SUMMARY

Lychnis alba Mill. is a plant which contains a saponin. Most of the saponin is in the root.

Saponin can be extracted with hot water or hot alcohol, concentrated and purified on a Florisil column, and eluted with 25% alcohol.

Three fractions of *L. alba* saponin have been prepared and it is very likely that they contain just one saponin.

Gelatin blood suspension gel is useful in the detection of saponin on paper chromatograms.

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Kinetics of Thiamine Hydrolysis

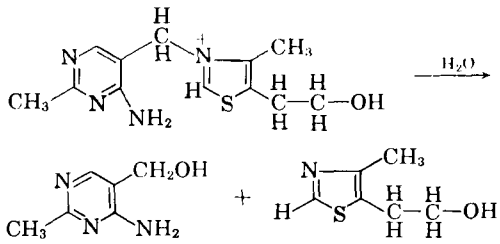
By JOHN J. WINDHEUSER and TAKERU HIGUCHI

The pH-rate profile of the hydrolytic cleavage of thiamine determined from kinetics measurements has been found to be unusually complex. Under essentially buffer-free conditions, the overall behavior suggests existence of at least four separate reactions: (*a*) acid catalyzed mechanism leading to the formation of oxythiamine at pH below 1; (*b*) water cleavage of protonated thiamine yielding a pyrimidine and a thiazole fraction between pH 1–6; (*c*) at pH 2–6.5, hydroxyl ion catalysis of protonated thiamine producing the same products found in reaction (*b*); (*d*) above pH 6.5, water cleavage of thiol thiamine to yield a pyrimidine diamine and other undetermined products. The ionic strength and temperature dependency of the reaction has also been determined. Contrary to earlier works, the hydrolytic reaction appears not to be subject to major negative or positive catalysis by amino acid species. The rate, however, is pronouncedly influenced by what appears to be general base catalysis by several buffer species.

ALTHOUGH hydrolytic cleavage of thiamine, an important pharmaceutical product, represents the major mode of anaerobic degradation of the vitamin, the kinetic dependency of this reaction on pH has not previously been reported in terms of the reactive species present. The present contribution is concerned with the results of an investigation designed to establish the pH profile of this important reaction, to reconfirm the degradation products under the conditions of the experiments and to correlate them with the kinetic observations, to determine whether agents other than hydroxyl and hydrogen ions can accelerate or inhibit the cleavage, and to establish its dependency on other kinetic variables.

Some doubt has been expressed previously on the question of whether the cleavage followed a strictly first-order kinetic expression with respect to the vitamin (1–3), although it appears generally agreed that the rate of hydrolysis was enhanced by increasing pH and the temperature (4–8). Others (9, 10) have also noted that buffers increased the rate of the reaction, although no attempts were made to evaluate the specific effects. McIntire and Frost (2), studying the effects of various compounds on the hydrolytic rate, claimed to have found inhibitory and catalytic effects but did not establish mechanisms for the observed results.

Several cleavage products have been recovered and identified. Watanabe (11), working with the reaction products resulting from the hydrolysis of a 0.5% thiamine solution heated at 140° for 12 hours at approximately pH 3.5, the pH resulting from a solution of the salt itself, was able to isolate and identify the degradation products and proposed the following reaction



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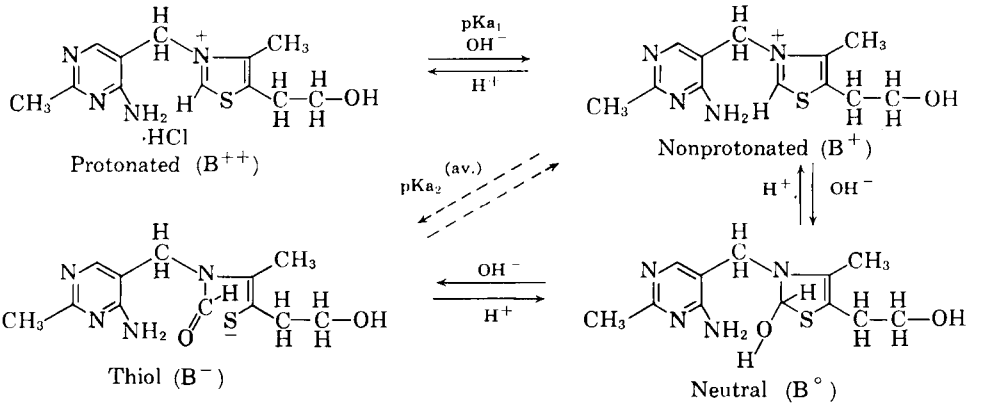
In a recent publication, Watanabe (12), reviewing the physical chemical properties of thiamine, indicated that in alkaline solution the products of the degradation became more complex and differed from those found in the acidic region. Under strongly acidic conditions the products of the degradation again change. Rydon (13) was able to prepare and isolate oxythiamine by refluxing thiamine in 6 *N* hydrochloric acid for 6 hours. This compound appeared to be formed by the hydrolysis of the 4-amino group on the pyrimidine ring.

Any detailed kinetic analysis of the hydrolytic rate must take into account the existence of different thiamine species in solution. The vitamin undergoes several transformations under conditions of increasing pH. Williams (14) found, when titrating thiamine chloride hydrochloride with base, that after one mole equivalent of base, the pH did not continue to rise rapidly on further addition. The addition of two more mole equivalents of base were required for complete neutralization. He further observed that the last two moles of base reacted at a finite rate, indicating an internal rearrangement, which he postulated as follows



Back titration with acid showed the reaction to be reversible. As it was not possible to determine the concentration of the neutral intermediate, it was necessary to calculate an average equilibrium constant for the conversion of the nonprotonated thiamine to the thiol form, rather than the constants for the individual steps. This constant was designated as the average second dissociation constant. From his data, Williams calculated pKa_1 to be 4.8 and pKa_2 to be 9.0 at 25°. These values are in good agreement with those found by later workers (15, 16).

RESULTS AND DISCUSSION

Reaction Order with Respect to Thiamine.—The results of the present investigation clearly indicate that the hydrolytic reaction proceeds at rates directly proportional to the thiamine concentration in accord with the recent suggestion of Watanabe (12) and in contrast to his earlier work (1) and that of Farrer (3) and McIntire and Frost (2). A plot of the logarithm of the residual thiamine concentration *vs.* reaction time yielded a straight line in each case. The calculated first-order rate constants were found to be identical within experimental limitations. The observed half-lives of the reaction at varying thiamine concentrations are shown in Table I. For our studies, solutions were prepared at constant ionic strength, constant buffer concentration, constant pH, but with varying amounts of thiamine.

TABLE I.—EFFECT OF INITIAL THIAMINE CONCENTRATION ON THE OBSERVED HALF-LIFE OF THE REACTION AT pH 6.37 IN SOLUTIONS DEGRADED AT 96.4°

Initial Thiamine, mg./100 ml.	$t_{1/2}$, hr.
200.0	5.4
100.0	5.5
50.0	5.4
25.0	5.4
12.5	5.5
5.0	5.4
1.0	5.4
0.5	5.5

The previously reported changes of the reaction order might be attributed to changes of pH and also variations of ionic strength of the system. At some pH ranges, the ionic strength apparently has a marked influence on the rate of the reaction.

Dissociation Constants of Thiamine.—As previously stated, the evaluation of the reactions of thiamine requires a detailed knowledge of the species present in the system. For this purpose the dissociation constants were determined at varying temperatures and ionic strength such as to permit species analysis under the conditions of the kinetic runs. From spectrophotometric measurements, based on the method of Flexer (17), pKa_1 was estimated to be 4.0 at 96.4° and pKa_2 (av.) to be 7.8. These values were actually obtained by extrapolation of data determined at lower temperatures from Arrhenius-type plots as shown in Fig. 1 since the instability of the compound precluded deter-

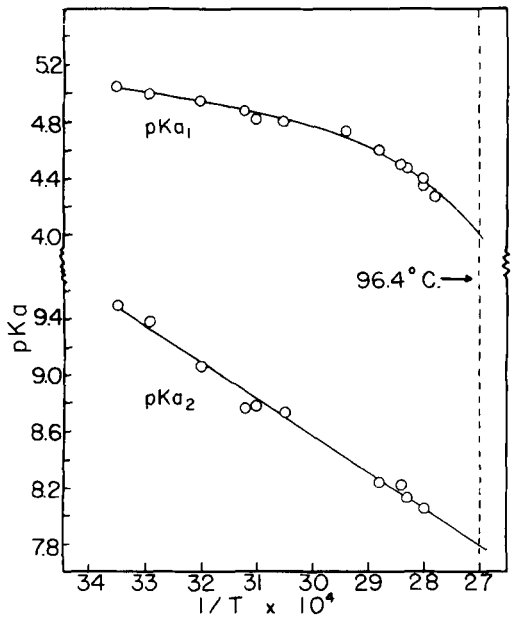


Fig. 1.—Effect of temperature on spectrophotometrically determined pKa_1 and pKa_2 of thiamine at ionic strength 0.5.

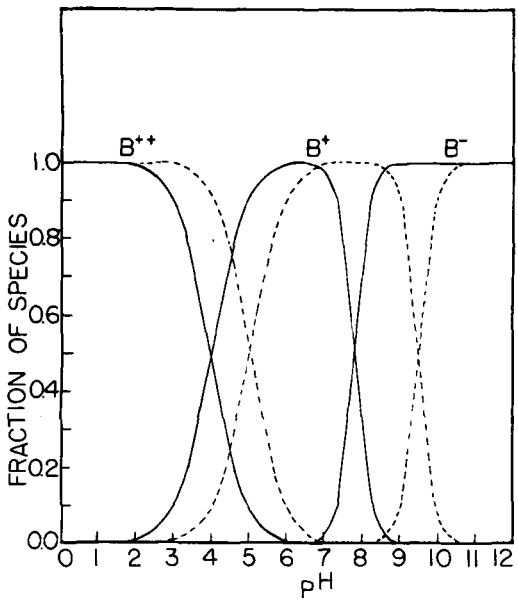


Fig. 2.—Fraction of thiamine species present in solution as functions of pH at 96.4° (—), and 25.0° (---) as calculated from the experimentally determined pKa_1 and pKa_2. B^{++} represents protonated thiamine, B^+ nonprotonated thiamine, and B^- thiol thiamine.

mination at the higher temperature. Figure 2 shows the variations of the thiamine species as a function of pH at 25 and 96.4°. The symbols B^{++} to denote protonated thiamine, B^+ for nonprotonated thiamine, and B^- for the thiol form will be used throughout this presentation.

Identification of the Cleavage Products.—Based on the present findings, and those of others cited, three hydrolytic reactions appear to be responsible for degradation under nonoxidative conditions, the relative importance of each being dependent on the pH of the solution. (*a*) In strongly acid solutions, thiamine apparently degrades by replacement of the primary amino group on the pyrimidine ring by a hydroxyl group to yield oxythiamine. (*b*) The singly protonated and nonprotonated forms which exist in the weakly acid to neutral range seem to undergo simple hydrolytic cleavage at the methylene bridge between the pyrimidine and thiazole groups. (*c*) Under alkaline conditions, where thiamine is present as the thiol form, the end products suggest multiple pathways of degradation. One mechanism resulted in the formation of a pyrimidine diamine and the others resulted in products which have not yet been clearly identified.

To establish the identities of the degradation products over a broad pH range, solutions sealed in ampuls under nitrogen were subjected to hydrolysis at pH values varying from 0.4 to 10.0. The products were separated by a modification of the paper chromatographic technique reported by Lhoest, *et al.* (18). Figure 3 is a composite representation of the chromtographs obtained from the degraded samples along with spots from known compounds. The spots are shown at the average R_f value for each compound and are represented by circles for illustrative purposes.

The results in neutral and acidic media confirmed those of Watanabe (11), in that the end products in this range were 2-methyl-4-amino-5-hydroxymethyl pyrimidine (D) and 4-methyl-5β-hydroxyethyl thiazole (F).

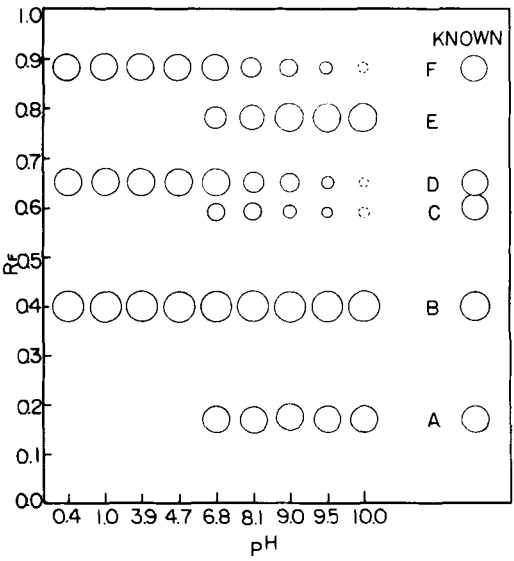
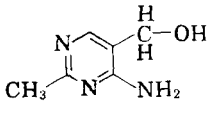
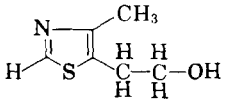


Fig. 3.—Composite representation of paper chromatograms from solutions degraded at 96.4° at various pH's. The spots represent the following compounds: A, 2-methyl-4-amino-5-aminomethyl pyrimidine; B, thiamine; C, thiochrome; D, 2-methyl-4-amino-5-hydroxymethyl pyrimidine; E, unidentified; and F, 4-methyl-5β-hydroxyethyl thiazole. The circles are representative of the size and position of the spots found, not the shape.

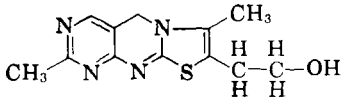


2-Methyl-4-amino-5-hydroxymethyl pyrimidine (D)



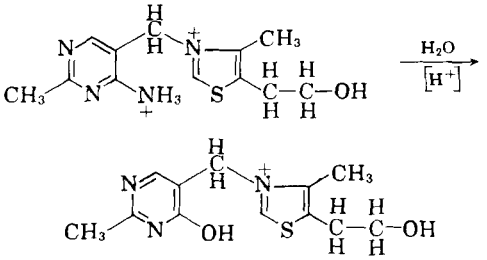
4-Methyl-5β-hydroxyethyl thiazole (F)

These compounds were apparently formed as a result of the hydrolytic cleavage of thiamine at the methylene bridge. Although traces of thiochrome (C) were found on some of the chromatograms, electrofluorometric measurements indicated that the amount formed was negligible. This compound, which is an oxidation product of thiamine may have been produced when the ampuls were opened for removal of sample. At high pH the thiochrome production probably ceased due to the inability of the thiol form of thiamine to yield this compound (15).



Thiochrome

In systems of acid concentration higher than 1 *N* acid, a different route of degradation appeared to become dominant. Rydon (13) found that after refluxing thiamine in 6 *N* hydrochloric acid for 6 hours, a new compound was formed in which the primary amine on the pyrimidine ring was replaced by a hydroxyl group according to the following reaction

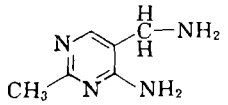


The oxythiamine which was formed did not give the thiochrome reaction.

Chromatography of a solution of thiamine hydrolyzed under Rydon's conditions, indicated that the oxythiamine produced had the same R_f value as thiamine (B) but gave a negative test with ferricyanide reagent. The fact that both thiamine and oxythiamine have the same R_f values in this system may explain why no additional spot was found on chromatographs of solutions degraded at low pH. Only faint traces of the products of the methylene bridge cleavage were found in highly acidic solutions, indicating that the formation of oxythiamine was the main product of the reaction in this range.

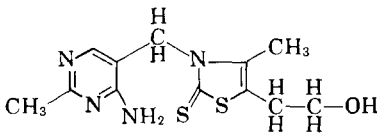
As the pH of the system was increased above 7, the pyrimidine (D) and the thiazole (F) fractions again decreased in intensity. Their decrease was accompanied by the appearance of two new spots, (A) and (E). This progressive change in the nature of the degradation products suggested that the open ring thiol form degraded in a different manner than the closed ring form.

Study of its ultraviolet absorption and paper chromatographic characteristics and reaction to Tollen's reagent and to bromphenol blue-silver nitrate solution suggests that compound (A) is 2-methyl-4-amino-5-aminomethyl pyrimidine, a previously detected degradation product (1, 19).



2-Methyl-4-amino-5-aminomethyl pyrimidine

The nature of the compound giving rise to spot (E) was not clearly established. Other investigators (20, 21) had indicated that at high pH and 100°, thiamine thiothiazolone was formed along with the pyrimidine diamine (A).



Thiamine thiothiazolone

Their conclusions were based on the nature of the ultraviolet absorption spectrum of the degraded solution when adjusted to pH 2.7. It was noted that under these conditions, an absorbance peak was found at 320 mμ which initially increased with time and then decreased, indicating secondary degradation. This absorbance was ascribed to thiamine thiothiazolone as this compound exhibits a marked absorption peak at this wavelength in solutions varying in pH from 2.7 to 9.0. No other thiamine derivative has shown an absorbance peak in this range.

In an effort to identify the compound represented by spot (E), the area was cut out of the paper and the compound eluted with distilled water and the ultraviolet absorption spectrum was determined in solutions at pH 2.8 and 8.5. In the acidic solution a peak was found at 325 and at 243 mμ, the 243 mμ peak being characteristic of the pyrimidine structure. In basic solution, a hypsochromic shift of the spectrum was observed with a new maximum at 305 mμ. Similar results were obtained when samples of the degraded solution were passed through ion exchange columns containing Amberlite IRA-400[1] resin, in the base form, to remove the undegraded thiamine. The eluate adjusted to pH 2.8 and 8.5 exhibited the same spectral characteristics as the samples eluted from the paper. The absorbance at 325 mμ was followed in a kinetic manner, and the results indicated that the compound which was responsible for the observed absorbance appeared to be formed directly from thiamine, based on no observable lag in the time between the initiation of degradation and increase of absorbance. These results were also borne out by following the kinetic procedure by paper chromatographic separations.

The shift of the spectrum of the isolated com-

[1] Rohm and Haas Co.

pound as compared to the stability of known thiamine thiothiazolone possibly casts some doubt on the assumption that the thiothiazolone compound is one of the major degradation products of the reaction under the conditions of this study.

Effect of Ionic Strength on the Reaction Rate.—As might be expected from the various possibilities of thiamine to exist in a charged state, it was found that the rate of the reaction was influenced by ionic strength. The salt effects varied as a function of the thiamine species present and of the mechanism of degradation. It is of pharmaceutical interest, that where the salt effect was operative, an increase of ionic strength resulted in a lower rate of degradation. As pointed out earlier, this effect might also have been a contributing factor to the apparent changes in the order of the reaction found by others (1–3).

Solutions were prepared at pH 2.0, 3.90, 6.40, 6.95, and 10.0 and all degraded at 96.4° except the pH 10.0 samples which were run at 75.0° due to the rapidity of the reaction. The results shown in Fig. 4 only depict the changes found at pH 3.90, 6.40, and 6.95, because at pH 2.0 and 10.0 the reaction was found to be independent of ionic strength. In all cases where changes in rates were observed, it was noted that a plateau existed over which the rates were invariant in respect to ionic strength. For the purpose of accuracy, all subsequent work was conducted at ionic strength 0.5 as this value corresponded to the middle of the plateau at each pH.

To conduct these studies, solutions were prepared at constant thiamine concentration, constant buffer concentration, constant pH, but at varying ionic strength. The ionic strength was controlled by the addition of sodium chloride.

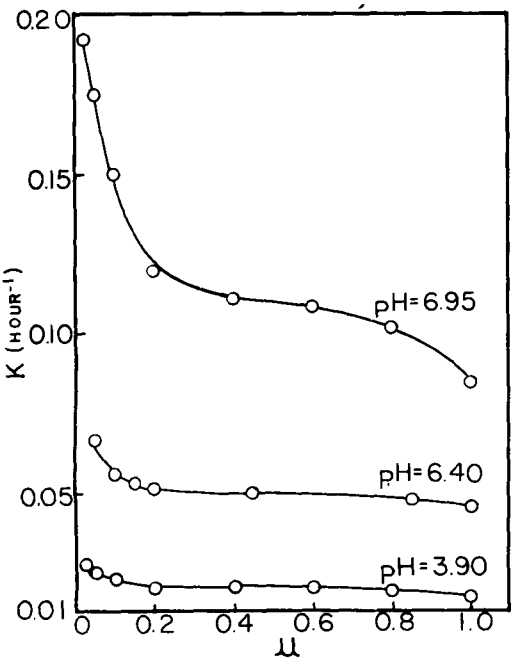


Fig. 4.—Effect of ionic strength on the observed overall rate of hydrolysis at 96.4° at several pH values.

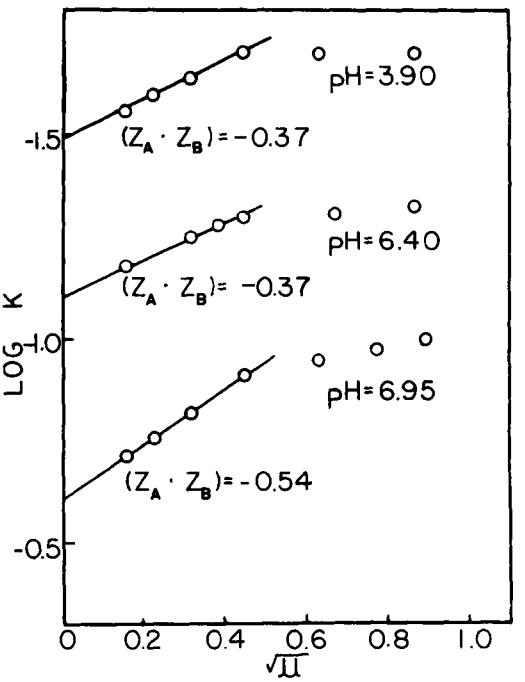


Fig. 5.—Bronsted-Bjerrum-type plot of the logarithm of the observed rate constants against $\sqrt{\mu}$ to estimate the charges on the interacting species at 96.4°.

The data were analyzed by utilization of the Bronsted-Bjerrum equation (22), which relates the primary salt effect on the observed rate to the ionic strength. Figure 5 shows the results of the treatment of the data in this manner. As might be expected, the experimental values deviate rapidly from linearity as this relationship was derived on the assumption of low ionic strength, but the values obtained from the initial slopes were of interest. The finding of nonintegral values for $(z_a \cdot z_b)$ might be due to high ionic strength, the occurrence of more than one reaction at the point of determination, or to a significant contribution by the secondary salt effect. A more complete correlation of these effects will be taken up under the discussions leading to a possible rate equation describing the pH profile of the reaction.

Effect of Buffers as General Acid or General Base Catalysts.—Although earlier studies utilizing phosphate, acetate, and citrate buffers have shown that hydrolysis of thiamine was apparently hastened in their presence, no serious analysis of this effect was made. The findings of the present study indicated that the vitamin was subject to general base catalysis but not to general acid catalysis, the effect being functions of both the buffer species and the forms of thiamine present in the solution. Figures 6–9, representing the results of these studies, are shown as plots of the reciprocal of the half-lives of the reactions *vs.* buffer concentrations under conditions of constant pH. In these studies, solutions were prepared at constant ionic strength, constant pH, and constant thiamine concentration, with only the buffer concentration varying at a given pH.

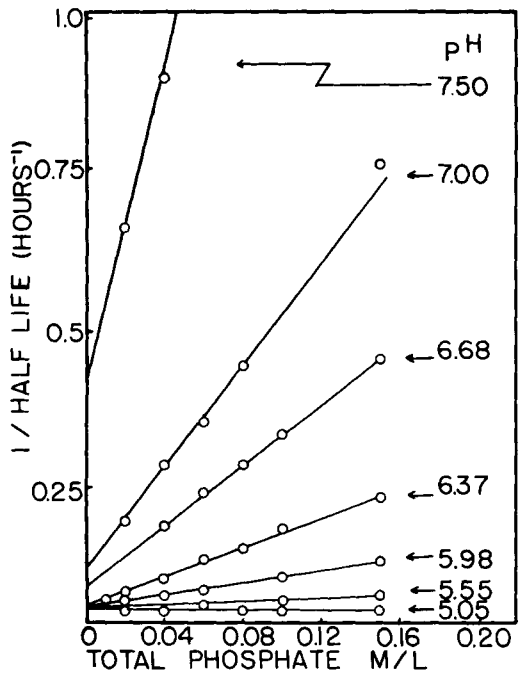


Fig. 6.—Reciprocal of the half-life of thiamine hydrolysis in a phosphate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

This was repeated at a number of pH values within the effective range of the buffers employed.

It was not possible as a part of this investigation to separate clearly the observed total buffer catalysis into the specific contributions of the several general acids and bases present acting on any or several of the thiamine species in solution. If the catalytic activities of these systems are largely limited to their base forms, as apparently indicated by the increasing slopes of the lines in the plots at higher pH, it can be readily shown that

$$[H^+] = Ka \cdot a(1/m) - Ka$$

where Ka is the apparent dissociation constant of the buffer at the temperature of the study; a is a proportionality constant between the slope of the line and the concentration of the conjugate base; m is the slope of the line resulting from the plot of the reciprocal of the half-life against buffer concentration at constant pH. The equation is, as indicated, based on the assumption that only the conjugate base of the buffer is catalytic and that the concentration of the effective substrate is constant. It predicts that a plot of $[H^+]$ against the reciprocal of the slope should yield a straight line with a slope of $Ka \cdot a$ and having an intercept of $-Ka$.

Figure 10 shows the data obtained from the acetate system plotted in accordance with the above equation. The linearity of the experimental points in the higher pH range where essentially only nonprotonated thiamine exists is in conformance with the derived equation. At lower pH, a deviation is noted which indicates that protonated thiamine is perhaps slightly more prone to acetate catalysis. Extrapolation to the intercept yields the dissociation constant of the buffer 1×10^{-5}, which is in good agreement with the experimentally determined value at 96.4°.

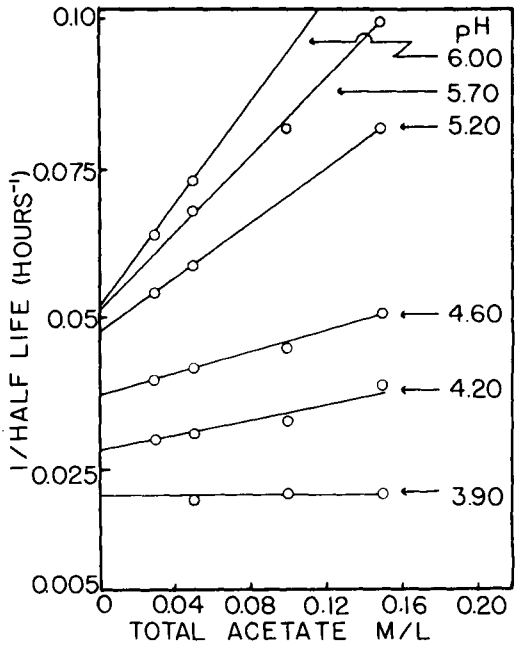


Fig. 7.—Reciprocal of the half-life of thiamine hydrolysis in an acetate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

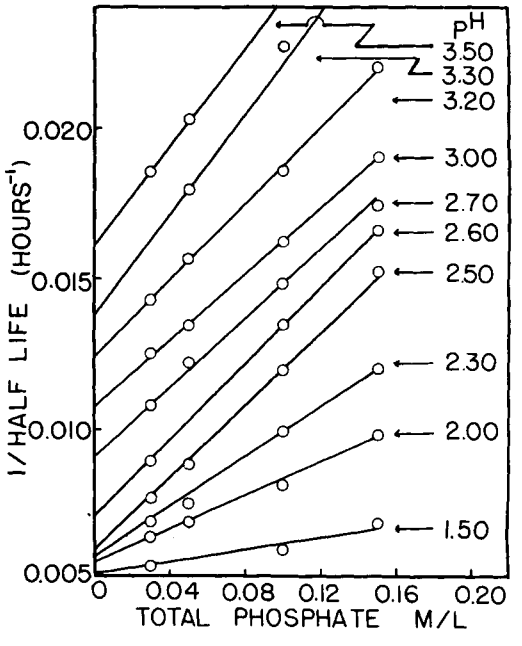


Fig. 8.—Reciprocal of the half-life of thiamins hydrolysis in a phosphate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

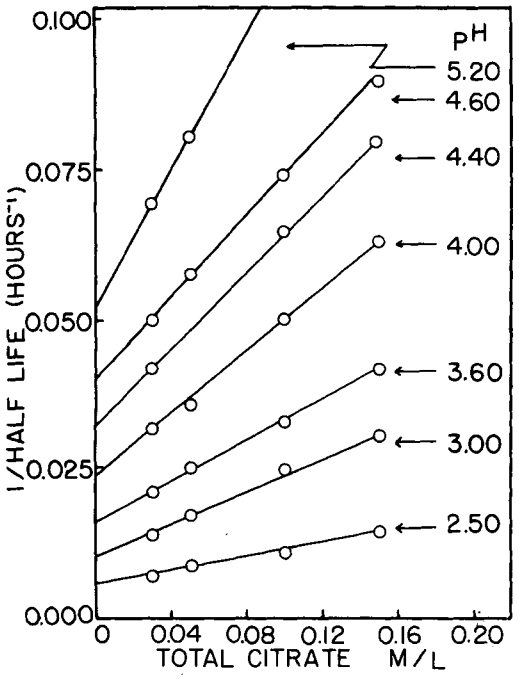


Fig. 9.—Reciprocal of the half-life of thiamine hydrolysis in a citrate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

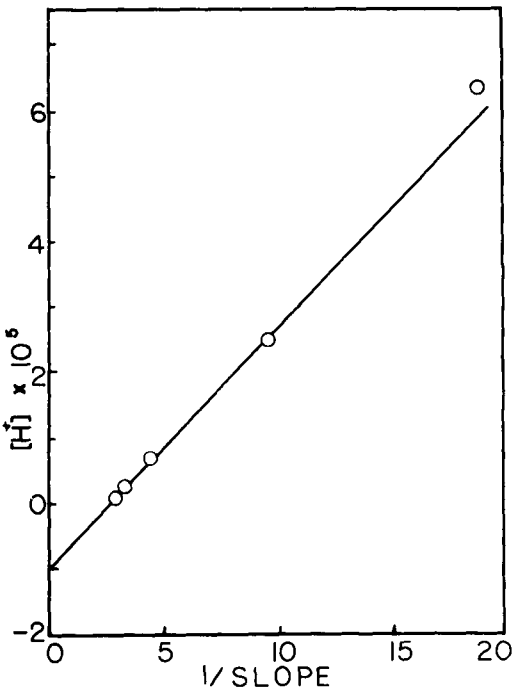


Fig. 10.—Plot of $[H^+]$ *vs.* the reciprocal of the slope of the lines from the acetate system of Fig. 7 in accord with the equation: $[H^+] = Ka\,(a)\,(1/m) - Ka$.

Analysis of the data presented in Fig. 7, depicting the results of degradation in a phosphate buffer system in the pH range 5–7.5, indicates that this reaction may be a function not only of the dibasic phosphate ion, but of the tribasic phosphate ion as well. In this pH range, the nonprotonated thiamine is in equilibrium with the thiol form, whose concentration will increase as the pH is increased. From studies at pH 10, it has been found that the thiol thiamine is not subject to general base catalysis. The essentially zero slope of the line at pH 5.05 indicates that the monobasic phosphate salt is noncatalytic with respect to the nonprotonated thiamine. From these observations it can be suggested that the results found are due to the following reactions

$$B_1 \text{ (nonprotonated)} + HPO_4^{=} \rightarrow \text{products}$$

$$B_1 \text{ (nonprotonated)} + PO_4^{\equiv} \rightarrow \text{products}$$

Mathematical treatment of the data based on the above reactions yields results which are in good agreement with the experimental findings up to pH 7. Above this value there is a negative deviation of the calculated values from the experimental. This deviation may be indicative that the reaction is more complex than anticipated in this region.

Further investigation of the phosphate catalysis was carried out in the pH range 1.5–3.5. The experimental data as shown in Fig. 8 indicate that protonated thiamine is catalyzed by monobasic phosphate ion and not attacked by the undissociated acid. Calculations based on this assumption, together with the previous supposition that the nonprotonated thiamine was not catalyzed by monobasic phosphate, were in good accord in the experimental findings.

The catalytic effects of a citrate system are shown in Fig. 9. From this plot it can be suggested that again the undissociated acid form of the buffer has no catalytic action, but that the buffer anions act as general base catalysts. The interactions are not postulated on a species basis, as this system is too complex to lend itself to this treatment.

Effect of Various Compounds on the Rate of Hydrolysis.—It has previously been reported authoritatively in the literature (2) that a number of compounds such as amino acids, amides, and amines had a marked and surprising effect on the rate of this mode of degradation. A concentration of 0.05 moles per L. of glycine added to a thiamine solution at pH 6.0 apparently produced a threefold decrease in the rate of hydrolysis at 100°. The addition of the same concentration of γ-aminobutyric acid under the same conditions, on the other hand, appeared to increase the rate of the degradation fourfold. Similar effects were found with other compounds and it was noted that in the case of the amino acids, α and β acids tended to stabilize thiamine, whereas those with the amino group further removed from the carboxyl group enhanced degradation. In the cases of the other compounds tested, the effects could not be so clearly related to structural changes.

The magnitude of the effects apparently observed at the low concentrations employed was surprisingly large, in fact so great as to be rationalized on the basis of complex formation between the thiamine and the added agent which might enhance or retard hydrolysis. This type of behavior has been re-

ported by other investigators (23, 24), compounds such as procaine and benzocaine having been stabilized by complexation with a number of xanthine derivatives. Because of the great theoretical significance of this effect, if real, a number of compounds studied earlier were reinvestigated. Table II summarizes the results of present investigations designed to retest this effect.

TABLE II.—EFFECTS OF A SERIES OF COMPOUNDS IN 0.05 *M* CONCENTRATION ON THE OBSERVED HALF-LIFE OF THIAMINE HYDROLYSIS AT pH 6.0 AND 96.4°

Compound Added	$t_{1/2}$, hr.
Control	9.5
Glycine	7.4
Alanine	7.5
Nicotinamide	7.1
Nicotinic acid	7.3
Isonicotinic acid	7.5
Picolinic acid	9.7
Salicylic acid	8.6

In obtaining these results, 0.05 mole per L. of the compound being tested was added to separate thiamine solutions maintained at pH 6.0 by a 0.1 molar phosphate buffer. The ionic strength of the system was maintained at 0.5 by the addition of sodium chloride. Data shown represent means of duplicate runs, reproducibility of the result being of the order of 0.1 hr.

Contrary to the previously published findings, glycine and alanine were found not to exhibit any stabilizing effects on the reaction, but instead showed slight catalytic activity. The other compounds listed, with the exception of picolinic and salicylic acids, exhibited approximately the same slight degree of catalysis. This limited study suggests that the previously reported remarkable changes brought about by the use of some of these compounds may well have been due to other kinetic variables such as ionic strength effects, pH changes, or general base catalysis.

pH-Rate Profile of Thiamine Hydrolysis.—Although the marked influence of hydrogen ion concentration on the rate of the hydrolytic degradation of thiamine was recognized by many early workers (4–8), no previous serious attempts clearly separating the influence of pH from the effects of buffer catalysis seem to have been made. Buffer-free rates at various pH values can, however, be readily picked from Figs. 6–9 since the intercepts of these plots represent the hydrolytic rates at zero buffer concentration. These rates expressed as first-order rate constants are shown in Fig. 11 as a function of pH.

The data shown for pH values above 7.5 were actually run at 75.0° since the hydrolytic rates were too fast at 96.4°, the temperature used for the more acid systems. Actual values obtained at the lower temperature are shown plotted in Fig. 12. These were corrected to the higher temperature by determining the thermal coefficient as shown in Fig. 13, and are represented on the profile by solid circles.

The profile suggested that the overall degradative rate represented a summation of a relatively large number of separate reactions. This is apparent in the analysis of the plot in terms of its observed slope over limited ranges as shown in Table III.

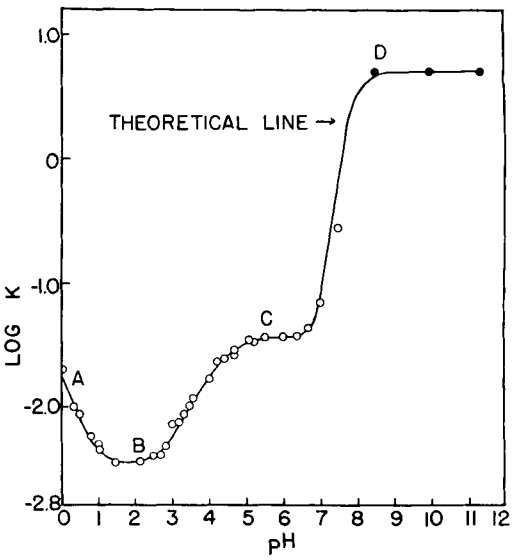


Fig. 11.—pH-rate profile of thiamine hydrolysis at 96.4°. The circles represent the experimental results and the line corresponds to that expected theoretically from the four proposed reactions. The solid circles were calculated from lower temperature studies.

As with all kinetic relationships, a number of different chemical reactions can lead to the same observed experimental dependencies. For the present system a series of four reactions such as

1. protonated thiamine (B^{++}) + [H^+] $\xrightarrow{k_{H^+}}$
2. protonated thiamine (B^{++}) + H_2O $\xrightarrow{k_{H_2O}}$
3. protonated thiamine (B^{++}) + OH^- $\xrightarrow{k_{OH^-}}$
4. thiol thiamine (B^-) + H_2O $\xrightarrow{k'_{H_2O}}$

would be sufficient to fit the experimental points. It is apparent that we can write

$$-dB_T/dt = k_{H^+}[B^{++}][H^+] + k_{H_2O}[B^{++}] + k_{OH^-}[B^{++}][OH^-] + k'_{H_2O}[B^-]$$

where k_{H^+} k_{H_2O} k_{OH^-} and k'_{H_2O} are the specific catalytic constants and B_T represents the total thiamine. By substituting for the thiamine species in terms of their equilibrium concentrations in the manner previously utilized by Higuchi, *et al.* (25), in their studies of procaine hydrolysis, the equation can be rewritten to yield the following

$$k_{obs} = k_{H^+}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right][H^+] + k_{H_2O}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right] + k_{OH^-}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right] \times \frac{k_w}{[H^+]} + k'_{H_2O}\left[\frac{Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right]$$

Ka_1 is the equilibrium constant for the conversion

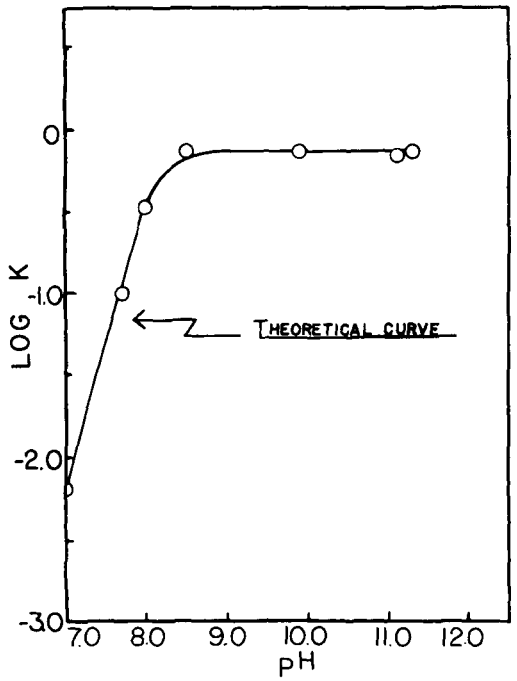


Fig. 12.—Logarithms of experimentally determined overall rate constants for the hydrolysis shown as functions of pH at 75.0°. The solid line corresponds to the theoretical function calculated from the observed rate at pH 9.9 and previously determined pKa values.

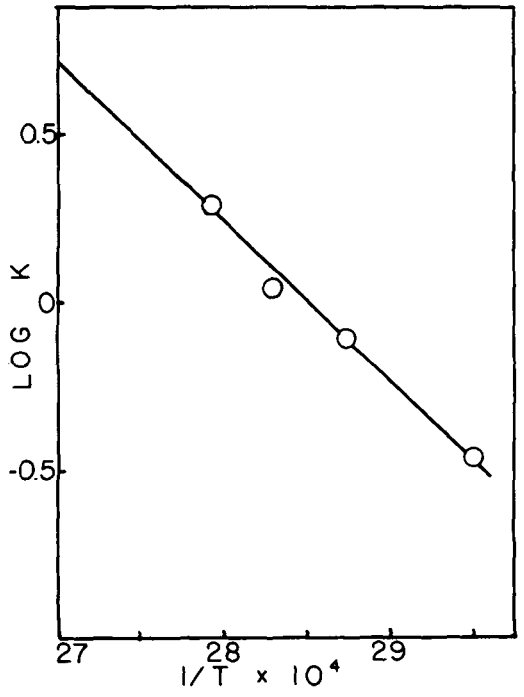


Fig. 13.—Arrhenius-type plot of the logarithm of the observed rate constants against the reciprocal of absolute temperature for the hydrolysis at pH 9.9 under conditions of constant pH. The line is extrapolated to 96.4°.

TABLE III.—APPARENT HYDROXYL ION DEPENDENCY OF THE HYDROLYTIC REACTION

pH Range	Apparent Slope
0–1	−1
1.5–2.5	0
3–4	0.4
5–6	0
7–7.5	2
9–11	0

of protonated thiamine to the nonprotonated form; Ka_2 is the average equilibrium constant for the conversion of the nonprotonated form to thiol thiamine.

It is evident that k'_{H_2O} and k_{H^+} can be readily evaluated from the two extremities of the profile. The remaining two rate constants can be easily calculated from any two points in the central part of the profile and from the two Ka values previously determined. When the constants thus obtained were substituted into the expanded equation, the functions shown as solid lines on Figs. 11 and 12 resulted.

The good agreement of the experimental data and the calculated theoretical profile does not unambiguously establish the nature of the interactions along all segments of the curve. The major reaction along the D portion of the curve on Fig. 11 could be formulated on the basis of nonprotonated thiamine being attacked by two hydroxyl ions or by the spontaneous cleavage of thiol thiamine by water. The choice of the latter interaction, reaction 4 above, as largely representing the D section of the curve is based on the absence of a primary salt effect and general base catalysis in this region; whereas in the alternative case, both of these effects would be expected.

Similar equivalences could be set forth for the dominant reaction along the C segment. Here, the observed effects could be due to protonated thiamine reacting with hydroxyl ion or due to water cleavage of the nonprotonated thiamine; these are kinetically indistinguishable. Again the choice as the most probable mechanism must be made on the basis of other supportive evidence. The negative dependency on ionic strength, as indicated in Fig. 5, points to the reaction of negatively and positively charged groups. The value of −0.37 for the product of the charges of the interacting species does not agree with the −2 predicted by the Bronsted-Bjerrum theory, but this discrepancy might be attributed to the high ionic strength of the experimental solutions as compared with the limitations of the equation. Further, the postulation of a cleavage of the nonprotonated thiamine by water alone would also indicate that B^+ reacted faster with water than B^{++}. This would not be in accord with the results found with buffered systems, where it was found that although the monobasic phosphate ion did not appear to catalyze the degradation of B^+, it was active in respect to B^{++}. In general, it appears that the protonated thiamine is more susceptible to nucleophilic attack than the nonprotonated species. Hence it would appear probable that the main reaction in the C segment can be attributed to B^{++} reacting with OH^-, reaction 3 above.

The failure to find any salt effect at pH 2.0 tends to support the suggestion that the most important

reaction in the B region is reaction 2, cleavage of B^{++} by water. The salt effects observed at pH 3.90 are in agreement with the proposed reactions, as in this region the observed effects are a composite of reactions 2 and 3.

In the strongly acid region designated by the letter A, the reaction is first order in respect to hydrogen ion. From the work of Rydon (13) and our own studies, it appears that the mode of degradation changes from the methylene bridge cleavage associated with reactions 2 and 3, to the hydrolysis of the 4-amino group on the pyrimidine ring. They night be postulated to involve ring protonation with subsequent attack by water.

Although it would appear from the above discussion that the series of four reactions adequately fit the data at hand, the possibility and perhaps the probability that the true situation may involve one or more substitute reactions is acknowledged. Kinetic analyses can readily eliminate many proposed reaction mechanisms, but they cannot establish the correct ones with absolute certainty.

Determination of the Apparent Activation Energies of the Hydrolytic Reactions.—The apparent activation energies of the reactions were determined by measurements of the observed rate constants at varying temperatures under conditions of constant pH. These determinations were made on solutions at pH 0.05, 1.70, 6.37, and 9.90. These points were picked because at each, only one term of the proposed rate equation for the pH dependency of the reaction was significant, therefore it was possible to estimate the temperature dependence of the individual reactions responsible for the profile. At pH 6.37, where buffers were employed, the rates were those obtained by extrapolation to zero buffer concentration.

The results shown in Fig. 13 were obtained from studies at pH 9.90 and changes at the other pH values followed similar linear relationships when log k was plotted against the reciprocal of the absolute temperature. The linearity of the points over the temperature range indicated the adherence of the observed reactions to the Arrhenius equation and allowed calculation of the apparent activation energies from the slopes of the lines. The results are summarized in Table IV.

These energy values appear to be within the expected magnitude based on the stability of the vitamin as found in our studies and by others. These high activation energies are of practical significance in the preparation of sterile thiamine solutions. Near 100°, the rate of the degradation increases by a factor of approximately 2.5 for every 10° rise in temperature. This change in the rate may be important when determining the most suitable conditions for sterilization.

TABLE IV.—APPARENT ACTIVATION ENERGIES FOR HYDROLYSIS DETERMINED UNDER SEVERAL CONSTANT pH CONDITIONS

Corresponding Reaction	pH of Experiment	E_a, Kcal./mole
1	0.05	24
2	1.70	29
3	6.37	25
4	9.90	21

EXPERIMENTAL

Reagents.—All chemicals used in the preparation of the buffers and for the preparation of the kinetic solutions were of analytical reagent grade except for the special compounds listed below: Thiamine chloride hydrochloride was obtained by double recrystallization of U. S. P. grade material from 95% ethanol and dried under vacuum at room temperature, m.p. 247–248°. 2-Methyl-4-amino-5-aminomethyl pyrimidine dihydrochloride was prepared by the acid degradation of thiamine disulfide according to the method of Zima and Williams (19), m.p. 264°. 2-Methyl-4-amino-5-hydroxymethyl pyrimidine hydrochloride was isolated from the hydrolysis of thiamine according to the method of Watanabe (1), m.p. 219°. 4-Methyl-5-hydroxyethyl thiazole hydrochloride was also isolated from thiamine hydrolysis in the manner described by Watanabe (1), m.p. 96°. Oxythiamine hydrochloride was prepared according to the method of Rydon (13), m.p. 193–194°.

Analytical Procedure.—Residual thiamine concentrations were determined by the thiochrome method of U. S. P. XVI (26). All compounds used in the kinetic studies were tested for interference with the analytical method.

Kinetic Procedure.—All solutions for kinetic studies were prepared with glass-distilled water to minimize metal catalyzed oxidation. Prior to the addition of the thiamine to the buffers, the solutions were heated to expel oxygen and cooled under an atmosphere of nitrogen. After the addition of the thiamine, the solutions were filled into ampuls and again flushed with nitrogen before sealing. The samples were then placed in a constant temperature bath which was maintained at the desired temperature, ±0.05°. After allowing 15 minutes for temperature equilibration, the zero-hour samples were removed and further samples were withdrawn at time intervals suitable to the nature of the system. The reaction was quenched by immersion of the samples in ice water.

Control of pH.—In all cases except where citrate buffers were used, the solutions were prepared by precalculation of the buffer concentrations utilizing experimentally determined apparent pKa values of the buffers. The pKa values were determined from pH measurements on solutions containing equal concentrations of the buffer acid and salt at the ionic strength desired. These determinations were made at various temperatures and the results extrapolated to 96.4°. In all cases, the ionic strength was adjusted by the addition of sodium chloride.

In the case of the citrate system, due to its complexity, it was not feasible to precalculate the solutions, therefore the literature pKa values were used to calculate ionic strength and the pH of the solutions were adjusted by the addition of base.

Determination of Thiamine Dissociation Constants.—The acid dissociation constants of thiamine were determined spectrophotometrically using the method of Flexer, *et al.* (17), which was modified by use of circulating apparatus for photometric titrations as described by Rehm, *et al.* (27). This modification allowed titration of the sample to a desired pH, and after recording the spectrum, titrating further without the necessity of preparing separate solutions. The method is based on the determina-

tion of the absorbance of a compound at a pH where it is completely unionized and then measuring the absorbance of the same concentration at a pH where it is completely ionized and at some intermediate point. The apparent pKa_1 was calculated from the following equation

$$pKa = pH - \log A_{B^{++}} - A/(A - A_{B^{+}})$$

where $A_{B^{++}}$ is the absorbance of the protonated thiamine, $A_{B^{+}}$ is the absorbance of the nonprotonated thiamine, and A the absorbance of a solution containing a mixture of the two species. The absorbance was measured at 255 mμ using a Cary recording spectrophotometer. This wavelength was used as it most readily reflected the changes of the species in the solution.

Although the above procedure was suitable for the determination of pKa_1, which is a simple one-step dissociation, modifications in the equation were required for the estimation of the second constant. As described in the earlier sections, the second dissociation constant is essentially an average value for a two-step mechanism, therefore a new model was used in the calculation. The model used was that of a dibasic acid in which the first and second dissociation constants were equal, only allowing measurement of the initial and final forms. Application of this relationship to the thiamine system, the equation derived was as follows

$$pKa_2\ (av.) = pH - {}^1/_2 \log A_{B^{+}} - A/(A - A_{B^{-}})$$

where $A_{B^{-}}$ is the absorbance of the solution when all of the thiamine is in the thiol form. The other symbols are as used above.

The thiamine concentration was maintained at 4 mg./100 ml. throughout the titrations and the ionic strength maintained at 0.5. The change of ionic strength during the titration was negligible due to the swamping effect of the added electrolyte. Solutions were allowed to equilibrate at least 15 minutes after each change of pH before recording the spectrum.

Chromatographic Separation of Degradation Products.—Solutions of thiamine in 1% concentration were prepared at various pH's employing the same methods as used with the kinetic samples, and degraded at 96.4° for a time equivalent to two half-lives of the reaction. Since the observed R_f value of a compound is dependent on the form in which it exists in a system, all solutions were readjusted to pH 3.5 prior to spotting on the paper to maintain constancy of species. The samples were chromatographed using the method described by Lhoest, *et al.* (18), with several modifications. Whatman No. 4 chromatography paper used by the previous workers and sample size was reduced from 0.1 ml. to 0.01 ml. to yield better definition of the spots. Development of the spots was carried out in the same manner as that of earlier workers, except that known degradation products were run along with the degraded solutions. The inclusion of the known compounds allowed identification of most of the spots found from the degraded solutions.

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