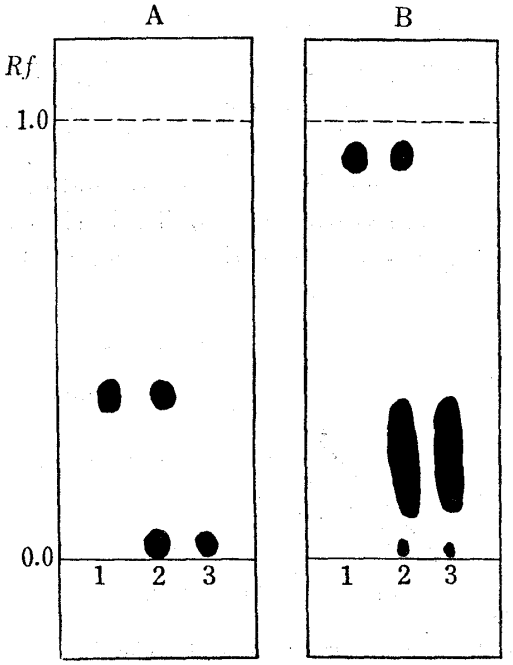

Fig. 9. TLC of Gut Homogenate extracted by Organic Solvent

1: propyl disulfide
2: cyclohexane extract of gut homogenate after circulation of TPD
3: cyclohexane extract of gut homogenate after circulation of Tyrode solution
developer A: *n*-heptane B: *n*-heptane–dioxane (95 : 5)

32) T. Kawasaki and T. Horio, *Vitamins* (Kyoto), **19**, 48 (1960).
33) J. Xan, E.A. Willson, L.D. Roberts, and N.H. Horton, *J. Am. Chem. Soc.*, **63**, 1139 (1941).

Although the quantitative relation is not clear, however, the evidence of propyl disulfide in gut wall would suggest the possibility of the reaction shown in Eq. (9) in the system.

It was concluded from the result obtained that the formation of thiamine in mucosal solution was caused by the coupled system between the chemical reaction and enzymic reaction shown by Eq. (2), (9), and (10).

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