Effect of pH and Dye Concentration on the Extraction of a Thiamine Dye Salt by an Organic Solvent

By V. DAS GUPTA*, DONALD E. CADWALLADER, HARVEY B. HERMAN†, and IRWIN L. HONIGBERG

The effect of pH and dye concentration on the extraction of thiamine dye salt by an organic solvent has been studied. An equation which expresses the dependence of the thiamine dye salt extracted with changes in pH was derived. The theoretical and experimental data were found to be in good agreement.

IN A previous report (1) concerning an acid dye assay method for thiamine using bromothymol blue, it was observed that the amount of thiamine dye salt extracted by chloroform varied with pH and dye concentration. The variation in the amount of salt extracted from an aqueous solution by an organic solvent with changes in pH and dye concentration is well documented (2). Schill (3, 4) has recently reported the effect of various experimental conditions on the extraction of bromothymol blue amine salts. The purpose of this study was to derive an equation that expresses the dependence of the thiamine dye salt extracted with changes in pH and dye concentration, and to prove the validity of this equation by comparing theoretical and experimental data.

THEORETICAL

With the use of a thermodynamic argument Divatia and Biles (5) showed that the alkyl amine salts of tropaeolin 00 in the organic phase were in equilibrium with the ionic forms of amine and dye in the aqueous phase. In their studies they called the equilibrium constant for such a phenomenon the apparent partition coefficient and expressed it as

$$K = \frac{S}{A^+ \times D^-}$$

where S = concentration of salt in the organic phase; A^+ = concentration of dissociated amine in the aqueous phase; and D^- = concentration of dissociated dye in the aqueous phase.

In the authors' studies an equivalent equation was expressed as follows:

$$K = \frac{S}{[HI^-]_a [TH^+]_a} \qquad \text{(Eq. 1)}$$

where S = concentration of thiamine dye salt in

the organic phase; $[Th^+]_a$ = concentration of thiamine in the aqueous phase (thiamine has one positive charge); $[HI^-]_a$ = concentration of dissociated dye in water.

The term equilibrium constant was preferred over the apparent partition coefficient because the latter gave the impression that the equilibrium was between the concentrations of the thiamine dye salt in two phases.

An equation relating the amount of thiamine dye salt extracted in a single portion of chloroform (when the volumes of immiscible solvents are equal) to changes in pH was derived using the following symbols:

S = Concentration of thiamine dye salt in the organic phase in moles/L. This also represents the concentration of dye or thiamine in the thiamine dye salt (Eq. 1).

$[C_{H_2I}]$ = Total concentration of the dye in both phases in moles/l.

$[C_{H_2I}]_a$ = Total concentration of the dye in aqueous phase in moles/l.

$[H_2I]_{ua}$ = Concentration of the undissociated dye in aqueous phase in moles/l.

$[H_2I]_c$ = Concentration of free dye in chloroform in moles/l.

$[HI^-]_a$ = Concentration of dissociated dye in water in moles/l.

A_0 = $[H^+]^2/([H^+]^2 + K_1[H^+] + K_1K_2)$ = fraction of $[H_2I]_{ua}$ at a given pH (6).

A_1 = $K_1[H^+]/([H^+]^2 + K_1[H^+] + K_1K_2)$ = fraction of $[HI^-]_a$ at a given pH (6).

K_1 = First acid dissociation constant of bromothymol blue.

K_2 = Second acid dissociation constant of bromothymol blue.

D = Distribution coefficient of the dye.

$[C_{Th}]$ = Total concentration of thiamine in both phases in moles/l.

$[C_{Th}]_a$ = Total concentration of thiamine in aqueous phase in moles/l.

$[TH^+]_a$ = Concentration of undissociated thiamine in aqueous phase in moles/l.

$[TH]_c$ = Concentration of free thiamine in chloroform phase in moles/l.

K_3 = Acid dissociation constant of thiamine.

K = Equilibrium constant (Eq. 1).

The equilibrium expression for the thiamine salt of bromothymol blue in chloroform and water is given by Eq. 1 which may be rearranged to give:

$$K[HI^-]_a [TH^+]_a - S = 0 \qquad \text{(Eq. 2)}$$

To solve for $[HI^-]_a$ in terms of $[H^+]$, K_1, K_2, and $[C_{H_2I}]$ let

$$[C_{H_2I}] = [C_{H_2I}]_a + S + [H_2I]_c \quad (Eq. 3)$$

and

$$[H_2I]_c = D[H_2I]_{ua} \quad (Eq. 4)$$

The expression for determining $[H_2I]_{ua}$ in an aqueous phase is

$$[H_2I]_{ua} = [C_{H_2I}]_a A_0 \quad (Eq. 5)$$

therefore, Eq. 4 can be expressed as

$$[H_2I]_c = D[C_{H_2I}]_a A_0 \quad (Eq. 6)$$

The expression for determining $[HI^-]_a$ in an aqueous phase is

$$[HI^-]_a = [C_{H_2I}]_a A_1 \quad \text{or} \quad [C_{H_2I}]_a = \frac{[HI^-]_a}{A_1}$$

$$(Eq. 7)$$

Substituting Eq. 6 and 7 in Eq. 3 and rearranging gives

$$[C_{H_2I}] - S = \frac{[HI^-]_a}{A_1} + D[HI^-]_a \frac{A_0}{A_1}$$

or

$$[C_{H_2I}] - S = [HI^-]_a \left[\frac{1}{A_1} + D \frac{A_0}{A_1} \right]$$

or

$$[C_{H_2I}] - S = [HI^-]_a \left[\frac{1}{A_1} + D \frac{[H^+]}{K_1} \right]$$

therefore,

$$[HI^-]_a = [[C_{H_2I}] - S] \frac{A_1 K_1}{K_1 + A_1 D[H^+]} \quad (Eq. 8)$$

To solve for $[Th^+]_a$ in terms of $[H^+]$, K_3 and $[C_{Th}]$ let $[C_{Th}] = [C_{Th}]_a + [Th]_c + S$. Since $[Th]_c$ is very small (Table IV), the equation becomes

$$[C_{Th}] = [C_{Th}]_a + S \quad (Eq. 9)$$

The expression for determining $[Th^+]_a$ in an aqueous solution is

$$[Th^+]_a = [C_{Th}]_a \frac{K_3}{[H^+] + K_3}$$

or

$$[C_{Th}]_a = [Th^+]_a \frac{[H^+] + K_3}{K_3} \quad (Eq. 10)$$

Substituting Eq. 10 in 9 we obtain $[C_{Th}] - S = [Th^+]_a [[H^+] + K_3/K_3]$ or

$$[[C_{Th}] - S] \frac{K_3}{[H^+] + K_3} = [Th^+]_a \quad (Eq. 11)$$

Substituting the values of $[HI^-]_a$ and $[Th^+]_a$ from Eqs. 8 and 11 into Eq. 2 we obtain the following final equation:

$$K \left\{ [(C_{H_2I}) - S] \frac{A_1 K_1}{K_1 + A_1 D[H^+]} \times \right.$$

$$\left. [(C_{Th}) - S] \frac{K_3}{[H^+] + K_3} \right\} - S = 0 \quad (Eq. 12)$$

To prove the validity of Eq. 12 known values were substituted for the various terms and the equation solved for S with the aid of an IBM 7094 computer.[1]

[1] Computer Center, University of Georgia, Athens, Ga.

The values used were as follows: $K = 3.0 \times 10^5$ l./mole (Table II). $[C_{H_2I}] = 2 \times 10^{-4}, 1 \times 10^{-4},$ and 0.5×10^{-4} moles/l. (same as the experimental concentrations). $[C_{Th}] = 1.186 \times 10^{-5}$ moles/l. (usual concentration used for the assay). $D =$ Distribution coefficient of the dye (Table III). $K_1 = 1$. Bromothymol blue is a strong acid dye and should have more than one pKa value. It was found that bromothymol blue undergoes a color change from deep red to deep yellow between theoretical pH values of -1 and 1, therefore, the first pKa value was estimated to be approximately zero.

$$K_2 = 1.0 \times 10^{-7} \quad (Reference 7)$$

$$K_3 = 1.58 \times 10^{-5} \quad (Reference 8)$$

$[H^+]$-different values ranging from 1×10^{-1} to 1×10^{-11}. Results are presented in Table I and Fig. 1.

DISCUSSION

Several factors strongly influenced the extractability of thiamine dye salt in the organic phase. A change in dye concentration affects the amount of

TABLE I—EFFECT OF pH AND DYE CONCENTRATION ON THE CONCENTRATION OF THIAMINE DYE SALT EXTRACTED IN CHLOROFORM (ACCORDING TO EQUATION 12)[a] WHEN THIAMINE CONCENTRATION IS 1.186×10^{-5} MOLE/l.

	Thiamine Dye Salt Concn. (mole/l.) $\times 10^6$ when Dye Concn. (mole/l.) $\times 10^4$ is		
pH	0.5	1.0	2.0
1.0	1.87×10^{-6}	3.75×10^{-6}	7.50×10^{-6}
2.0	1.87×10^{-4}	3.75×10^{-4}	7.49×10^{-4}
3.0	1.83×10^{-2}	3.66×10^{-2}	7.30×10^{-2}
4.3	3.35	5.27	7.34
5.2	9.66	10.70	11.30
5.6	10.40	11.10	11.50
6.0	10.60	11.20	11.50
6.2	10.60	11.30	11.50
6.6	10.50	11.20	11.50
7.0	10.10	11.00	11.40
7.4	9.20	10.40	11.10
8.0	6.43	8.46	9.93
10.0	1.74×10^{-1}	3.43×10^{-1}	6.67×10^{-1}
11.0	1.77×10^{-2}	3.54×10^{-2}	7.06×10^{-2}

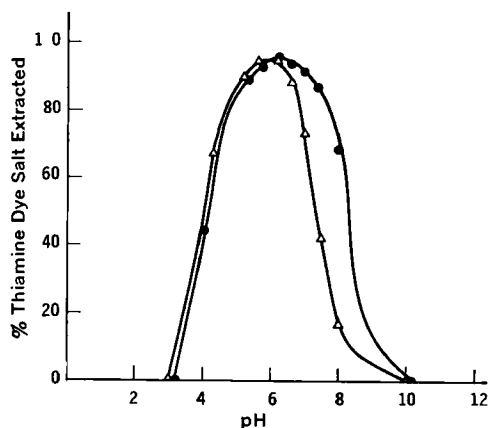[a] Similar results were obtained when solved quadratically.

Fig. 1—Effect of pH on the extraction of thiamine dye salt when dye concentration is 1×10^{-4} mole/l. Key: ●, theoretical; △, experimental. Data taken from Table II, Reference 1.

salt extracted by the first 10-ml. portion of chloroform (1). This can be explained by Eq. 1.

$$K = \frac{S}{[HI^-]_a\,[Th^+]_a}$$

From this equation it can be seen that if $[HI^-]_a$ decreases S will decrease to keep K constant.

The change in the amount of thiamine dye salt extracted by the first 10-ml. portion of chloroform with a change in pH (1) can also be explained by Eq. 1. Since K is constant it is apparent that a change in the concentration of either $[HI^-]_a$ or $[Th^+]_a$ will change the value of S.

Using Eq. 1, a theoretical equation (Eq. 12) was derived (*viz. Theoretical*) to determine the changes in the amount of thiamine dye salt extracted with changes in pH. This equation was programmed for an IBM 7094 computer, and when solved within the pH range of 1–11, the amount of thiamine dye salt predicted was in excellent agreement with the experimental data (Fig. 1). Between the pH range of 7.0 to 8.0, the theoretical values were higher than the experimental values probably due to an error introduced by using the reported value for K_2 (10^{-7}). A K_2 value of 10^{-6} for bromothymol blue gave better theoretical results. The K_1 value of bromothymol blue was not in good agreement with the value reported by Schill (9), however, changes in K_1 values on either side of the approximated value did not affect the results.

The experimental and theoretical data indicate that the extraction of thiamine dye salt with a single 10-ml. portion of chloroform, between the pH range of 5.2–6.6 is greater than 88.4% (Fig. 1). This pH range appears to be the best since three 10-ml. portions of chloroform will extract more than 99.8% of thiamine dye salt.

Although the above equation was derived specifically for a thiamine-bromothymol blue system, no such limitations are placed on this mathematical expression. Therefore, it is suggested that [Th] can be any base and [H₂I] can be any acidic dye. This would provide a theoretical foundation for the well-documented empirical observations of the dependence of the concentration of salt extracted on pH.

The site of reaction in thiamine may be either the amino group of the pyrimidine moiety or the quaternary nitrogen of the thiazole moiety. The amino group may be reacting with the sulfonic acid group of the dye since it is possible to form the salt by directly adding thiamine mononitrate and bromo-

thymol blue in chloroform. On the other hand Eq. 1 predicts the possibility of reaction between the quaternary nitrogen and the sulfonic acid group as follows:

$$HI^- + TH^+ = [HI^- \cdot TH^+]$$
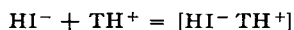
EXPERIMENTAL

Reagents—All chemicals and reagents used were either USP, NF, or ACS grade. Bromothymol blue was purchased from Fisher Scientific Company and was used without further purification.

Solutions—Various solutions were prepared using procedures as reported earlier (1). The ionic strength of the solutions was not adjusted but solutions were prepared in 0.05 M phosphate buffer.

Analytical Procedures—Procedures for determination of thiamine dye salt in organic phase, determination of dye in aqueous and organic phases, and determination of thiamine in aqueous and organic phases have been described (1).

Determination of Equilibrium Constant—The equilibrium constant was determined at three different pH values (5.6, 6.0, and 6.5). To run these experiments solutions containing 10 mcg./ml. of thiamine mononitrate were prepared in various buffer solutions and the thiamine dye salt determined according to *Procedure B* of a previous report (1). Thiamine in the aqueous phase was determined by the USP method (10) and the dye in the aqueous phase was determined by the method described earlier (1).

To determine the equilibrium constant the thiamine mononitrate present in the aqueous phase was assumed to be completely undissociated since the pKa of thiamine mononitrate is 4.8. The concentration of the dissociated dye $(HI^-)_a$ in the aqueous phase was calculated using Eq. 7. Results of the experiments are presented in Table II.

Determination of Distribution Coefficients—To determine the distribution coefficient (D) for bromothymol blue, 10.0 ml. of buffer solution of appropriate pH was vigorously shaken with 10.0 ml. of dye in chloroform solution ($6.7 \times 10^{-5}M$) in a 125-ml. separator for 1 min. The phases were allowed to separate and the dye content in both phases was determined using previously described methods (1). To determine the distribution coefficient the concentration of the undissociated dye in the aqueous phase was calculated using Eq. 5. Results are presented in Table III.

TABLE II—DISTRIBUTION OF THIAMINE AND DYE BETWEEN CHLOROFORM AND WATER (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF EQUILIBRIUM CONSTANT[a]

pH	Thiamine Dye Salt in Chloroform	Thiamine Mononitrate in Water	Dye in Water Total	Dye in Water Diss.[b]	Equilibrium Constant in (l./mole × 10^{-5})
		Concn. in mole/l. × 10^6			
5.6	11.3	0.6	53.0	50.9	4.3
6.0	11.3	0.6	82.0	74.6	2.7
6.5	11.0	0.86	85.0	64.7	2.0
				Av.	3.0

[a] Each value is an average of three experiments. [b] Calculated using Eq. 7.

TABLE III—DISTRIBUTION OF BROMOTHYMOL BLUE BETWEEN CHLOROFORM AND WATER (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF DISTRIBUTION COEFFICIENT[a]

pH	Chloroform	Water (Total)	Water (Undiss.[b] × 10^{-5})	$D \times 10^{-5}$[c]
	Concn. of Dye (Absorbance × 100) in			
6.0	6.40	38.70	3.52	1.80
5.5	15.35	29.50	9.05	1.69
5.0	25.00	19.90	19.90	1.24
4.0	41.20	4.00	40.00	1.03

[a] Each absorbance value is an average of three experiments. [b] Calculated using Eq. 5. [c] In the theoretical equation an average value of 1.5 × 10 (pH 5.0 to 6.0) was used.

TABLE IV—DISTRIBUTION OF THIAMINE HYDRO-
CHLORIDE AND MONONITRATE BETWEEN WATER
AND CHLOROFORM (AT VARIOUS pH VALUES) FOR
THE DETERMINATION OF DISTRIBUTION COEFFICIENT[a]

| | % Thiamine Salt in | | | |
| | Chloro- | Water | Water | |
pH	form[b]	(Total)	(Undiss.[c])	D
Thiamine HCl				
8	0.8	99.2	99.2	0.008
6	0.6	99.4	93.8	0.006
2	0.0	100.0	0.0	0.00
Thiamine Mono-				
nitrate				
8	1.2	98.8	98.8	0.012
6	0.8	99.2	93.6	0.008
2	0.0	100.0	0.0	0.00

[a] Each value is an average of three experiments. [b] Cal-
culated by difference. [c] Calculated using Henderson-
Hasselbach equation.

To determine the D for thiamine, 15 mg. of thia-
mine hydrochloride or thiamine mononitrate was
dissolved in 25.0 ml. of the buffer solution of ap-
propriate pH and shaken vigorously with 25.0 ml.
of chloroform in a 125-ml. separator for 1 min.
The phases were allowed to separate and the
thiamine content in the aqueous phase was deter-
mined using the method described earlier (1).
To determine the distribution coefficient, the con-
centration of undissociated thiamine in the aqueous

phase was calculated using the Henderson-Hassel-
bach equation, and the concentration of thiamine in
the chloroform phase was calculated by difference.
Results are presented in Table IV.

REFERENCES

(1) Gupta, V. D., and Cadwallader, D. E., *J. Pharm.
Sci.*, **57**, 112(1968).
(2) Mukerjee, P., *Anal. Chem.*, **28**, 870(1956).
(3) Schill, G., *Acta Pharm. Suec.*, **2**, 99(1965).
(4) *Ibid.*, **2**, 109(1965).
(5) Divatia, G. J., and Biles, J. A., *J. Pharm. Sci.*, **50**,
916(1961).
(6) Laitinen, H. A., "Chemical Analysis," McGraw Hill
Book Co., New York, N. Y., 1960, pp. 35–36.
(7) "The Merck Index," 7th ed., Merck & Co., Rahway,
N. J., 1960, p. 172.
(8) "Merck Service Bulletin on Vitamin B₁," Merck &
Co., Rahway, N. J., 1955, p. 4.
(9) Schill, G., *Acta Pharm. Suec.*, **1**, 101(1964).
(10) "United States Pharmacopeia," 17th. rev., Mack
Publishing Co., Easton, Pa., 1965, pp. 913–914.

Structure and Activity Relationships
in Molluscicides

By I. NABIH* and M. T. ELWASIMI

Noninfected snails of the *Biomphalari* and *Bulinus* types (intermediate hosts for
Schistosoma mansoni and *Schistosoma hematobium*, respectively) can oxidize instantly
and intensively *p*-phenylenediamine dihydrochloride and its derivatives to the cor-
responding colored trivalent nitrogen-free radicals. This reaction fails to occur
with snails infected with miracedia of *Schistosomes*. The reaction is inhibited through
inhibitors specific for enzymes of the protoporphyrin type, also through the potent
molluscicide (bayluscide). Studies revealed the enzyme involved in the reaction is
the peroxidase, along with the catalase. Both enzymes were found to be present in
both types of snails.

BILHARZIASIS is a world health problem; it in-
fects millions of people all over the world and
causes a tremendous loss in economy and man-
power.

To bring the disease under control different
aspects would be encountered, mainly the irradi-
cation of snails, the intermediate vector of the
parasite, through the application of molluscicides.
The chemotherapy of the disease is still inade-

quate particularly in the areas where infection is
widely distributed, due to the serious side effects
the available agents exhibit when given either
orally or by injection.

In the development and search for new mollus-
cicides, extensive work that included investiga-
tions of thousands of compounds (mostly of
phenolic type) was carried out (1–3). These
investigations showed that activity of these
phenolic compounds depends upon the nature of
substituents on the aromatic ring. The most
active were pentachlorophenol and 2,4-dinitro-6-