*The A. C. Polarography of Thiamine O-Monophosphate**

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Many investigations of such phosphates of thiamine (B_1) as the monophosphate (TMP), diphosphate (TDP, cocarboxylase) and triphosphate (TTP) of thiamine, have been reported from various points of view because of their importance in the biological and medical fields.

Polarographic studies of such phosphates of B_1 may not yet, however, be considered to have been established. Only a short communication on the polarographic behaviors of TDP has been reported[1], and no precise comparisons to the B_1 findings and no studies of the nature of its electrode process have been carried out.

Among the above three phosphates of B_1, TMP was thought to be the most stable and to have the simplest structure, therefore, it was obtained rather easily as pure crystals.

In this paper, the a. c. polarographic behavior of TMP and a discussion of the differences between the behavior of B_1 reported on in a previous work[2] and that of TMP will be presented.

The d. c. polarographic reduction waves of B_1 and its derivatives were generally ill-defined in the buffer solutions because of the unfavorable influences of hydrogen-discharging currents or pre-sodium waves, while their a. c. polarographic reduction waves were rather well-defined, as was described in the previous paper[2]. Similar results were also obtained in the case of TMP. Therefore, the a. c. method was mainly applied in this investigation.

Experimental

Apparatus and Reagents.—The polarograph used was Yanagimoto model PA-102. (D. C. & A. C.); A. C.: ΔE=15 mV. r.m.s., 50 c.p.s., sinusoidal. The characteristics of the dropping mercury electrode were m=1.003 mg. sec^{-1}, t=4.09 sec. (open circuit), and 3.76 sec. (at −1.273 V.).

The TMP used was prepared by the phosphorisation of B_1[3] and was then hydrolyzed to be monophosphate and purified by the column chromatograph (CG-50) until it gave only one spot on the paper chromatography.

Data for the purified TMP used were as follows: R_f=0.12 (Solvent, acetic acid : *n*-butanol ; water = 1 : 4 : 5) ; the content of TMP as determined by thiochrome reaction test was 99.1%, m. p. = ca. 200°C (decomp.). Microanalytical data :

Found : C, 31.48 ; H, 4.01 ; N, 11.77 ; H_2O, 8.24. Calcd. for $C_{12}H_{18}N_4O_4PS \cdot HCl \cdot Cl \cdot 2H_2O$: C, 31.80 ; H, 4.22 ; N, 12.36 ; H_2O, 7.95%.

The other reagents used were of reagent grade.

Experiments were carried out two or three times on the same kind of sample at 25±0.5°C, and the values obtained were averaged. All potentials and peak currents were expressed in V. vs. S. C. E. and μamp. r. m. s., respectively.

Results

The Influence of the pH Value on i_a and E_a.—Some of the a. c. polarograms of 5.00×10^{-4} mol./l. TMP in the buffer solutions of pH 0.90～10.65 (their ionic strengths were adjusted

* Polarographic Studies of Some Organo-sulfur Compounds, Part III.

1) I. Tachi, *Kagaku no Ryoiki*, Extra Edition, (Nankō-do), **2**, 37 (1949).

2) K. Okamoto, This Bulletin, **34**, 1063 (1961).

3) P. Karrer and M. Viscontini, *Helv. Chim. Acta*, **29**, 711 (1946).

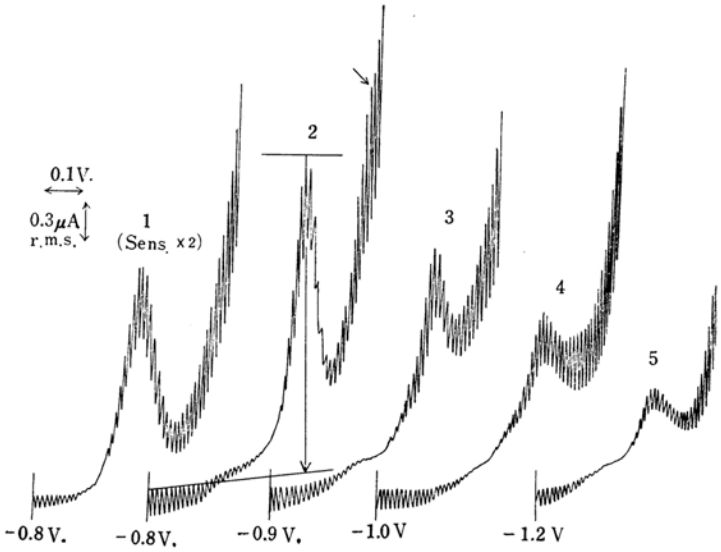

Fig. 1. Some of the A. C. polarograme of TMP.
pH; 1, 0.90; 2, 3.00; 3, 5.00; 4, 6.80; 5, 7.85, [TMP]$=0.500\times10^{-3}$ mol./l., at 25°C, air free, capacitance$=0\mu$F

TABLE I. THE VARIATION OF THE SUMMIT POTENTIALS E_s AND THE PEAK CURRENTS i_s WITH pH
TMP$=1.00\times10^{-3}$ mol./l., at 25°C

pH	Buffer*	$-E_s$ V. vs. S. C. E.	i_s μamp. r.m.s.
0.90	A	1.090	7.48
1.75	A	1.141	7.48
3.00	B	1.222	5.22
4.00	B	1.270	4.68
5.00	B	1.337	3.78
6.00	C	1.390	3.48
6.80	C	1.440	2.88
7.85	C	1.518	1.08
8.85	C	1.500 1.623	0.87 0.60
9.95	D	ca. 1.517	0.45
10.65	D	NW**	0

A, KCl+HCl
B, 0.25 M acetate buffer
C, 0.25 M phosphate buffer
D, 0.1 M borate buffer
* Ionic strength was adjusted to 0.50 constant by the addition of KCl.
** No wave

to 0.50 by the addition of potassium chloride) and also the method of the measurement of wave height are shown in Fig. 1.

The ralations of the peak currents, i_s, and of the summit potentials, E_s, to the pH value are both summarized in Table I and in Figs. 2 and 3.

One well-defined peaks current was observed at the pH values ca. 2 and ca. 4~8. One peak and an indefinite shoulder (as indicated by an

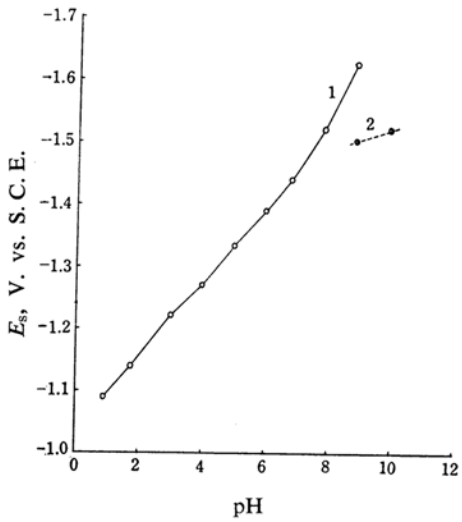

Fig. 2. The relation of pH to E_s.
1, 1st wave 2, pre-wave (indifinite)

arrow in Figs. 1—2.) were observed at the values of pH 1.75 and 3.00.

The behavior of a. c. reduction waves of TMP were not similar to that of B_1[2], as is shown in Fig. 1. Especially the attitude of the second wave of TMP was found to be different from that of B_1; that is, in the case of B_1 a rather definite second peak current was placed at more positive potentials and appeared at the pH value of 4.75, while that of TMP was not observed in the the same pH value region, but at the pH values of 1.75 and 3.00, at more negative potentials.

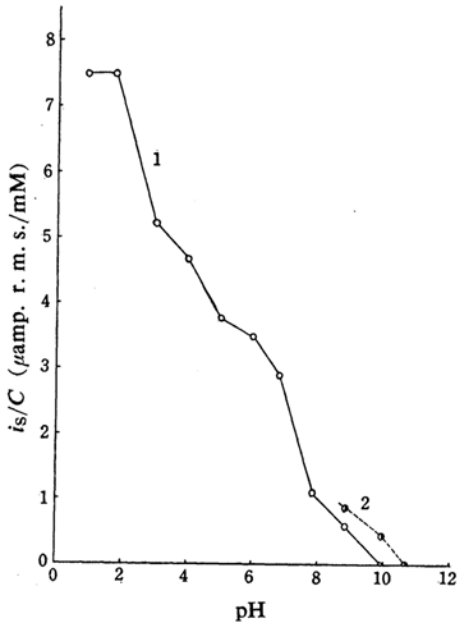

Fig. 3. The relation of pH to i_S.
1 : 1st wave. 2 : pre-wave (indifinite)

The variation of the E_S of TMP was very similar to the E_S of the first wave of B_1 within the pH value range of 4.0~7.0 (Fig. 2).

The peak currents of TMP increased according to the decrease pf pH values, as is shown in Fig. 3, and its heights and that of the first wave of B_1 ware not equivalent, although that of the latter was smaller than the former over all the pH value range studied.

The behavior of the peak current of TMP in basic solutions was almost the same as that of B_1 ; they both disappeared completely at a pH value of about 10.

The Relation of the Concentration of TMP to i_S and E_S.—In order to learn whether or not the peak current was due to the reduction of TMP itself, the relation between the concentrations and i_S was observed in two kinds of buffer solution, those with pH values of 0.90 and of 4.00. The results obtained are shown in Table II and in Fig. 4.

TABLE II. THE RELATIONS OF THE CONCENTRATIONS TO i_S AT TWO pH VALUES
measured at 25°C

Concn. $\times 10^{-3}$ mol./l.	pH=0.90*		pH=4.00**	
	i_S μamp. r.m.s.	$-E_S$ V. vs. S.C.E.	i_S	$-E_S$
0.05	0.24_5	1.130	0.20_7	1.271
0.10	0.60_5	1.092	0.45_8	1.270
0.25	1.86_5	1.103	1.17_0	1.268
0.50	3.54_8	1.109	2.28_0	1.276
0.75	4.71_0	1.116	3.24_0	1.283
1.00	6.22_5	1.124	4.18_5	1.290

* KCl+HCl (μ=0.50) ** 0.25 M acetate buffer (μ=0.50)

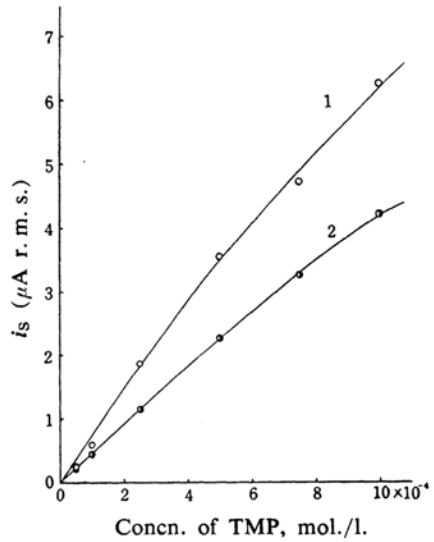

Fig. 4. The relation of the concentrations to i_S.
1, pH 0.90 2, pH 4.00

Almost linear relations between the concentrations and i_S were found for both samples; therefore, the first wave of TMP seemed to be the real reduction wave of TMP itself.

The summit potential, E_S, slightly shifted to negative potentials with the increase in the concentration of TMP.

This variation in of i_S and E_S according to the concentration change was very similar to that of B_1.

The Temperature Coefficients of i_S.—The temperature coefficient of the wave height was obtained as 2.17% between 18~32°C on acetate buffer solutions (μ=0.50) with a pH value of 4.00, containing 5.00×10^{-4} mol./l. of TMP.

The coefficient was larger than the theoretical values, 1.0~1.3%, for a. c. palarographic waves. This was thought to be mainly caused by the irreversible tendency of the electrode process of TMP, not by its kinetic properties.

The Influence of Mercury Heights H to i_S.—The relationship of H to i_S was studied by plotting H (corrected with the reverse pressure of H) against i_S at 25±0.5°C on the acetate buffer solution with a pH value of 4.00, containing 5.00×10^{-4} mol./l. of TMP.

The linear relation obtained may be formulated as follows:

$$i_S = 0.011H + 1.694$$

In the case of a. c. polarography, the i_S values were said to be independent of the H

values. The above relation almost coincided with the theory.

Electrocapillary Curve.—The electrocapillary curve of 5.00×10^{-4} mol./l. of TMP in the acetate buffer with a pH value of 4.00 is drawn in Fig. 5. Over the whole potential range, the drop time, t, in the sample solution was smaller than in the base solution, but at the electrocapillary zero, ca. -0.5 V., the difference was only 0.01 sec. A small step was observed at the potential $-1.25\sim-1.30$ V. corresponding to the summit potential of TMP, -1.273 V. A weak adsorption seemed to take place at the surface of DME over the whole potential range except at electrocapillary zero, but this could not have any remarkable effect upon the apparent a. c. waves.

When the electrocapillary curve of TMP was compared with that of B_1, the adsorption of TMP in the positive side to electrocapillary zero was the most different phenomenon.

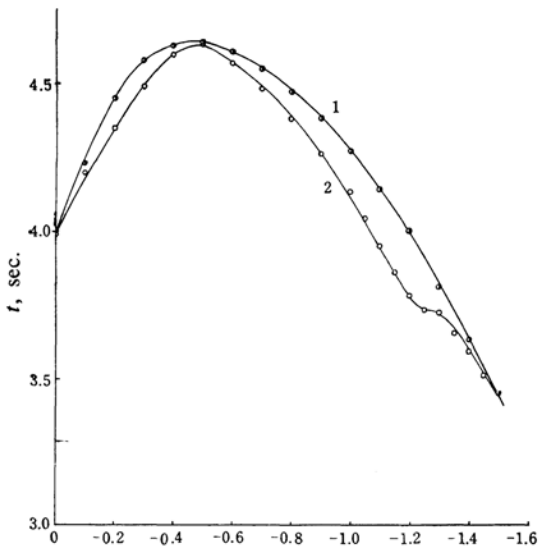

Fig. 5. Electrocapillary curves.
1, Base solution: acetate buffer of pH 4.00, $\mu=0.50$
2, 0.500×10^{-3} mol./l. of TMP in 1

Millicoulometric Determination (D. C. Method) of the *n*-Value.—The electrode reactions of TMP or B_1 have not yet been defined because of their complexity, but comparatively precise investigations of the reduction processes of B_1 have been reported by Asahi[4] recently. Asahi has suggested that B_1 is reduced by DME at the pyrimidine and also that the thiazol ring consumed about 10 electrons.

It was thought that the polarographic reduction process of TMP was similar to that of B_1 considering the reducible groups in a molecule.

Millicoulometric measurement of the n-values, therefore, was employed in order to confirm the number of electrons discharged, but the macro-scale-controlled potential electrolysis with a wide-surface-area mercury cathode was not applied, because the conditions of such a macro-coulometry were thought to be different from those of polarography.

The cell for millicoulometry used was of the Meites type[5], with the external S. C. E. anode separated from the cathodic part by a fine-sintered glass wall and an agar-agar bridge.

The coulometry was carried out on three kinds of bufier solutions, with pH values of 1.45, 3.00 and 4.00. d. c. potentials were applied, and the decrease in the d. c. wave height after electrolysis was measured.

When the pH value of the solution rose above about 4, the d. c. polarogram of TMP came to be very ill-defined, so that in the region with higher pH values than 4, millicoulometric studies could not be carried out.

The results obtained are summarized in Table III.

Table III. The results of millicoulometry

pH	$-E_{applied}$	n-Value
1.45	1.15	9.00
3.00	1.25	8.77
4.00	1.30	7.86

[TMP] $=5.371\times10^{-4}$ mol./l. Volume of solution, 0.25 ml. Electrolysis time, 100 min. Temp., 25 ± 0.5°C. Air was removed by nitrogen gas bubbling.

As is shown in Table III, the n-value of TMP was estimated to be 9 at a pH value of 3.0, but it seemed to decrease at pH values $>$ 4, in accordance with the gradual decrease of the peak current of the a. c. polarogram with the pH value increse (Fig. 3).

The n-values obtained were found nearly to coincide with Asahi's estimation.

Discussion

As has been described in the previous sections, comparatively well-defined a. c. polarographic waves of TMP observed in acidic buffer solutions, were not simple tensammetric waves, but were reduction waves with a few tensammetric properties.

The most remarkable difference between the a. c. polarogram of TMP and that of B_1 was

4) Y. Asahi, *J. Pharm. Soc. Japan* (*Yakugaku Zasshi*), **80**, 1222 (1960).

5) T. Meites and L. Meites, *Anal. Chem.*, 23, 1893 (1951).

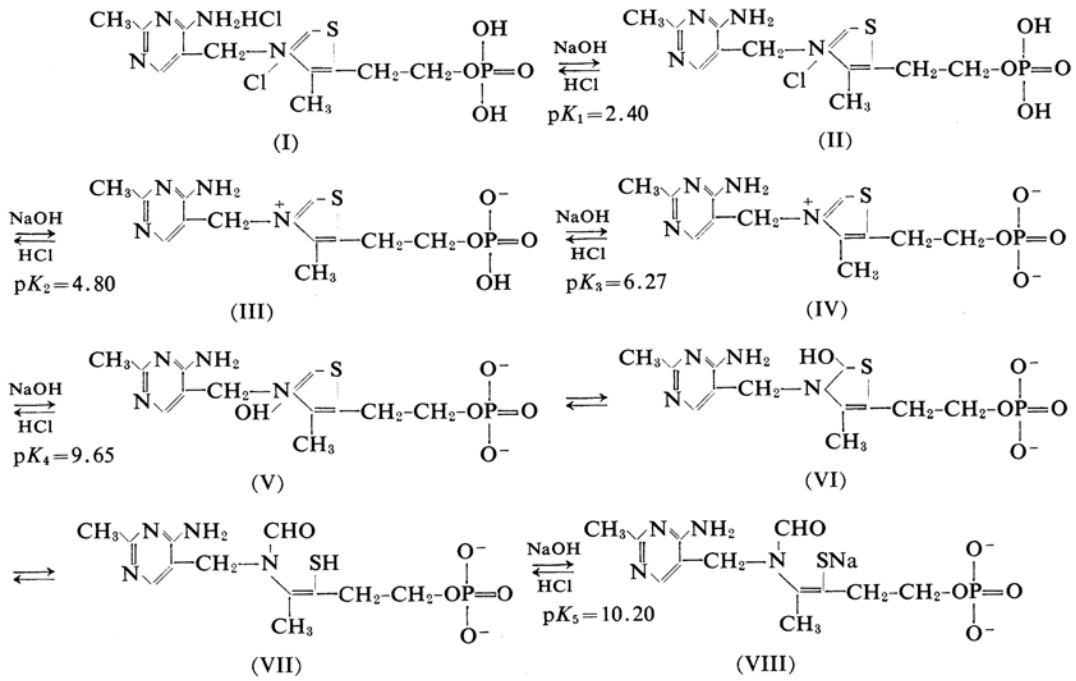

Fig. 6. Acid-base equilibrium of TMP.

the behavior of the second wave, which appeared rather definitely for B_1 at pH values>4.75, while it could not be observed in the same pH value region for TMP, but only as an obscure shoulder in the pH-value region of 1.75~3.

An other different result was the weak adsorption of TMP at the electrocapillary positive range, while the lack of it for B_1 could be observed on the electrocapillary curves.

This different behavior of TMP and of B_1 is thought to be caused by their different acid-base equilibrium processes.

The dissociation equilibrium of TMP when titrated with a sodium hydroxide solution can be shown as in Fig. 6, while its experimental titration curve is as in Fig. 7.

Definite pH-value changes were found at 1 mol. equiv. and at 3 mol. equiv., while an indefinite one was found at about a 5 mol. equiv. of sodium hydroxide.

The pK-values can be approximately estimated on the titration curve as $pK_1=2.4_0$, $pK_2=4.8_0$, $pK_3=6.2_7$, $pK_4=9.6_5$, and $pK_5=10.2_0$.

The obscure shoulder of the a. c. polarograms of TMP which appeared in the region of 1.75 ~3.00 pH values was, therefore, thought to be related to II species, but it could not be defined because of its ill-defined form.

The gradual decrease in the wave height of the a. c. polarograms according to the pH increase was caused by the comparatively continuous transition of the ionic species of

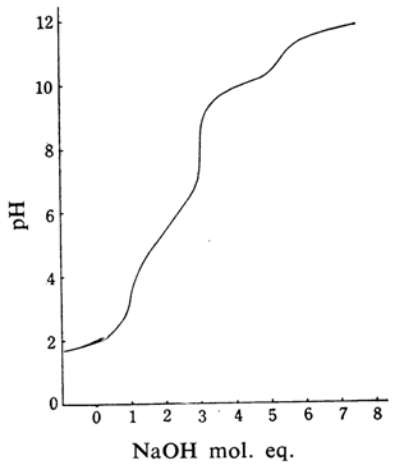

Fig. 7. Potentiometric titration curve of TMP.

TMP and by the complete disappearance of the wave at a pH value of about 10 was due to the disappearance of the thiazol ring as illustrated in Fig. 6.

The electron number discharged was confirmed for I species as 9, and as ca. 8 for II species, but that for other species could not be measured. Such a decrease in the n-values for each ionic species was thought to be mainly due to the reducibility of the pyrimidine rings with or without a proton; the wave height of I species was, therefore, the highest, as is shown in Fig. 3.

When the approximate assignment of the species of TMP in the solution of a certain pH value were made using the titration curve, species I was thought to exist in pH values <2, II in pH values ca. 3.5, III in those ca. 5.5, and IV in those ca. 8.

The comparisons, therefore, of the wave heights at each pH value seemed almost exactly to indicate the reducibility or the distinctive in electrode process of each species. The ratios of i_s at a pH value of 2 and at a pH value of 3.5, and of i_s at a pH value of 3.5 and at a pH value of 5.5 were $i_s^{3.5}/i_s=0.67$ and $i_s^{5.5}/i_s^{3.5}=0.74$ respectively. The apparent reducibility of a pyrimidine ring without hydrogen chloride was lower by about 67% than that of a ring with hydrogen chloride, while that of a thiazol ring without chlorine was also lower by about 74% than that of a ring of with chlorine. The reduction process was considered to be different for each ionic species according to the difference in the state of the amines in them.

The reduction mechanisms of TMP at DME could not be defined in this work, but it was suggested that the reduction may take place at both the pyrimidine ring and the thiazol ring, consuming 8 or 9 electrons.

Summary

A. C. polarographic studies of TMP, as well as a discussion of the polarographic behavior of TMP compared with that of B_1 reported on in a previous paper[1], were carried out.

TMP showed well-defined peak currents of almost the same E_s values as the second peak current of B_1 in the region of pH values <10, and obscure second waves were also observed at pH values between 1.75 and 3.00. The relation of the i_s of the first wave of TMP to the pH value was also similar to the second wave of B_1. The wave corresponding to the first wave of B_1, however, was not recognized for TMP under any of the conditions studied.

The nature of the first wave of TMP was determined to be that of the diffusion currents, not of the adsorption, kinetic, etc.

The number of electrons discharged could be ascertained by the millicoulometric technique as 9 at pH values <3 and 8 at a pH value of 4. The electrode reaction, therefore, of TMP was considered to be the same as that of B_1.

Some explanation of the relation of i_s to the pH value was attempted by reference to the pK values of TMP obtained from its experimental potentiometric titration curve.

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