Therefore, in the Compound Ⅲ, the equilibrium is not simply between the *cis*-thioenol and the thioxo forms, and the *trans*-thioenol form should be taken into consideration. The difference in the tautomerism is considered to reveal the difference in the chelating property as reported previously,*1 between the Compound Ⅱ and Ⅲ.

As previously reported in nuclear magnetic resonance spectrum of Compound I,[1] the signals corresponding to the thioxo form were not observed. In nuclear magnetic resonance spectra, the mercapto group was presumed to be hydrogen bonded with the carbonyl group intramolecularly, because the signal ($\tau=2.02$) based on the mercapto group was not influenced by the dilution. This result also supports the above mentioned conclusions obtained from the investigation by the ultraviolet spectroscopy.

The authors extend their gratitude to Prof. T. Uno of Kyoto University for his helpful advices. They are also indebted to Dr. T. Shingu of Kyoto University for the measurement of the nuclear magnetic resonance spectra.

[Chem. Pharm. Bull. 15(3) 295~302 (1967)] UDC 615.739-03 : 577. 16B

36. Hideyo Shindo, Koichi Okamoto, and Jun-ichi Totsu : Transport of Organic Compounds through Biological Membranes. I. Accumulative Uptake of S-Benzoylthiamine by Human Erythrocytes.

(*Central Research Laboratories, Sankyo Co., Ltd.**1)

The accumulative uptake of S-benzoylthiamine (SBT) by human red cells was demonstrated and the detail of the transport mechanism was investigated. From the studies on i) the transport of SBT into the red cells and into the ghosts, ii) the decomposition of SBT in the red cell homogenate and in an aqueous medium, iii) the effect of the concentration gradient on the uptake, and iv) the effect of the extracellular pH on the uptake, it was concluded that the penetration of SBT into the red cells proceeds through two steps; the first, a passive diffusion of SBT through the cell membrane, and the second, a rapid decomposition of SBT to undiffusible thiamine and the resultant its accumulation in the cell. In a series of substituted S-benzoylthiamines, the rate of their decomposition to thiamine in the red cell was found to be a predominant factor determining the rate of the penetration into the red cells, both being a linear function to Hammett's σ-constants of the substituents.

An important role of SBT in the intestinal absorption of S-benzoylthiamine monophosphate and the followed high blood thiamine concentration and its duration was pointed out.

(Received March 25, 1966)

In this laboratories, it has been found[1] that S-benzoylthiamine monophosphate (BTMP),*2 I, is much more readily absorbed from intestine than thiamine, and the oral administration results in higher thiamine and co-carboxylase levels in blood and in organs; moreover these levels last for a longer period of time. It has been well

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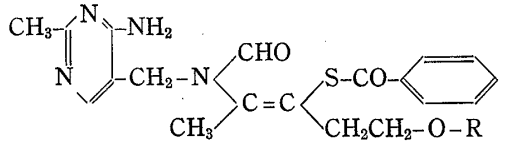
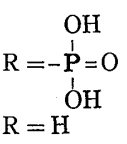
*2 The following abbreviations will be used in this paper. BTMP : S-Benzoylthiamine monophosphate, SBT : S-Benzoylthiamine, OBT : O-Benzoylthiamine, TMP : Thiamine monophosphate, TDP : Thiamine diphosphate.

1) T. Wada, H. Takagi, H. Minakami, *et al.* : Science, **134**, 195 (1961); T. Wada, H. Takagi, S. Miyazawa, *et al.* : Vitamins (Japan), **22**, 342 (1961).

established,[2] recently, that thiamine is absorbed from intestine in only a limited amount. As a probable mechanism of the absorption of BTMP, it has been suggested[3] that after hydrolysis of BTMP by phosphatase at a mucosal surface, the afforded S-benzoylthiamine (SBT), Ⅱ, might be transported through intestine into blood stream.





As is well accepted,[4] intestinal boundary is lipoidal in nature and red cell membrane, one of the typical lipoid-rich boundary, seems to be a convenient model for the epithelial boundary of intestine. On the other hand, a high blood thiamine concentration and its duration on the administration of BTMP seems to suggest that there might be some specific mechanism for accumulation of the thiamine compounds in red cells. In the present investigations, the red cells have been employed for the two purposes: the one, as a possible model for the intestinal boundary, and the other, to find any possibility of their role in the storage of thiamine compounds. In the present paper, an accumulative uptake of SBT by human erythrocytes is demonstrated and the detail of the transport mechanism is investigated.

Experimental

Human red cells were obtained from blood stored for less than two weeks at 4° in acid-citrate-dextrose solution. Whole blood was centrifuged, the plasma and buffy layer were removed, and the packed cells were washed three times with isotonic NaCl-phosphate buffer of pH, 7.4. The proportion of the cells in the suspension was estimated by way of determining the Hematocrit value.

Transport experiments were performed usually in the following way. The packed red cells were suspended in the isotonic phosphate buffer containing a given amount of SBT or other thiamine derivatives to approximately 40% and the suspension was incubated at 37° under a constant shaking.*3 At an appropriate interval, 5 ml. of the suspension was pipetted out, cooled and centrifuged at 4°. Two milliliter of the supernatant was carefully pipetted out and the "cell part" including approximately 1 ml. of the supernatant was hemolysed with 5 ml. of $N/5$ hydrochloric acid. The protein was precipitated with 3 ml. of 15% trichloroacetic acid, the mixture was centrifuged and the precipitates were extracted again with 8 ml. of 5% trichloroacetic acid. The combined extracts were shaken with two successive 20 ml. portions of cold ether to remove the trichloroacetic acid and lipids. The final solution and the two milliliter aliquot of the supernatant, which was, when necessary, also deproteinized in the same way as above, were diluted to an appropriate amount and were assayed for "free thiamine," total thiamine and, if necessary, OBT.

The concentrations of thiamines in the medium (C_m) were obtained directly from the analysis of the supernatant. Those in the cell (C_c) could be calculated according to the following equation, from the concentrations of thiamines in the "cell part" (C_c') and the Hematocrit value of the suspension (Ht).

$$C_c = \frac{C_c' \times 3 - \left(3 - \frac{5}{100} Ht\right) C_m}{\frac{5}{100} Ht}$$

The permeability was expressed in terms of the cell-to-medium concentration ratio at an equilibrium state or after a constant incubation period.

The red cell ghosts were prepared by a modified method of the Hoffman's.[5] One volume of the washed packed red cells was rapidly injected into ten volumes of distilled water at 4° under vigorous stirring.

*3 Extremely slow rotation of 100 strokes in the first 1 hour could suppress the hemolysis below 1.0% during 6 hours of incubation.

2) J. A. Campbell, A. B. Morrison: Am. J. Clin. Nutr., **12**, 162 (1963).
3) M. Yamazaki, T. Wada: The 13th Meeting of Japan Vitamin Society (1961). Abstract: Vitamins (Japan), **23**, 285 (1961).
4) L. S. Schanker: "Advances in Drug Research," Vol. 1, pp. 71 (1964). Academic Press, New York.
5) J. F. Hoffman: J. Gen. Physiol., **42**, 9 (1958); **45**, 837 (1962).

The mixture was kept at 4° for 30 minutes under mild stirring and then an appropriate amount of 4.0*M* NaCl solution was added under vigorous stirring to make the total solution isotonic. After 5 minutes, the ghosts were isolated by centrifugation and washed three times with isotonic NaCl–phosphate buffer of pH, 7.4. The percent hemolysis was 87.70±0.48 (S. E.) % for five different experiments. Hemoglobin was determined from the optical density at 540 mμ and the 100% hemolysis was estimated from the hemolysate of the same blood in 500 volumes of water. The volume of the ghosts was within ±10% of the original intact cells and the Hematocrit value under 60% was confirmed to be a linear measure of the cell number. The transport experiments were performed in the same manner as in the case of the intact cells.

Thiamine was fluorometrically measured by the thiochrome method.[6] Samples were oxidized with bromocyanide at pH 4.5, and the solution was made strongly alkaline by adding sodium hydroxide to afford thiochrome which was extracted with *n*-butanol. Both thiamine and OBT are estimated together as "free thiamine" in this method and each of them were roughly estimated by the method reported by Fujiwara, *et al.*[7] The principle is that the oxidized mixture was made weakly alkaline with ammonium hydroxide to form O-benzoylthiochrome which can be extracted selectively by ethylacetate. Total thiamine was measured by the thiochrome method after an incubation of a sample with a large excess of Takadiastase at 37° and at pH 5.6 overnight.

The organic solvent/water partition coefficients were calculated from the distribution of the compound after shaking a solution in phosphate buffer with chloroform or ethylacetate at 37°. Ionic character of a compound was estimated from the pKa values of the compound by the total sum of the degree of ionization of all ionizable groups in the molecule.

Results and Discussion

Permeability of Red Cell Membrane to Thiamine Derivatives

The relative rates of the penetration of BTMP and its possible metabolites; SBT, OBT, thiamine, TMP, and TDP, into the cell were determined by measuring the uptake by the cells after incubation of red cell and ghost suspensions containing 0.3 μ*M* / ml. of the thiamine derivatives at 37° for 1 hour.*4 The results are shown in Table I with some of the physical properties of the compounds. From the table, it is obvious that the red cell membranes are almost impermeable to BTMP and thiamine as well as to TMP and TDP. On the other hand, among these compounds, only SBT was found to penetrate into the cells in remarkable amount.

Table I. Permeability of Red Cell Membranes to Thiamine Derivatives and some Physical Properties of the Compounds

Compound	$C_{cell}/C_{medium} \times 10^2$ (Total thiamine)		Partition coefficient ($CHCl_3$/Buffer)	Ionic character at pH 7.4
	Red cell suspension	Ghosts suspension		
BTMP	0.58	0.78	0.004	1.735
SBT	112.26	92.33	37.351	0.033
OBT	15.22	8.17	2.830	0.991
Thiamine	0.74	2.88	0.001	0.991
TMP	0.29	1.68	0	2.587
TDP	0.40	1.95	0	2.856

These results could be roughly explained by the physical properties of the compounds. As can be seen from the table, the permeability increases with the partition coefficient of the compound to an organic phase and decreases with the ionic

*4 In these experiments, except for SBT, the concentrations in the cell were determined after washing the cells twice with a small amount of cold isotonic phosphate buffer, since the amounts of passage into the cells were too small to be estimated by the method described in the experimental part.

6) M. Fujiwara, K. Matsui: Anal. Chem., **25**, 810 (1953); R. Strohecker, H. M. Henning: "Vitamin Assay," pp. 65 (1965). Verlag Chemie, GmbH, Weinheim.

7) M. Fujiwara, Y. Sasakawa, *et al.*: Vitamins (Japan), **31**, 340 (1965).

character of them. These are in good agreement with the requirements[8] for a passive diffusion of organic compounds through lipoidal membranes.

From these results, it can be anticipated that as far as a lipoidal boundary is concerned, BTMP might not be transported through intestinal boundary to any appreciable extent and that SBT must be the most probable intermediate for the intestinal absorption of BTMP, in consistent with the fact that BTMP was preferably transferred to SBT in rat intestine homogenates.[3]

Accumulative Uptake of S-Benzoylthiamine by Red Cells

To investigate the time course of the transport, the red cell suspension containing 0.3 μM/ml. of SBT was incubated at 37° and the concentrations in the cell and in the medium were determined at appropriate intervals. One of the typical results obtained is shown in Fig. 1 and Table II. The concentrations of total thiamine in and outside of the cell became equal after about 15 minutes and an equilibrium state was reached after 180 minutes, where the cell-to-medium concentration ratio was approximately 2.8.*5 This result indicates that SBT can be accumulated in the red cells up to 2.8 times higher in concentration than in the medium, against the concentration gradient.

It was found, however, that SBT was readily decomposed to "free thiamine"*6 both in the cell and the medium; after 180 minutes more than 90% of the total thiamine were found as "free thiamine." But, as can be seen from the figure, at the earlier stages of incubation, a much greater part of the total thiamine in the cell was found to be "free thiamine" than that in the medium. Moreover, it was found that in the cell, thiamine was a main component of "free thiamine" throughout the course of incubation, while in the medium, a greater part of it was OBT.

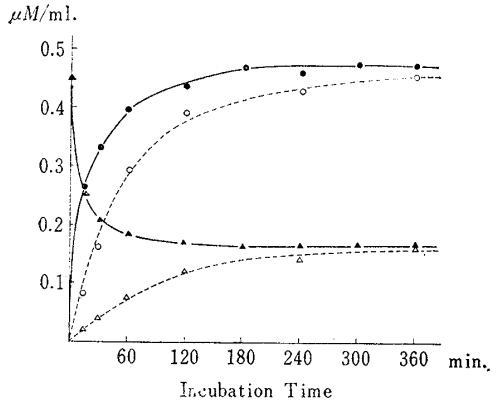


Fig. 1. Time Course of the Uptake of SBT by Human Red Cells (ca. 40% Cell suspension, at 37°, pH 7.4)

—●— Total thiamine in the cell
------○------ "Free thiamine" in the cell
—▲— Total thiamine in the medium
-----△------ "Free thiamine" in the medium

Table II. Uptake of S-Benzoylthiamine by Human Red Cells and the Ghosts

Incubation time (min.)	C_{cell}/C_{medium} (Total thiamine)	
	Red cells	Ghosts
15	1.10	0.73
30	1.61	0.92
60	2.12	1.01
120	2.52	1.09
180	2.93	1.19
240	2.78	1.12
300	2.78	1.18
360	2.79	1.01

These results were confirmed by the separate experiments on the decomposition of SBT in the bnffer of pH 7.4 and in the red cell homogenate at 37°. As shown in Fig. 2, in the buffer, the product was found to be mainly OBT with only about 15%

*5 The ratio is 3.9 if the water content of the red cells is assumed to be 72 (w/v) % (ref. 8).
*6 The word, "free thiamine," will be used to represent the thiazol type compounds which are extractable as thiochrome with *n*-butanol.
8) L. S. Schanker, P. A. Nafpoliotis, J. M. Johnson : J. Pharmacol. Exptl. Therap., **133**, 325 (1961); L. S. Schanker, J. M. Johnson, J. J. Jeffrey : J. Physiol., **207**, 503 (1964).

of thiamine, while in the red cell homogenate, not only the rate of decomposition was much faster than that in the buffer, but also the product was mainly thiamine with only about 15% of OBT. It can be concluded, therefore, that the decomposition of SBT in an aqueous medium is mainly due to an acyl-migration from sulfur to oxygen, while that in the red cells mainly to deacylation either by hydrolysis with S-esterase[9] or by reduction with intracellular SH compounds, *e.g.*, glutathione.[10] The red cell extracts, after deproteination with trichloroacetic acid or after inactivation of enzymes by heating at 100°, kept one half to one third of metabolizing activity of the red cell homogenates to SBT and, therefore, both hydrolysis and reduction are expected to be involved in the transfer of SBT to thiamine in the cell.

These results indicate that SBT entered in the red cells is reduced to undiffusible thiamine very rapidly and the resultant accumulation of it in the cells seems to be mainly responsible for the observed up-hill transport of SBT.

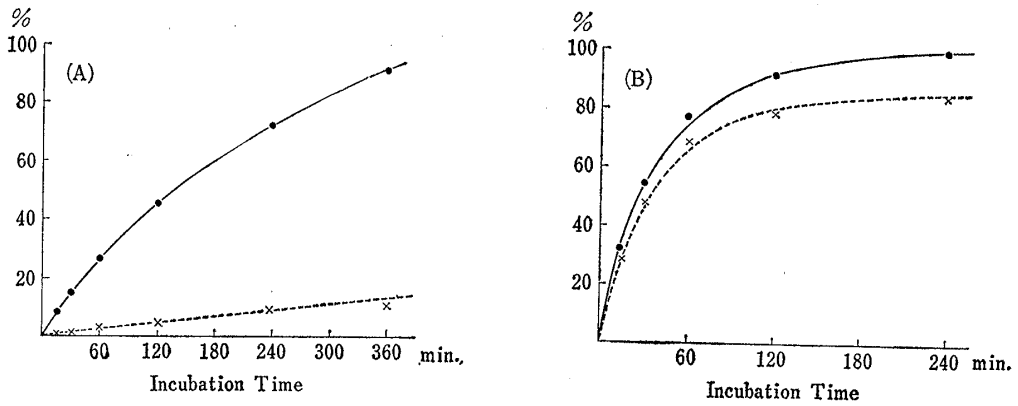


Fig. 2. Decomposition of SBT (0.3 m*M*/L.) in (A) : Phosphate Buffer, pH 7.4 and (B) : 80% Red Cell Homogenate (pH 7.0), at 37°

—●— "Free thiamine" (Thiamine+OBT)
------×------ Thiamine

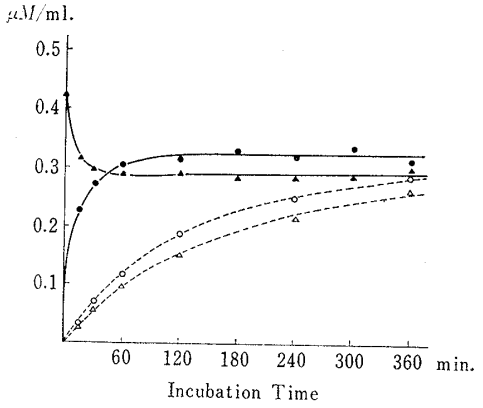


Fig. 3. Time Course of the Penetration of SBT into the Red Cell Ghosts (ca. 30% Cell suspension, at 37°, pH 7.4)

—●— Total thiamine in the cell
------○------ "Free thiamine" in the cell
—▲— Total thiamine in the medium
------△------ "Free thiamine" in the medium

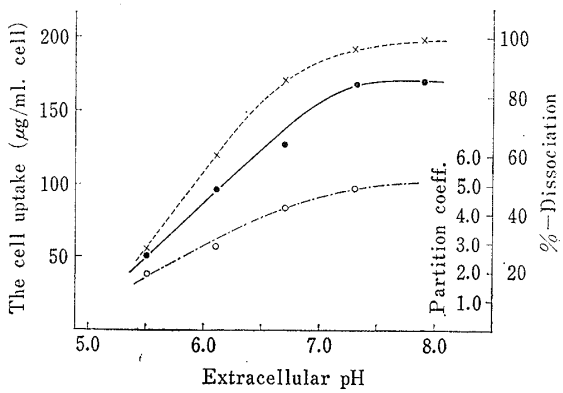


Fig. 4. Effect of Extracellular pH on the Rate of the Red Cell Penetration of SBT

—●— The uptake of SBT by the red cells during 30 minutes at 37°, ca. 40% cell suspensions in 100 μg./ml. SBT
------×------ Percent dissociation of SBT calculated from the pKa value, 5.93
·-·-○-·-·- Partition coefficients of SBT between ethylacetate and the buffer, shaken for 30 minutes at 37°

9) Z. Suzuoki, T. Suzuoki : J. Biochem. (Tokyo), **49**, 599 (1953).
10) L. Eldjarn, J. Bremer, H. C. Börresen : Biochem. J., **82**, 192 (1962); A. Ito, W. Hamanaka, K. Okamoto, T. Wada : Vitamins (Japan), **22**, 358 (1961).

In order to clarify further the mechanism of SBT passage through the cell membrane, the transport experiments were performed in the ghost suspensions under the same experimental conditions as those in the red cell suspensions. As shown in Table II and Fig. 3, the concentrations of total thiamine in the cell and the medium reached an equilibrium state after 120 minutes, where the final cell-to-medium concentration ratio was only around 1.15. Since the red cell ghosts used contain about 10% of the residual hemoglobin —— thus the same extent of other intracellular components, the value is quite reasonable as representing the 1:1 concentration equilibrium across the cell membranes. In this case, a greater part of the "free thiamine" in both the cell and the medium was found to be OBT, being similar to the decomposition in the buffer. It can be concluded, therefore, that the mechanism of passage of SBT through the red cell membranes is essentially a simple passive diffusion.

If the substance penetrates the cell by diffusion, the amount of entry must be proportional to the concentration gradient across the cell membrane in accordance with Fick's law. The red cell suspensions were incubated in the varying initial extracellular concentrations of SBT from 0.04 to 8.08 mM/L. As shown in Table III, the amount of the uptake by the cells was found to be proportional to the initial concentrations of SBT and their ratio was approximately constant for the different concentrations. Assuming the difference between the amounts of the total thiamine and the "free thiamine" to be that of SBT, the concentration of SBT species in the cell was also proportional to the initial concentration of SBT in the medium.

Table III. Relation between the Concentration Gradient and the Uptake of S-Benzoylthiamine by the Red Cells

Initial SBT concentration (μM/ml. medium)	Uptake by the cells (Total thiamine, μM/ml. cell)		Uptake/initial concn.		
			Total thiamine		SBT
Incubation (min.)	30	120	30	120	30
0.040	0.030	0.042	0.74	0.93	0.56
0.404	0.361	0.421	0.89	1.04	0.46
2.020	1.824	2.083	0.90	1.03	0.46
4.040	3.485	4.232	0.86	1.05	0.45
8.079	6.711	8.140	0.83	1.01	0.48

Concerning the effect of varying the extracellular pH on the uptake of SBT by the red cells, the experiments were performed at pH 5.5, 6.1, 6.7, 7.3 and 7.9. As shown in Fig. 4, the rate of the penetration was found to diminish markedly with decreasing pH of the medium. The apparent partition coefficients of SBT to ethylacetate and the calculated percentages of the unionized form of SBT at the different pHs were also plotted to the pH values in Fig. 4, and it can be seen that there is a close agreement among the three curves each other. The results indicate that only unionized molecules of SBT can penetrate into the cell and the rate of the penetration is governed by the apparent lipid solubility of SBT, providing a further proof for the passive diffusion mechanism of the tansport of SBT. It is also notable that the rate of the penetration of SBT into the cells reaches maximum at pH 7.4. A similar relations have been reported on the uptake of thiamine methyldisulfide by chick blood cells.[11]

11) Z. Suzuoki, T. Suzuoki: J. Biochem. (Tokyo), **40**, 11 (1953).

No appreciable effect was observed on the uptake of SBT by the red cells by adding either glucose, Mg^{++} and Ca^{++} ions or 2,4-dinitrophenol (10^{-3} M/L.) or ouabain (10^{-3} M/L.) in the suspending medium.

Comparative Study on the Red Cell Penetration of Substituted S-Benzoylthiamines

From the foregoing results, it was clarified that the penetration of SBT into the red cells proceeds through two steps; the first, a passive diffusion of SBT through the cell membrane and the second, a rapid decomposition of SBT to thiamine, which accumulates in the cell. The first step is expected to depend largely on the lipid solubility of the compound, while the second one on the electronic effect on the reaction site. Thus, a series of SBT derivatives with various substituents on the phenyl ring were synthesized and their rates of the red cell penetration were investigated.

The results are shown in Table Ⅳ with π-constants[12] and Hammett's σ-constants[13] of the substituents. As can be seen from the table, it was shown that the rate of the red cell penetration of SBT is much affected by an introduction of substituents and, as shown in Fig. 5, there is a distinct linear relation between the permeability and the σ-constants of the substituents. The rate of the penetration increased with electron-attracting power of the substituents. On the other hand, it has no relation to the π-constants of the substituents —— the lipid solubility of the compounds.

Table Ⅳ. The Red Cell Permeability to Substituted S-Benzoylthiamines and the Physical Constants of the Substituents

No.	Substituents	π-Constants[a]	σ-Constants	C_{cell}/C_{medium} [b]
1	p-CN	−0.31	0.628	2.30
2	m-NO_2	−0.05	0.710	2.23
3	H	0.00	0.000	1.15
4	p-NO_2	0.02	0.778	2.90
5	p-OCH_3	0.08	−0.268	0.66
6	p-CH_3	0.42	−0.170	0.78
7	p-Cl	0.87	0.227	1.70
8	3,4-Cl_2	1.70	0.600	2.50

a) The values are those obtained form mono-substituted benzoic acids (ref. 12).
b) The red cell suspensions (ca. 40%) containing 0.3 μM/ml. of the compounds were incubated at 37° for 30 minutes and the total thiamine concentrations were determined in the cell and the medium.

The rate of decomposition of these compounds to thiamine in the red cells were estimated from the formation of "free thiamine" in the red cell homogenates after 30 minutes of incubation. As shown in Fig. 6, a linear relation was found also between the rate of the decomposition in the cell and the σ-constants of the substituents, the rate increasing with the σ-values of the substituents. In the buffer of pH 7.4, the rate of the decomposition also increased with the σ-values of the substituents, but not in a linear way (Fig. 6). This difference in the behaviors might be originated in the difference in the mode of decomposition involved, as described before.

From these results, it can be concluded that at least in this series of compounds a predominant factor determining the rate of the penetration into the red cells is the rate of their decomposition to thiamine in the red cells. It seems to be noteworthy, however, that two compounds which showed an appreciable deviations down from the

12) C. Hansch, T. Fujita : J. Am. Chem. Soc., **86**, 1616 (1964); T. Fujita, J. Iwasa, C. Hansch : *Ibid.*, **86**, 5175 (1964).
13) H. H. Jaffe : Chem. Revs., **53**, 191 (1953).

straight line in Fig. 5, have a substituent of negative π-values, that is, of lowering the lipid solubility of the compound, suggesting that the change in the lipid solubility has also a minor effect on the rate of the cell penetration.

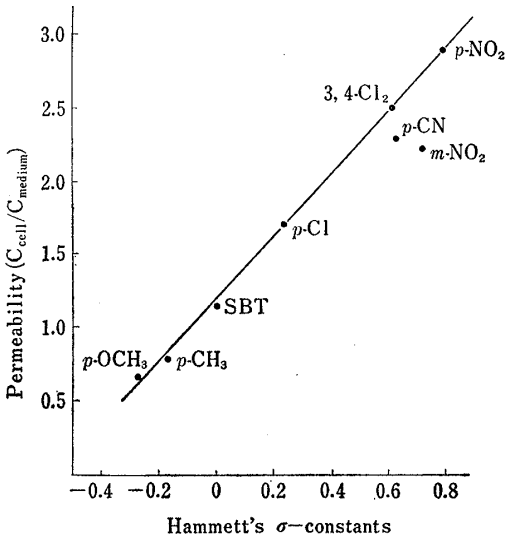


Fig. 5. Relation between the Red Cell Permeability to Substituted S-Benzoylthiamines and Hammett's σ-Constants of the Substituents

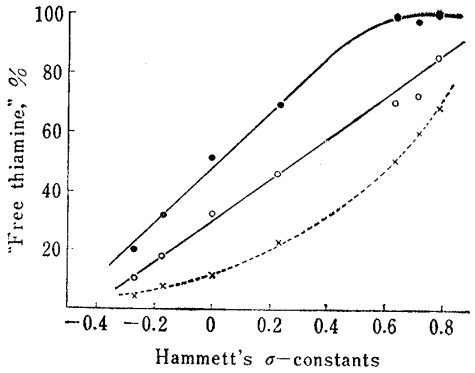


Fig. 6. Relation between the Rate of Decomposition of Substituted S-Benzoylthiamines and Hammett's σ-Constants of the Substituents

—●— In 80% red cell homogenate, 0.3 m*M*/L. SBT derivatives, incubation at 37° for 30 minutes
—○— In 40%red cell homogenate
------×------ In phosphate buffer, pH 7.4

The authors express their deep gratitudes to Dr. G. Sunagawa, director of this laboratories, for his helpful advices and kind encouragement throughout the course of this work, and to Drs. I. Iwai and A. Ito of this laboratories for their helpful discussions and encouragements. Thanks are also due to Dr. M. Nagawa and Mr. M. Kataoka of this laboratories for the preparation of substituted S-benzoylthiamines.

[Chem. Pharm. Bull. 15(3) 302~306 (1967)]

UDC 547.94.02 : 582.761

37. Tohru Kikuchi and Shoichiro Uyeo : Pachysandra Alkaloids. Ⅳ.*1 Structure of Pachysamine-A and -B.*2

(*Faculty of Pharmaceutical Sciences, Kyoto University**3)

Pachysamine-A and -B are minor alkaloids of *Pachysandra terminalis* Sieb. et Zucc., a Buxaceous plant. The structures of both alkaloids were discussed herewith and assigned to the formulas Ⅲ and Ⅷ, respectively.

(Received May 27, 1966)

In Part Ⅱ[1] and Ⅲ*1 of this series, we reported the structures and stereochemistry of pachysandrine-A (Ia), -B (Ib), -C (Ⅱa), and -D (Ⅱb), which had been isolated from *Pachysandra terminalis* Sieb. et Zucc. (Japanese name : Fukki-so) and belong to the

*1 Part Ⅲ. T. Kikuchi, S. Uyeo, Jr. : This Bulletin, **15**, 207 (1967).
*2 Preliminary communication of this work appeared in Tetrahedron Letters, No. 25, 1641 (1964).
*3 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto (菊池 徹, 上尾庄一郎).
1) M. Tomita, S. Uyeo, Jr., T. Kikuchi : This Bulletin, **15**, 193 (1967).