EFFECT OF pH ON THE REACTIVITY BETWEEN THIOL-TYPE THIAMINES AND OXIDIZED EGG ALBUMIN¹

ISAMU UTSUMI, KIYOSHI HARADA AND KEIICHI KOHNO²

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.,
Higashiyodogawa-ku, Osaka*

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It was reported in the previous paper that thiamine and *O*-benzoylthiamine (OBT) reacted with iodine-oxidized egg albumin resulting in formation of protein-thiamine mixed disulfide (1). The formation of protein-bound thiamine is dependent on the pH of the reaction mixture and the amount of bound thiamine was much greater at pH 9.0 than at pH 7.3 possibly due to the difference of the amount of the thiol form of thiamine which really took part in the reaction. Further, the reactivity of thiamine was different from that of OBT at the same pH levels (7.3 and 9.0), the latter being more reactive with protein than the former. In the present experiment, the reactivity at various pH levels was investigated for further confirmation. It was found that the formation of bound thiamine increased gradually from pH around 6, but rapidly at pH around 8.0, showing the similar tendency of the relationship between pH and the formation of thiol-type thiamine. OBT was confirmed to be more reactive with protein than thiamine at various pH levels. Therefore, the equilibrium concentrations of the thiol-type thiamine and OBT were compared by measuring the anodic wave height of polarography at constant pH levels and it was found that the anodic wave height of OBT was remarkably higher than that of thiamine at pH 7 to 8, showing the greater production of the thiol form. Thus the main factor of the difference in the reactivity of both the compounds with oxidized egg albumin was confirmed.

EXPERIMENTAL

1. Reaction with Oxidized Protein and Determination of Bound Thiamine

According to the methods described in the preceding papers, 250 mg of iodine-oxidized egg albumin and the thiamine derivative equivalent to 4 mg thiamine were allowed to react in the buffer solutions of various pH levels (McIlvain buffer for pH 4—6, 0.5 *M* phosphate buffer for pH 7.0—8.0, Clark-Lubs buffer for pH 8.2—10, Kolthoff buffer for pH 11—12), at 37° for one hour and after metaphosphoric acid precipitation (1) the amount of protein-bound thiamine was

¹ Studies on Interaction of Thiamine or its Related Compounds with Protein V.

² 内海 勇, 原田 清, 河野啓一.

estimate.

2. Measurement of the Anodic Wave Height of Thiol-Type Thiamine

An aliquot of thiamine or OBT was dissolved to make 1×10^{-2} M solution in 0.1 M phosphate buffer (pH 7.0–8.0) or Clark-Lubs buffer (pH 8.2–8.8) and to 25 ml of this solution was added 0.5 ml of saturated KCl solution and 0.25 ml of 0.5 % gelatine solution. The mixture was kept under nitrogen at $20 \pm 1^\circ$ for 60 minutes and was used as a sample. However, OBT at pH 8.2 was determined with 2×10^{-3} M solution and thiamine and OBT at pH 8.8 with 1×10^{-3} M solution. The anodic wave height of polarography was measured according to Watanabe *et al.* (2), whereby a saturated mercurous chloride electrode was used as an anode and connected with the test solution by a KCl bridge. The anodic wave height was corrected with the residual currents taking as a reference the polarogram of corresponding buffer solutions without containing thiamine or its derivatives.

RESULTS AND DISCUSSION

1. Reaction pH and the Amounts of Bound Thiamine

The amounts of bound OBT and thiamine with oxidized egg albumin at various pH levels (bound thiamines are all disulfide forms) are shown in Table I and

TABLE I
Relationship between Reaction pH and Amount of Bound Thiamine

pH	Bound thiamine	
	Thiamine	OBT
	$\mu\text{g}/250 \text{ mg protein/m}$	
3.9	3	41
6.0	8	150
7.3	66	292
8.0	131	520
8.2	197	745
8.6	456	1,180
9.0	505	1,230
9.6	570	1,190
10.0	615	1,170
11.0	660	1,120
12.0	645	960

Thiamine derivative equivalent to 4 mg thiamine was used per 250 mg protein. The mixture was incubated at 37° for 60 minutes.

FIG. 1 Amount of Bound Thiamine and pH
●, thiamine; ○, OBT

Fig. 1. The reaction of thiamine with the protein occurred from pH about 7.0, increasing rapidly at pH 8.2, and reached to the maximum value at pH 10–12. On the other hand, OBT reacted with the protein at pH levels as low as 6.0, reaching the maximum value at pH 9.0, but at pH 11–12, the tendency to decrease was observed. Tachi *et al.* (3) demonstrated polarographically the presence of thiol-type thiamine at pH greater than 9.0 by measuring the oxidation wave.

Later Watanabe *et al.* (2) proved the partial existence of the thiol-type thiamine at the physiological pH about 7. They found further that the formation of thiol-type thiamine at pH 7 to 8 was less than 1%, assuming the formation at pH 10 to 11.6 to be 100%, but at pH 9.35, the pH near the pK_a of thiamine-thiol at 25°, the formation was as high as 50%. In order to produce protein-thiamine mixed disulfide in the reaction of thiamine with oxidized egg albumin, thiamine is considered to take a thiol form. The relationship between the pH and the formation of thiol form shows the similar tendency of the relationship between the pH and the reactivity observed by the experiment. It is therefore assumed that the formation of the thiol form varies according to the reaction pH, resulting in the change in the amount of the bound form. The comparison of the reactivities with protein between thiamine and OBT showed, as described in the previous paper, that OBT was more reactive than thiamine at each pH level, even at a weakly alkaline range of pH 7 to 8, a considerable amount was found to be bound. On the contrary, thiamine produces little thiol form at pH 7 to 8, and thus very low reactivity is expected. That OBT was 3 to 4 times as much bound as thiamine at weakly alkaline range seemed to show the difference in the formation of the thiol form. (Even in the reaction at pH above 9.0, the recovery of the total thiamine, *i.e.*, bound plus unbound thiamine, was in all the cases above 95%, showing that very little degradation other than disulfide form has taken place. It cannot be ascribed to the difference in stability between thiamine and OBT). Therefore, the equilibrium concentrations of the thiol forms of thiamine and OBT at various pH levels were then compared by determining the anodic wave heights of polarography.

2. Comparison of Anodic Wave Heights

The solutions of thiamine and OBT dissolved in buffer solutions of various pH levels were kept at $20 \pm 1^\circ$ for 60 minutes in nitrogen and the anodic wave heights of polarography were determined and the results are shown in Fig. 2. The plot of the logarithm of the anodic wave height against pH is shown in Fig. 3. The samples were measured after keeping at room temperature for 60 minutes, taking into consideration the rate of conversion to the thiol form. The anodic wave heights of thiamine and OBT increased gradually with the lapse of time within 30 minutes after dissolution, showing the opening to the thiol form. All the half-wave potentials, $E_{1/2}$, were -0.38 ± 0.03 V (against saturated mercurous chloride electrode). However, in the case of OBT solution at pH 8.8, a maximum wave appeared at around -0.18 V, and the wave heights could not be measured. The anodic wave height corresponding to the equilibrium concentration of the thiol form increased proportionally to the rise in pH from 7 to 8, in both

FIG. 2 *Anodic Wave Heights of Polarography of Thiamine and of OBT in Various Buffer Solutions*

a, buffer; *b*, 1×10^{-3} M thiamine; *c*, 1×10^{-3} M OBT; *d*, 1×10^{-2} M thiamine; *e*, 2×10^{-3} M OBT; *f*, 1×10^{-2} M OBT.

FIG. 3 *Anodic Wave Heights of Thiamine and of OBT at Various pH Levels*

○, OBT; ●, thiamine
(0.01 M buffer; sensitivity, 1×10^{-2} A/mm; $20 \pm 1^\circ$)

thiamine and OBT, but the wave height of OBT was higher than that of thiamine in each pH, showing more formation of the thiol form of OBT than that of thiamine. The anodic wave heights of OBT rose suddenly at pH 8.0 to 8.2, whereas that of thiamine seems to rise suddenly at pH above 8.2. Comparison of the results with the relationship between pH and reactivity at a weakly alkaline range given in Fig. 1, show quite the same tendency. The main difference in the reactivities of thiamine and OBT seems therefore to be due to the difference in production of the thiol forms of both compounds. It is also consistent with the considerations of electronic theory of both chemical structures.

However, both thiamine and OBT take the thiol forms in a strongly alkaline medium. Assuming that the reactivity depends solely on the formation of thiol

forms, the amounts of the bound forms of thiamine and OBT are expected to be about the same, since the amounts of the thiol forms are considered to be about the same at that pH range. The experiment showed, however, a remarkable difference in the amounts of the bound forms between thiamine and OBT at pH above 10. In the case of OBT, the reactivity at pH 11–12 was lower than that at pH 9–10. These findings cannot be explained by the difference in the formation of thiol forms and the effect of *O*-benzoyl group on the reactivity of OBT remains to be studied.

SUMMARY

1. The amounts of protein-thiamine mixed disulfide produced by the reaction of thiamine or *O*-benzoyl thiamine (OBT) with oxidized egg albumin at various pH levels were determined. The formation of the bound form of thiamine was detected at pH about 7.0, increasing suddenly at pH above 8.2, and reaching a nearly constant value at pH above 10.0. The pH-curve was in good agreement with the curve of pH and formation of the thiol form, showing the parallelism between the production of the bound form and the concentration of the thiol type thiamine.

2. OBT showed already the reactivity at pH around 6.0, and more amounts were bound with protein than thiamine at any pH levels.

3. The equilibrium concentrations of the thiol form of thiamine and of OBT were compared in weakly alkaline range by measuring the anodic wave height of polarography. The anodic wave height of OBT was found to be far higher than that of thiamine, showing that OBT was more liable to take the thiol form. These findings suggest that the difference in the reactivities of thiamine and OBT in weakly alkaline range is mainly due to the difference in the formation of the thiol form.

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REFERENCES

1. Utsumi, I., Harada, K. and Kohno, K., *J. Vitaminol.* **10**, 13 (1963).
2. Watanabe, A. and Asahi, Y., *Yakugaku Zasshi* **75**, 1050, (1955).
3. Tachi, I. and Koide, S., *J. Agr. Chem. Soc. Japan* **25**, 145, (1951); **26**, 243, (1952).