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Pharmacokinetics of Thiamine Derivatives. I.[1] Formation of Thiamine from Thiamine Propyl Disulfide by Erythrocytes of Rat[2]

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The processes of uptake of TPD (thiamine propyl disulfide) and formation of thiamine were investigated in detail, using the suspension of rat erythrocytes, since erythrocytes were most effective to form thiamine from TPD in blood.

1) The ratio of amount of thiamine formed in erythrocytes to that in the suspension medium was 1:1 after incubation of TPD in erythrocytes suspension. This ratio did not change remarkably, even when amount of erythrocytes was increased by 100 fold.

2) It was verified that TPD was converted to thiamine in the membrane under the influence of reducing compounds in erythrocytes and thiamine, which was formed there, went out and into erythrocytes with equal probability.

3) Process of formation of thiamine from TPD by erythrocytes was expressed by second order rate reaction kinetics, and its rate constant was determined.

The thiamine derivatives of disulfide type are rapidly reduced by thiol compounds in various parts of body. The formed thiamine is efficiently utilized in body. The processes of reduction in various parts of body have been investigated by many workers.[4]

Thiamine derivatives of disulfide type are reduced with glutathione (reduced form) or protein thiol in small intestine,[5] and hemoglobin[6] or glutathione[7a] in erythrocytes. The reduction of the disulfide to thiol is followed by decreasing of glutathione (reduced form) in erythrocytes,[7b] that is, the conversion of glutathione reduced form to oxidized form through GSSR.[8] Utsumi, *et al.*[9] reported about the kinetics of reaction of various thiamine derivatives of disulfide type with hemoglobin, plasma albumin and glutathione (reduced form). In comparison with the each rate constant of their reactions, they showed that glutathione would be an important thiol source which reduced thiamine derivatives in erythrocyte.

On the other hand, Nogami, *et al.*[10] investigated the kinetics of the reactions of thiamine derivatives of disulfide type with cysteine and other thiols to elucidate the mechanism of reaction.

1) This paper forms Part XVII of "Studies on Absorption and Excretion of Drug," by H. Nogami; Part XVI: H. Nogami, M. Hanano, S. Awazu and K. Imaoka, *Yakugaku Zasshi*, **90**, 378 (1970).
2) This work was presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969, being taken in part from the thesis of Tohru Fuwa for the degree of Doctor of Pharmaceutical Sciences, University of Tokyo, 1969.
3) Location: *Hongo, Tokyo.*
4) Reviewed in the introduction of previous paper: H. Nogami, J. Hasegawa and K. Noda, *Chem. Pharm. Bull.* (Tokyo), **17**, 219 (1969).
5) H. Nogami, J. Hasegawa and K. Noda, *Chem. Pharm. Bull.* (Tokyo), **17**, 219 (1969); H. Nogami, J. Hasegawa, S. Nakatsuka and K. Noda, *ibid.*, **17**, 228 (1969); H. Nogami, J. Hasegawa and K. Noda, *ibid.*, **17**, 234 (1969).
6) M. Hamada, T. Hayakawa, T. Yamaguchi and M. Koike, *Vitamins* (Kyoto), **35**, 474 (1967).
7) *a*) I. Utsumi, K. Kohno, Y. Kakie and M. Mizobe, *Vitamins* (Kyoto), **35**, 339 (1967); *b*) F. Honda, *ibid.*, **36**, 452 (1967).
8) S-Propylmercapto glutathione.
9) I. Utsumi, K. Kohno, Y. Kakie and M. Mizobe, *Vitamins* (Kyoto), **37**, 264 (1968).
10) H. Nogami, J. Hasegawa and N. Ikari, *Chem. Pharm. Bull.* (Tokyo), **15**, 685 (1967); H. Nogami, J. Hasegawa and N. Ikari, *ibid.*, **15**, 693 (1967); H. Nogami, J. Hasegawa, T. Suzuki and K. Hirata, *ibid.*, **16**, 1273 (1968); H. Nogami, J. Hasegawa and K. Okazaki, *ibid.*, **16**, 1732 (1968); H. Nogami, J. Hasegawa, N. Ikari and K. Takeuchi, *ibid.*, **17**, 1541 (1969).

However, the reaction of disulfides with erythrocytes was so rapid that their kinetics were hardly studied. Moreover, there has been no authentic rule about the processes of the transport of thiamine derivatives into erythrocytes and the formation of thiamine from thiamine derivatives by erythrocytes. Then, the authors studied the kinetics of the formation of thiamine from thiamine propyl disulfide (TPD) by erythrocytes, which is typical compound of thiamine derivative of disulfide type, as a part of pharmacokinetic research for the behavior of it in body.

Experimental

Experimental Procedure——The arterial blood was obtained from carotid arteries of male albino rats (Donryu), not fasted, weighing 250—300 g after their cervixes were dissected under the anaesthesia with ether. It was intactly used for the reaction of TPD in the whole blood.

The preparation of the erythrocytes suspensions was as follows. The both plasma and buffy coat layer were first removed. Erythrocytes were washed three times with 0.9% NaCl solution. Erythrocytes were suspended with Krebs Ringer phosphate buffer solution (pH 7.3) to have the same hematocrit number (*ca.* 43) as the original blood. In various experiments, this erythrocytes suspension was suitably diluted on each time.

Ghosts were prepared as reported by Shindo, *et al.*[11] and text book.[12] The washed erythrocytes were poured into purified water which was cooled in ice-water, and shaked violently. The hemolysate obtained was made isotonic by addition of 4M NaCl solution. Then, ghosts were obtained by repeating mutually several times washing and centrifuging, until the upper layer became colorless. Ghosts obtained as above were suspended with Krebs Ringer phosphate buffer solution just before use.

Formation of Thiamine from TPD in the Whole Blood——500 μg TPD (equivalent to thiamine hydrochloride) were added to 10 ml blood in 50 ml Erlenmeyer's flask with glass stopper, and then it was shaken in the incubator (TAIYO INCUBATOR M-1) at $37\pm0.1°$. At various times later, a certain volume of blood was sampled by microsyringe. It was poured into 2 ml of cooled 10% HPO_3 solution in 10 ml measuring flask to be deproteinized and free thiamine was determined as described later.

Formation of Thiamine from TPD by Blood Components——The each component of blood was diluted 100 fold against the original blood with Krebs Ringer phosphate buffer solution. 50 μg TPD were added to 5 ml blood component suspension and it was shaken in the incubator at $37\pm0.1°$.

Uptake of TPD and the Formation of Thiamine by Erythrocytes——5 ml of suspensions in which erythrocytes were diluted according to the experimental purpose were first taken in twelve pieces of 8 ml polyethylene tube using for the cooling centrifugal separator, and then all tubes were simultaneously kept warm in the incubator at $37\pm0.1°$. Later, same amount of TPD was added into each tube at intervals of the appointed time. After the definite time, all the tubes were simultaneously centrifuged under cool to cease the reaction by precipitation of erythrocytes. The difference between the time of addition of TPD and the time of centrifuging shows the period of reaction. Thiamine and TPD which were contained in the upper phase were separately determined as described later. The amount of thiamine in the upper phase indicates thiamine formed in the medium of erythrocytes suspension, and the difference between the initial amount of TPD and the sum of amounts of both TPD and thiamine remained in the upper phase shows amount of thiamine taken up into erythrocytes.

Formation of Thiamine from TPD by Ghosts——The 100 fold diluted suspension of ghosts was used, and TPD was incubated according to the same procedure as erythrocytes.

Material——TPD was supplied by Takeda Chemical Industries, Ltd.

Determinations of Thiamine and TPD in Various Samples——Determination of Free Thiamine: Free thiamine in whole blood was determined by the modified method of Fujiwara's one.[13] 5 ml of 0.1N H_2SO_4 solution was added at 20 minutes past after deproteinization of blood by 10% HPO_3 as described above. Later 30 minutes, purified water was added up to 10 ml, and then the 8 ml was drawn up in other 10 ml measuring flask. Next, the drawn solution was adjusted to pH 4.5 by adding 0.6 ml of 4M sodium acetate and then acetate buffer solution (pH 4.5) was added up to 10 ml.

Of these 8 ml was poured into permutite column and then thiamine adsorbed was eluted with boiling 25% KCl–0.1N HCl solution till volume of desorbing solution reached to 25 ml. Of these 8 ml was used for determination. 3 ml of BrCN solution and 2 ml of 30% NaOH solution were added in the order into the solution. Thiochrome formed was fluorometrically determined at 360 mμ (exciter wavelength) and 425 mμ (analyzer wavelength) by HITACHI 203 Fluorescence Spectrophotometer after extraction into

11) H. Shindo, K. Okamoto and J. Totsu, *Chem. Pharm. Bull.* (Tokyo), **15**, 295 (1967).
12) T. Onishi, "Seitaimaku Jikken Gijutsu," Nankodo, p. 168.
13) M. Fujiwara, *Vitamins* (Kyoto), **9**, 148 (1955).

20 ml of *n*-butanol. 8 ml of 25% KCl–0.1N HCl containing a definite amount of thiamine was used for the standard. Blank was made by adding 30% NaOH solution and BrCN solution into 8 ml of thiamine solution in the order. Thiochrome was not formed in the blank.

Thiamine amount (c) in 8 ml of 25% KCl–0.1N HCl solution was calculated by Eq. 1.

$$c = S_A \times \frac{U - B_U}{S_A - B_S} \tag{1}$$

where S_A and U were amounts of standard and unknown thiamine respectively, and B_S and B_U were blanks of standard and unknown respectively. Thiamine amount (d) in whole blood taken for determination was calculated by Eq. 2, considering the dilution in determining procedure.

$$d = c \times \frac{25}{8} \times \frac{10}{8} \times \frac{10}{8} = \frac{2500}{512} c \tag{2}$$

Cocarboxylase formation was not found in whole blood by usual method which used diastase.

Determination of free thiamine in erythrocytes suspension and blood component solution were carried out by the similar procedure as described above. But permutite treatment was excluded.

Separatory Determination of Free Thiamine and TPD: The determination of TPD was based on the fission of –SS– bonding of TPD by cysteine at pH 7.0. TPD amount was calculated from the difference between total thiamine and free thiamine. The sum of free thiamine and thiamine formed from TPD was expressed as total thiamine. The reagents and procedures for determination of free thiamine and total thiamine in supernatant of erythrocytes suspension were shown in Chart 1.

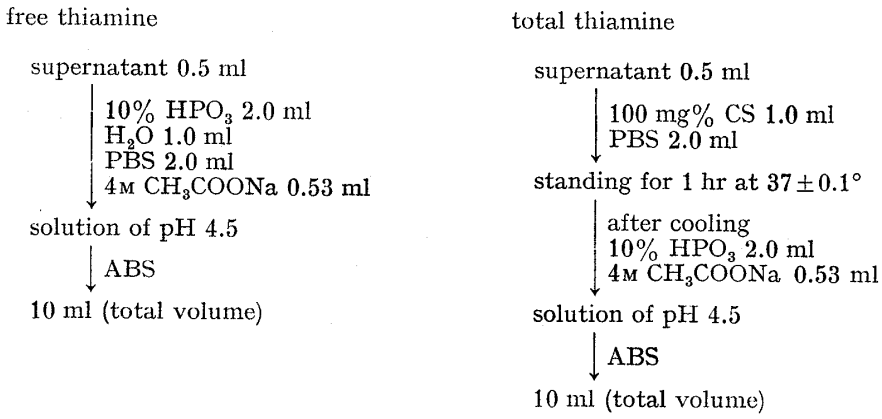

Chart 1. Separatory Determination for Free Thiamine and Thiamine Propyl Disulfide

PBS: phosphate buffer solution (pH 7.0)
ABS: acetate buffer solution (pH 4.5)
CS: cysteine solution

Permutite treatment was excluded in this case. 3 ml of BrCN solution and 2 ml of 30% NaOH were added in the order into 5 ml of solution obtained (pH 4.5) to form thiochrome. 2 ml of 30% NaOH and 3 ml of BrCN solution were added in the order into other 5 ml to obtain blank.

Results and Discussion

Formation of Thiamine from TPD by Blood Components

The whole blood formed thiamine from TPD so rapidly as shown in Fig.1 that diluted blood components were used to know clearly the difference of effeeto of components on the reduction of TPD. Each component was diluted 100 fold with Krebs Ringer phosphate buffer solution (pH 7.3). As a result of the incubation of TPD in diluted blood components, it was found that the erythrocytes was the most effective on the reduction of TPD, but the plasma and ghosts were almost ineffective (Fig. 2). Consequently, erythrocytes must predominant lyparticipate in the reduction of the disulfide. These were consistent with the results reported by Hamada, *et al.*[6] who used the human blood.

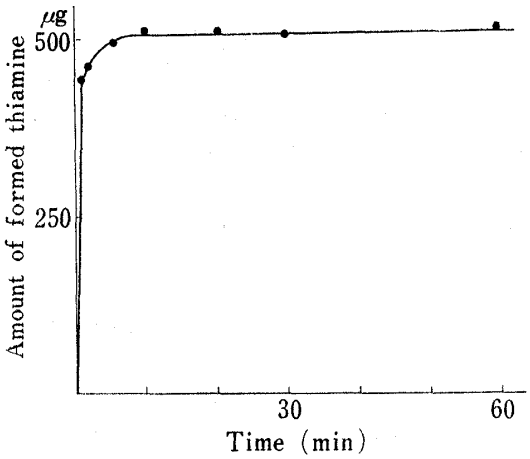

Fig. 1. Formation of Thiamine from Thiamine Propyl Disulfide in Rat Blood at 37±0.1°

initial amount: 500 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)
initial blood volume: 10 ml

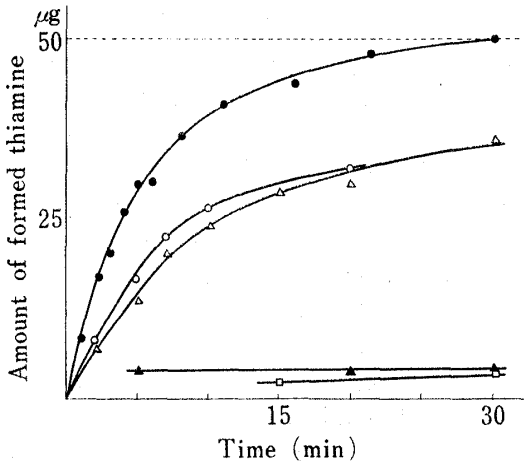

Fig. 2. Formation of Thiamine from Thiamine Propyl Disulfide by Blood Components Diluted 100 Fold with Krebs Ringer Phosphate Buffer Solution (pH 7.3) at 37±0.1°

initial amount : 50 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)
initial volume: 5 ml
—●—: erythrocyte —○—: hemolysate+ghost
—△—: hemolysate —▲—: plasma
—□—: ghost

Uptake of TPD and Formation of Thiamine by Erythrocytes

Since it was found that TPD was mainly converted to thiamine by erythrocytes as shown above, this process was studied kinetically. TPD was incubated in the 100 fold diluted ery-

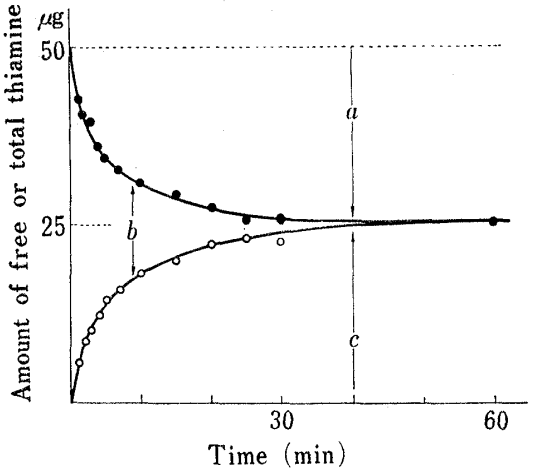

Fig. 3. Uptake of Thiamine Propyl Disulfide and Formation of Thiamine by Erythrocytes in 100 Fold Diluted Erythrocytes Suspension at 37±0.1°

initial amount: 50 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)
initial volume: 5 ml
a: amount of thiamine taken up by erythrocytes
b: amount of thiamine propyl disulfide remained in the medium
c: amount of thiamine formed in the medium
—●—: total thiamine (thiamine+thiamine propyl disulfide) remained in the medium
—○—: free thiamine formed in the medium

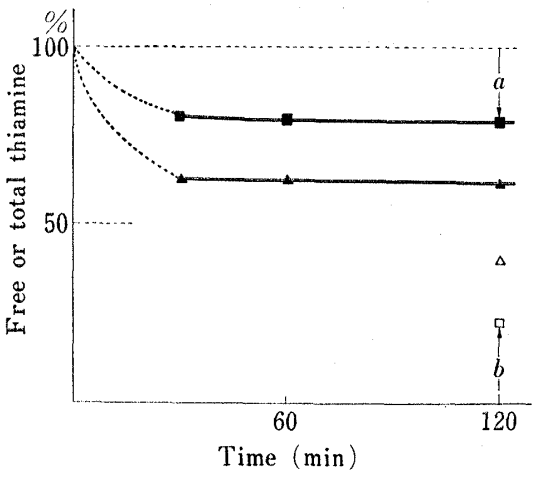

Fig. 4. Uptake of Thiamine Propyl Disulfide and Formation of Thiamine by Erythrocytes in 100 Fold Diluted Erythrocytes Suspension at 37±0.1° in Various Initial Amount of Thiamine Propyl Disulfide

initial volume: 5 ml
a: percentage of thiamine taken up by erythrocytes
b: percentage of thiamine formed in the medium
—■—, —□— } 250 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)
—▲—, —△— } 100 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)

throcytes. The typical example was shown in Fig. 3, where 50 μg TPD was incubated in 5 ml erythrocytes suspension. The upper curve in the figure shows the time course of the total amounts of both TPD remained and thiamine formed in the suspension medium, and the lower curve shows the time course of the formation of thiamine in it. About thirty minutes later, TPD was almost completely converted to thiamine. And it was found that half amount of thiamine formed was in the medium and the other half was in erythrocytes.

When the incubated amount of TPD was increased, TPD remained unchanged (Fig. 4). Namely, the reduction of the disulfide by erythrocytes was saturated beyond the certain amount of it. But even in this case, it was found that the ratio of the amount of formed thiamine was also 1:1 between in the medium and in erythrocytes.

The relationship between amount of thiamine formed at 120 minutes and initial amount of TPD was shown in Fig. 5. The asymptotic values of two curves give the maximum amounts of thiamine formed in the medium and erythrocytes respectively. The sum of two amounts obtained above was considered to be the apparent amount of the reducing compounds in erythrocytes. The amount of it was 115.5 μg, *i.e.* the sum of 55.0 μg (in erythrocytes) and 60.5 μg (in the medium) in terms of the amount of thiamine hydrochloride. This meant that 1 ml whole blood could contain 2310 μg reducing compounds in terms of the amount of thiamine hydrochloride. They can be considered to be glutathione-like thiols as described in the introduction.

When the amount of erythrocytes in the suspension was increased up to the same amount as the intact blood, that is, 50 μg TPD were incubated in the suspension containing the same amount of erythrocytes as the intact blood (hematocrit number, *ca.* 43), the ratio of amount of thiamine formed in erythrocytes to that in the medium increased to about 3:2. Thus, the ratio changed very slightly, though the amount of erythrocytes was increased 100 fold. This could not be explained by the simple partition between erythrocytes and the medium, because the partition mechanism predicts remarkable change of ratio when the amount of erythrocytes was increased 100 fold.

Thiamine in the medium cannot be caused by passing of thiamine out of erythrocytes through the membrane, since the membrane of erythrocyte is almost impermeable to thi-

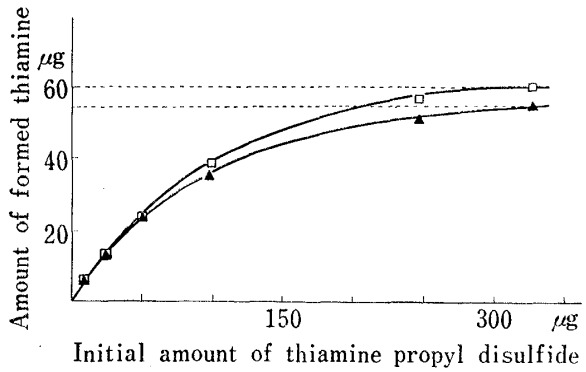

Fig. 5. Tendency of Saturation of Uptake and Formation of Thiamine by Erythrocytes in 100 Fold Diluted Erythrocytes Suspension at 37±0.1°

initial volume: 5 ml
—□—: thiamine formed in the medium
—▲—: thiamine taken up by erythrocytes

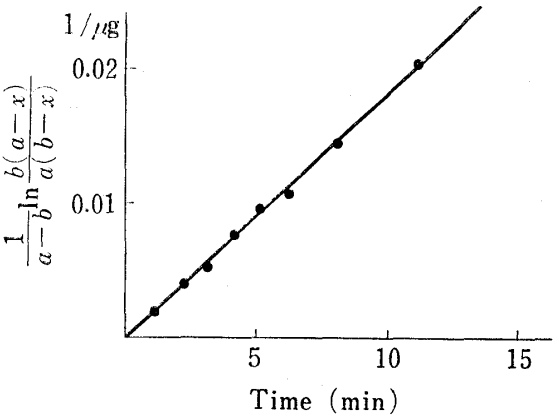

Fig. 6. Second Order Plot[a] of Formation of Thiamine from Thiamine Propyl Disulfide by Erythrocytes

dilution ratio of erythrocytes: 1/100 (a=115.5 μg)
initial amount: 50 μg thiamine propyl disulfide (equivalent to thiamine hydrochloride)
initial volume: 5 ml
a: initial amount of thiol compound in erythrocytes
b: initial amount of thiamine propyl disulfide (equivalent to thiamine hydrochloride)
x: amount of thiamine formed at time t
a) Plots until 11 minutes obeyed second order rate equation.

amine.[14] Furthermore, thiamine cannot be formed from TPD in the medium, since ghosts and washings of erythrocytes are hardly able to form thiamine.

Consequently, it will be assumed that lipophilic TPD is liable to stay at lipoid portion of the membrane after uptake, until the disulfide is reduced to thiamine there by thiol compounds in erythrocytes. Thiamine formed in the membrane rapidly goes out or into erythrocytes from the lipoid portion with probabilities P_1 and P_2, since thiamine is lipophobic.

Thus, the process of the formation of thiamine by erythrocytes was devided into that of chemical reaction in the membrane and that of the rapid movement of the formed thiamine. If P_1 is nearly equal to P_2, the ratio of the amount of thiamine in erythrocytes to that in the medium will be nearly 1:1 in all the cases, that is, in varying the amount of erythrocytes, in starting from the different amount of TPD, and even in the case of saturation of TPD. The proposed scheme is expressed by Chart 2.

In Chart 2, k is the reaction rate constant in forming thiamine, and R–SH and VB_1 express reducing compounds in erythrocytes and the whole thiamine formed by erythrocytes respectively. VB_1 in and VB_1 out are thiamine formed in erythrocytes and the medium respectively. It was assumed that thiamine was formed by the reaction of TPD and thiol compounds in the membrane of erythrocyte and later, the thiamine was rapidly released out of membrane with probabilities P_1 and P_2.

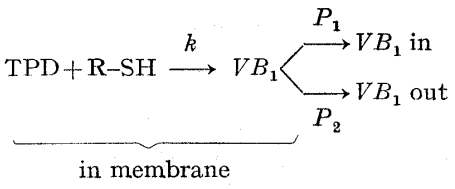

Chart 2. Model of Formation of Thiamine from Thiamine Propyl Disulfide by Erythrocyte

TPD: thiamine propyl disulfide
R-SH: thiol compounds
VB_1: thiamine
VB_1 in : thiamine in erythrocyte
VB_1 out: thiamine in the medium

Experimentally, amounts of VB_1 in and VB_1 out are separately obtained as shown in Fig. 3. Amount of VB_1 is the sum of both amounts of VB_1 in and VB_1 out. The time course of the formation of VB_1 was shown in Fig. 2. This process was apparently expressed by second order reaction as follows.

$$k_{obs}t = \frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)} \qquad (3)$$

where k_{obs} is observed second order rate constant, a and b are amounts of the apparent thiol compounds as obtained above and TPD at time 0 respectively, and then $(a-x)$ and $(b-x)$ are amounts of thiol compounds and TPD at time t respectively. When $a=115.5$ μg (equivalent to thiamine hydrochloride) and $b=50.0$ μg (in the case of 5 ml erythrocytes suspen-

Table I. Second Order Rate Constants of Formation of Thiamine from Thiamine Propyl Disulfide in Erythrocytes Suspension Diluted with Krebs Ringer Phosphate Buffer Solution (pH 7.3)

Dilution ratio of erythrocytes	a (μg)	b (μg)	k_{obs} (1/μg/min)
1/100	115.5	50.0	1.91×10^{-3}
1/100	115.5	10.0	2.18×10^{-3}
1/500	23.0	10.0	2.12×10^{-3}

a: initial amount of thiol compound in erythrocytes
b: initial amount of thiamine propyl disulfide (equivalent to thiamine hydrochloride)
k_{obs}: second order rate constant

14) T. Matsukawa, S. Yurugi, H. Kawasaki, Y. Aramaki and J. Suzuoki, *Ann. Rep. Takeda Res. Lab.*, 12, 1 (1953).

sion diluted 100 fold), the plotting of Eq. 3 gave a straight line and k_{obs} was obtained (Fig. 6).

In other conditions, that is, a=115.5 μg and b=10.0 μg, or a=23.1 μg (the erythrocytes suspension diluted 500 fold) and b=10.0 μg, second order rate plots gave the good straight lines and each k_{obs} was calculated from Eq. 3. The formation of thiamine in the erythrocytes suspension was clearly verified to be the second order rate process, since the values of every k_{obs} were similar to each other as seen in Table I.

If the formation of thiamine is rate-determining process and the formed thiamine is rapidly released out of membrane in the scheme as shown in Chart 2, Eq. 4 and Eq. 5 express the rates of the formation of thiamine in erythrocytes and in the medium respectively.

$$\frac{d(VB_1\ \text{in})}{dt}=k_1(\text{TPD})(\text{SH}) \tag{4}$$

$$\frac{d(VB_1\ \text{out})}{dt}=k_2(\text{TPD})(\text{SH}) \tag{5}$$

where (VB_1 in), (VB_1 out), (TPD) and (SH) are amounts of thiamine in erythrocytes, thiamine in the medium, TPD and virtual thiol compounds in erythrocytes respectively, and then k_1 and k_2 are the rate constants.

Since k_1 and k_2 are equal to P_1k and P_2k respectively, the following equation is given.

$$k_1+k_2=P_1k+P_2k=k \tag{6}$$

If the ratio of $d(VB_1\text{in})/dt$ to $d(VB_1\text{out})/dt$ is derived from Eq. 4 and Eq. 5, Eq. 7 is obtained.

$$\frac{d(VB_1\text{in})}{d(VB_1\text{out})}=\frac{k_1dt}{k_2dt} \tag{7}$$

The integration of Eq. 7, accordingly, gives Eq. 8.

$$\frac{(VB_1\text{in})}{(VB_1\text{out})}=\frac{k_1}{k_2}=\frac{P_1k}{P_2k}=\frac{P_1}{P_2} \tag{8}$$

If $(VB_1\text{in})/(VB_1\text{out})$ is 1:1, P_1/P_2 becomes 1:1. Since in all the experiments $(VB_1\text{in})/(VB_1\text{out})$ was 1:1, thiamine formed in membrane went out and into erythrocyte with equal probability. However, k, the second order rate constant of the reaction in the membrane cannot be obtained from k_{obs}, since volume of the lipoid portion in the membrane and partition coefficient of TPD between the membrane and the medium of erythrocytes suspension are unknown.

In regard to the mechanism of the formation of thiamine in the medium, Hamada, *et al.*[6] reported that it occurred by hemoglobin's thiol through the membrane of erythrocyte, and Utsumi, *et al.*[15] reported that propylmercaptane which formed from TPD in erythrocytes permeated out to the medium and then reduced TPD which remained in there.

However, the present study concluded that the membrane of erythrocyte hardly affected the formation of thiamine from TPD, since the reactivity of the ghost with TPD was low in the case of the suspension diluted 100 fold as seen in Fig. 2. Moreover, TPD did not form thiamine in the supernatant obtained from 100 fold diluted erythrocytes suspension in which TPD was incubated in advance, when the similar experiment as Utsumi, *et al.* was carried out. The mechanism which were proposed by Utsumi, *et al.* was applicable to the experiment using the suspension which contained much more erythrocytes (*ca.* twenty five times), but it was not applicable to the case of the 100 fold diluted suspension. It was ascribed to thiols which came from erythrocytes components that thiamine was formed from TPD in the

15) I. Utsumi, K. Kohno, I. Saito and M. Mizobe, *Vitamins* (Kyoto), 37, 257 (1968).

membrane.

The rate of formation of thiamine from TPD by the propylmercaptane will be slow, since the amount of mercaptane in the medium is much less than the amount of thiols in erythrocytes. Furthermore, if it is considered that propylmercaptane permeated out from erythrocytes form thiamine from TPD in the medium, over-all reaction of TPD with erythrocytes is not probably expressed by second order rate process at initial time of reaction. It is also difficult to explain that this mechanism causes to form thiamine from TPD in erythrocytes and the medium at ratio of 1:1.

In contrast to that, assuming that TPD is converted to thiamine by thiol compounds existent in erythrocytes in the middle of the membrane, all the results can be clearly explained.

Reduced form of glutathione is converted to its oxidized form, as the thiamine derivatives of disulfide type are reduced to thiamine in erythrocytes and reduced form of glutathione is restored to the former state by the addition of glucose.[7b] The restoration of glutathione (reduced form) could be ignored in present study, since glucose was not added. Accordingly, the process of formation of thiamine from TPD by erythrocytes was treated by second order rate process, since glutathione (reduced form) decreased as the reaction proceeded.

The results as above can be applicable to the elimination of TPD from blood after intravenous injection of TPD.

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