Oxidation of IB.—The solution contained 0.1600 mmole of IB and 0.1875 mmole of perbenzoic acid. The average rate constant was found to be 0.29 ± 0.03 l./mole-min.

Oxidation of IC.—The solution contained 0.4355 mmole of IC and 0.9035 mmole of perbenzoic acid. After an almost instantaneous loss of one equivalent of perbenzoic acid the rate of oxidation approached that of the rate of oxidation of IA, and during the interval of 400–800 minutes the rate of oxidation coincided with the rate reported above for IC.

Ultraviolet Spectra.—The ultraviolet spectra were determined in 95% ethanol by means of a Beckman DU spectrophotometer. The absorption curves are reproduced in Fig. 2.

PITTSBURGH 19, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Structures of Thiamine in Basic Solution[1]

BY GEORGE D. MAIER AND DAVID E. METZLER

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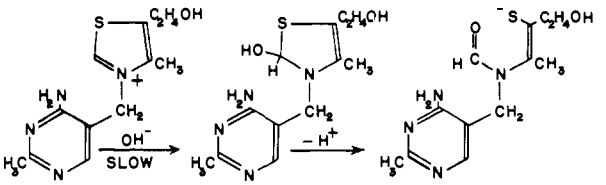
Thiamine in basic solution is instantly converted to a yellow form which fades within a few minutes in water but which persists for several hours in methanol. The *p*H dependence of the zero-time concentration of this yellow form indicates that it arises by the simultaneous loss of two protons from the neutral form of thiamine with an apparent average pK value of 11.6 at 19°. In methanol the two dissociation steps are distinct and an intermediate can be detected. This intermediate non-ionic form of thiamine has been isolated as a white crystalline solid. It apparently arises through the intramolecular addition of the amino group of thiamine to the thiazolium ring with loss of one proton; opening of the thiazole ring and loss of a second proton produce the yellow form. The *p*H dependence indicates that fading of the yellow color results from formation of the pseudo-base and open-ring thiol forms. The possible biochemical significance of these unstable forms of thiamine is considered.

Thiamine (I) reacts slowly with hydroxyl ions to form a pseudo-base II and a thiol form III in which the thiazole ring has been opened.[2–4] In addition, it has been known for many years that thiamine in alkaline solutions develops a transient yellow color.[5,6] We have investigated this latter phenomenon in the belief that it might shed some light on the mechanism of the catalytic action of this vitamin.



I II III

Pseudo-Base and Colorless Thiol Forms.—Figure 1, curve A shows the ultraviolet absorption spectrum of the neutral form of thiamine (I) and (curve B) that of thiamine at *p*H 10.4, 90 minutes after its addition to this basic buffer. The latter represents the spectrum of the thiol, III, which forms slowly over a period of about an hour at this *p*H. An identical spectrum is observed for the crystalline sodium salt of III[6] dissolved in 0.02 N KOH. (Addition of base to simpler thiazolium salts such as 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium chloride causes very similar absorbancy increases at all wave lengths.)

The two reactions by which the neutral form of thiamine is converted to the thiol form III can be regarded as formally equivalent to the stepwise dissociation of a diprotic acid with characteristic acid dissociation constants which can be measured titrimetrically. Williams and Ruehle[2] have reported the apparent pK_{av} (average of the apparent pK's for the two steps) of 9.0, and Watanabe and Asahi,[4] of 9.33 at 25°. We have estimated pK_{av} spectrophotometrically as 9.3 for thiamine and 10.3 for the simpler 3,4-dimethyl-5-(2-hydroxyethyl)- and 3-benzyl-4-methyl-5-(2-hydroxyethyl)-thiazolium salts.

The Yellow Form of Thiamine.—The addition of thiamine to solutions of *p*H 11 and above leads to the instantaneous production of a yellow color, which fades rapidly in aqueous media,[5] but which is clearly visible, depending on the basicity, for an hour or more in methanol. Figure 1C shows the spectrum of thiamine in 0.1 N NaOH extrapolated to zero time (see Experimental) and Fig. 4A the spectrum in methanolic KOH. Since these spectra are not further changed at higher base concentrations, we believe that they represent a single substance. No other forms of thiamine absorb light above 325 mμ; hence we have used the absorbancy at 349 mμ to measure the concentration of this yellow form in mixtures. In water the yellow form decays within several minutes to a substance having a spectrum identical to that of the thiol form III (Fig. 1B). A similar but much slower decay occurs in methanol. The change leading to color formation appears to be completely and rapidly reversible as indicated by the quenching of the yellow color and the immediate appearance of the spectrum of thiamine hydrochloride upon addition of hydrochloric acid to a solution containing the yellow form. The yellow color can then be regenerated quantitatively (97% or more of the original amount) by addition of more base.[5]

Figure 2 shows the increase in zero time absorbancy at 349 mμ with increasing *p*H of an aqueous

(1) This study was supported in part by the National Science Foundation. Journal Paper No. J-3174 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 1259.

(2) R. R. Williams and A. E. Ruehle, THIS JOURNAL, **57**, 1856 (1935).

(3) H. T. Clarke and S. Gurin, *ibid.*, **57**, 1876 (1935).

(4) A. Watanabe and Y. Asahi, *J. Pharm. Soc. Japan*, **75**, 1046 (1955).

(5) R. A. Peters and J. H. L. Philpot, *Proc. Roy. Soc. (London)*, **B113**, 48 (1933).

(6) O. Zima and R. R. Williams, *Ber.*, **73**, 941 (1940).

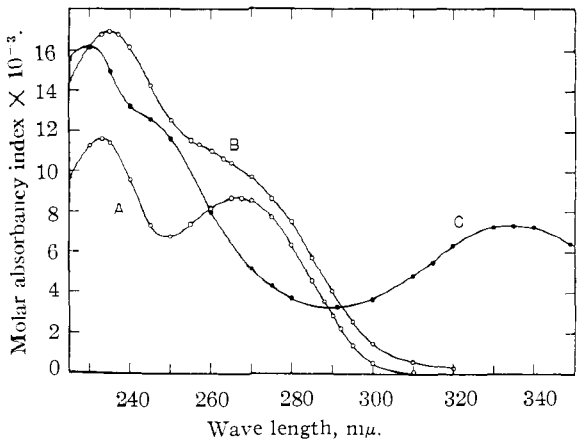


Fig. 1.—Spectra of thiamine in aqueous solutions: A, thiamine at *p*H 6.8, phosphate buffer; B, thiamine in *p*H 10.4 piperidine buffer after standing 90 minutes, or in 0.02 *N* KOH after standing 30 minutes, and white sodium salt of thiamine in 0.02 *N* KOH; C, thiamine in 0.1 *N* NaOH at time of mixing (from extrapolation).

solution of thiamine. The curve is an S-shaped "titration curve" similar to that ordinarily observed when a spectral change results from the dissociation of an acidic group. Curve A (Fig. 2) has been constructed to pass through the experimentally observed mid-point at *p*H 11.6 and to approach the limiting absorbancies at the high and low *p*H ends; it has the shape theoretically expected for the dissociation of a monoprotic acid or a diprotic acid in which $K_1 >> K_2$. However, the experimental points do not fit this curve, but do fit curve B which has a mid-point slope of twice that of curve A and which represents the theoretical shape expected for a diprotic acid in which the two protons dissociate simultaneously with no detectable amount of an intermediate singly dissociated form ($K_1 << K_2$) and $pK_{av} = {}^1/_2(pK_1 + pK_2) = 11.6$ (the apparent pK_{av} for an ionic strength of 0.2) at 19°. This unusual type of behavior is known for several compounds reported by Schwarzenbach[7] and for benzothiazole methiodide.[8] It is evident from the shapes of the published titration curves that the conversion of thiamine to the colorless thiol form III is also a reaction of this type. In thiamine solutions the pseudo-base form II is never present in amounts great enough to affect the shape of titration curves,[2,4] nor can II be detected through oxidation to the thiazolone.[9]

From the foregoing results we conclude that the yellow form arises from neutral thiamine by a reaction involving the removal of *two* protons. Zima and Williams in 1940 isolated a crystalline yellow sodium salt of thiamine by treatment of thiamine hydrochloride in ethanol with three moles of sodium ethoxide.[6] We have prepared this salt and find its spectrum and fading behavior in methanol and water to be identical to those of the yellow form described here.

(7) G. S. Schwarzenbach, *Helv. Chim. Acta*, **26**, 418 (1943); G. S. Schwarzenbach and R. Sulzberger, *ibid.*, **26**, 453 (1943).

(8) W. H. Mills, I. M. Clark and J. A. Aeschlimann, *J. Chem. Soc.*, **123**, 2353 (1923).

(9) P. Sykes and A. R. Todd, *ibid.*, 534 (1951).

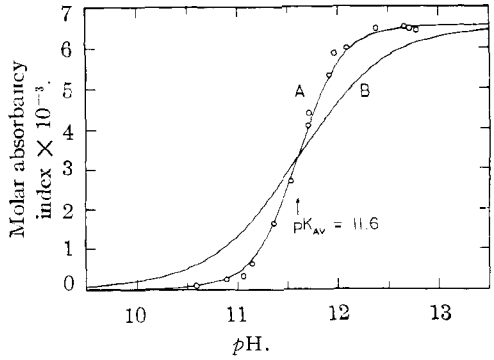


Fig. 2.—Absorbancy of thiamine at 349 mμ *versus* *p*H. Data are extrapolated back to the time of mixing. The lines are theoretical curves constructed for: A, a monoprotic acid; B, a diprotic acid with no detectable singly dissociated intermediate.

The Intermediate between the Neutral and Yellow Forms.—The development of the yellow color in methanol shows a fundamental difference from that in water. In Fig. 3A the absorbancy at 349 mμ is plotted against the logarithm of the excess, unreacted KOH concentration. The solid line is a curve of the theoretical shape for a monoprotic acid and in this case fits the experimental points well. This suggests that in methanol a more normal situation prevails in which $K_1 > K_2$ and that at intermediate basicities the thiamine should exist largely in the form of a singly dissociated intermediate. The spectra of the yellow and neutral forms exhibit points of equal absorbancy at 263 and 290 mμ. The absorbancies of thiamine in methanolic KOH of basicities intermediate to those in which either the neutral and yellow forms predominate were measured at these wave lengths. The presence of an intermediate was clearly indicated at 290 mμ by a marked increase in absorbancy (to an a_M of 5.55×10^3 at log [KOH] = −2.86) and subsequent decrease at higher base concentrations, and at 263 mμ by a lower absorbancy at intermediate base concentrations. (Similar measurements in *water* at 289 mμ indicate that no more than 5% of the intermediate is ever present, that amount being present at *p*H 11.6.)

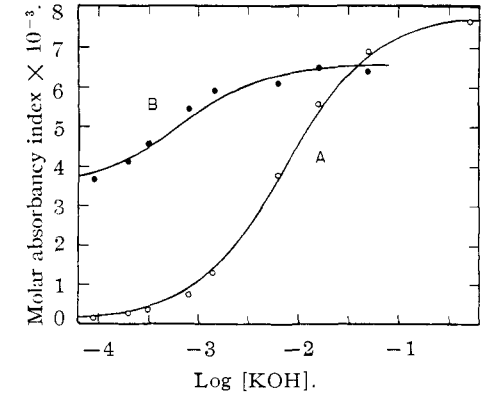


Fig. 3.—Ultraviolet absorbancy of thiamine *versus* concentration of methanolic KOH: A, molar absorbancy index at 349 mμ; B, molar absorbancy index of the intermediate plus free base forms at 290.3 mμ (see text). The lines are theoretical curves for dissociation of monoprotic acids.

The amount of the intermediate at any base concentration was computed as follows: the concentration of the yellow form was calculated from the absorbancy at 349 mμ. Then the contribution of the yellow form to the absorbancy at 290.3 mμ was subtracted from the observed absorbancy at that wave length and the difference used to compute the absorbancy index, a_M (neutral + intermediate), of the remaining mixture of neutral plus singly dissociated forms. The plot of a_M (neutral + intermediate) *versus* log [KOH] (Fig. 3B) is in effect a titration curve for the production of the intermediate. The solid line is again a theoretical curve for a monoprotic acid with a mid-point at log [KOH] = −3.2. From Fig. 3A, the mid-point of the second dissociation step is estimated as log [KOH] = −2.15 and it follows that K_1 is about 16 times K_2. These constants are sufficiently far apart that the lower points in Fig. 3A should deviate from the constructed curve by only a few per cent. From the spectrum of a solution of log [KOH] = −3.1 in which 58% of the thiamine exists as the intermediate, the spectrum of the intermediate was computed (by subtracting the absorbancy of the neutral and yellow forms at each wave length) Fig. 4B shows the spectrum of the intermediate form in methanol.

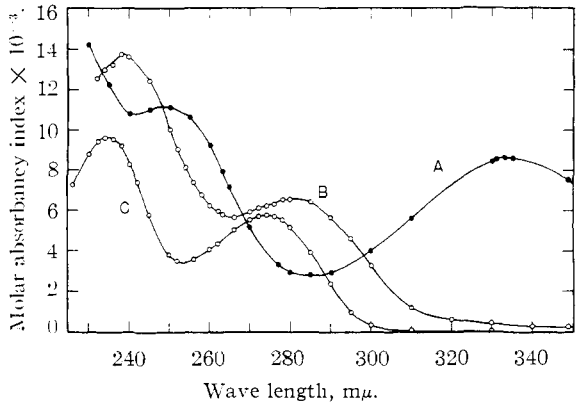


Fig. 4.—Ultraviolet spectra of thiamine in methanol: A, thiamine, 1 *N* KOH, 1–6 minutes after addition of KOH; B, calculated spectrum of the intermediate tricyclic form; C, spectrum of dihydrothiamine, VI.

By treatment of thiamine hydrochloride suspended in ethanol with two equivalents of sodium ethoxide we have been able to isolate the intermediate form of thiamine as a white crystalline solid free of both sodium and chloride ions. It dissolves in ethanol or butanol to give a nearly colorless solution but disproportionates partially in methanol and completely in water to the neutral and yellow forms of thiamine. In water the intense yellow color fades quickly as expected. The spectrum of the isolated compound in butanol is closely similar to that calculated (Fig. 4B) for the spectrum in methanol but is shifted about 3 mμ to shorter wave lengths.

The Structures of the Yellow and Intermediate Forms.—The following compounds which are structurally related to thiamine do not undergo spectral changes in 0.1 or 5.4 *N* methanolic KOH corresponding to those observed with thiamine: 2-methyl-4-amino-5-aminomethylpyrimidine dihydrochloride, 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium chloride and an equimolar mixture of these substances which nearly correspond to the two "halves" of the thiamine molecule, 3-benzyl-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride and oxythiamine (3-(4-hydroxy-2-methyl-5-pyrimidyl-methyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride). Neopyrithiamine (1-(4-amino-2-methyl-5-pyrimidylmethyl)-2-methyl-3-(2-hydroxyethyl)-pyridinium bromide hydrobromide) gave no color in 0.1 *N* KOH but did give a very transient color with a maximum absorbancy at about 360–370 mμ in 5.4 *N* methanolic KOH. The extreme base concentration and experimental difficulties make this behavior difficult to interpret. Thus it appears that production of the yellow form requires the presence of the primary amino group on the pyrimidine ring and the presence of the thiazolium ring in the same molecule.

We propose that the amino group of thiamine adds, with simultaneous loss of a proton, to the thiazolium ring to yield the intermediate, tricyclic, dihydrothiachromine form IV in a manner analogous to the addition of a hydroxyl ion to form the pseudo-base II. The intramolecular nature of the reaction with the amino group helps to explain its rapidity as compared to the reaction of a hydroxyl group. The ionization of IV with an opening of the thiazole ring leads to the yellow thiol form V. This structure for the yellow form was proposed by Zima and Williams[6] for their isolated compound and is supported by their analyses.

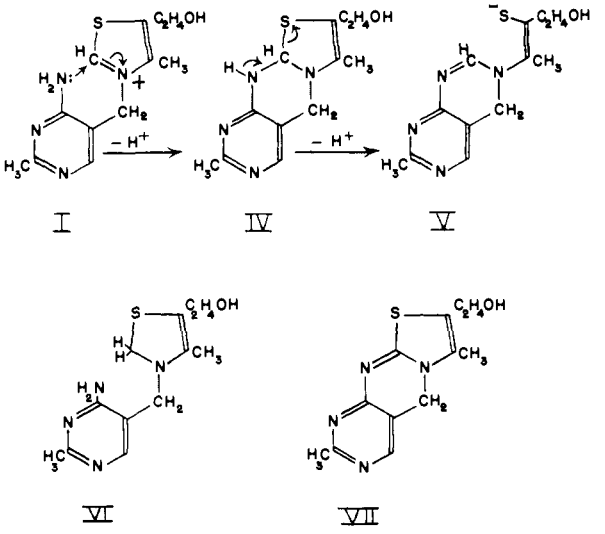
Additional support for the tricyclic structure IV for the intermediate comes from a comparison with the spectra of other aminopyrimidines. The absorption band of the intermediate near 280 mμ is shifted about 10 mμ to a longer wave length than that of the corresponding pyrimidine absorption band of dihydrothiamine (VI, Fig. 4C). Similarly, Brown and Short[10] have shown that methylation of 4-aminopyrimidines leads to bathochromic shifts of 8–9 mμ. However, IV absorbs more strongly around 240 mμ than does dihydrothiamine. It is quite possible that in IV the carbon–sulfur bond which breaks to yield V is already weakened and that this polarization of the molecule results in a higher absorption around 240 mμ, similar to that observed with the thiol form III (Fig. 1B).

The tricyclic form, IV, is closely related to thiochrome (VII) and in alcoholic solutions is gradually oxidized by air to VII as evidenced by the development of an intense blue fluorescence.[11]

(10) D. J. Brown and L. N. Short, *J. Chem. Soc.*, 334 (1953).

(11) Thiamine is oxidized readily to thiochrome by alkaline ferricyanide [G. Barger, F. Bergel and A. R. Todd, *Ber.*, **68**, 2257 (1935)]. However, we find that if the thiamine stands in base long enough for the yellow form to disappear before addition of the ferricyanide, no thiochrome is formed, nor is thiochrome formed from the white sodium salt of the thiol III (except for a trace which arises from the small amount of yellow form V in equilibrium with III. See the succeeding section of this paper for a discussion of this equilibrium). The thiol III apparently is oxidized to thiamine disulfide instead of thiochrome. On the other hand, the yellow sodium salt of V and the tricyclic form IV are oxidized readily to thiochrome by alkaline ferricyanide in accord with Syke's and Todd's view (reference 9) that V is the precursor of thiochrome.

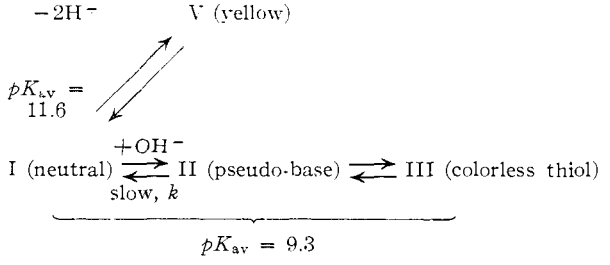
Structure IV has also been suggested as an intermediate in the de-



I IV V

VI VII

The Kinetics of Decay of the Yellow Color.—The yellow color decays in aqueous solutions at all *p*H values, with a half-life of less than one minute at 19° in some cases. By plunging a portion of thiamine into an alkaline buffer in a spectrophotometer cell and measuring the absorbancy at 349 mμ as a function of time, we were able to determine the rates of decay with satisfactory accuracy. At all *p*H values this decay was accurately first order with respect to the yellow form. First-order rate constants were calculated, and their logarithms are plotted against *p*H in Fig. 5. The yellow form does not exist to an appreciable extent below a *p*H of about 10.6; hence, no points could be obtained at lower *p*H values. However, the rate of reaction of thiamine with hydroxyl ion to form the colorless thiol form III could be measured in the *p*H range 10 to 10.8 by following the increase in absorbancy at 255 mμ. Again the rate was first order, this time with respect to the neutral form of thiamine. Rate data for this second reaction are also plotted against *p*H in Fig. 5. The points fall on the *same* hyperbola-like curve that describes the *p*H dependence of the rate of fading of the yellow color suggesting that the fading is caused by the *slow* reaction of hydroxyl ion with the neutral form I which is in a *rapid* equilibrium with the yellow form V. The final product is the more stable colorless thiol form III. From the pK_{av} values of 11.6 for the conversion to the yellow form



V and of 9.3 for conversion to the thiol form III, it follows that at equilibrium, the ratio of the colorless thiol form to the yellow form should be 200

composition of thiamine disulfide (ref. 9) and as a product of the reduction of thiochrome by hydrosulfite [R. Kuhn and H. Vetter, *Ber.*, **68**, 2375 (1935)].

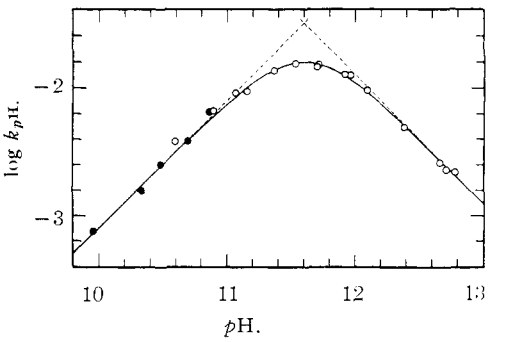


Fig. 5.—Rates of fading of the yellow color: O, apparent first-order rate constants for the disappearance of the yellow form of thiamine in buffers of 0.2 ionic strength; ●, apparent first-order rate constants for conversion of neutral thiamine to the thiol form. The line represents the theoretical *p*H dependence (see text).

and the disappearance of the yellow color will be almost complete.

This scheme leads to the prediction that the apparent first-order rate constants k_{pH} for the fading of the yellow color and for reaction of the neutral form to give the thiol form should have the *p*H dependence

$$\log k_{pH} = \log kK_w - pH - \log (K_{av}^2 + [H^+]^2)$$

where k is the *second-order* rate constant for the reaction of the neutral form of thiamine with hydroxyl ion; K_w is the ion-product of water, and K_{av} is the average of K_1 and K_2 for the dissociation of thiamine to form the yellow compound V. The solid line in Fig. 5 is a theoretical curve constructed to fit the foregoing equation using values of log $kK_w = -13.1$ moles liter^{-1} sec.$^{-1}$ and $pK_{av} = 11.6$ at 19.2°. The excellent fit of the experimental points to this theoretical curve provides additional support for the assumption of a two-proton dissociation of thiamine to form the yellow substance.

Significance with Respect to Enzymic Catalysis.—The mechanism of the catalytic action of thiamine is still uncertain although Breslow[12] has recently presented a plausible new theory. Consideration should also be given to the possibility that at some stage in the reaction sequence by which thiamine functions, structures analogous to IV or V occur. It may be objected that a *p*Kav of 11.6 for the formation of these structures is too high to permit their function in a biological system. However, a prior reaction with a substrate such as pyruvate might decrease *p*Kav greatly. If an intermediate analogous to V were formed after reaction with pyruvate (*e.g.*, as visualized by Breslow[12]) its decarboxylation would be assisted by the conjugation with the electron-accepting centers of the pyrimidine ring in a manner quite similar to that suggested in the decarboxylation of α-amino acids by vitamin B_6-containing enzymes.[13,14]

Acknowledgments.—We gratefully acknowledge our thanks to Marcella Vermeersch and to Frances

(12) Ronald Breslow, THIS JOURNAL, **79**, 1762 (1957).

(13) A. E. Braunstein and M. M. Shemyakin, *Biokhimia*, **18**, 393 (1953).

(14) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

Roddy for technical assistance, to Dr. Joseph Picken for a gift of 4-methyl-5-(2-hydroxyethyl)-thiazole, to Dr. Samuel Aronoff for the use of recording equipment and to the Institute for Atomic Research, Ames, Iowa, for the use of the Cary spectrophotometer.

Experimental

Chemicals.—Merck thiamine chloride hydrochloride was dried in a vacuum desiccator over magnesium perchlorate and used without further treatment. Recrystallization of this material led to no change in its absorption spectrum. Neopyrithiamine and oxythiamine were purchased from the California Foundation for Biochemical Research, Los Angeles, and 2-methyl-4-amino-5-(2-aminomethyl)-pyrimidine from Nutritional Biochemicals Corp., Cleveland. 3,4-Dimethyl-5-(2-hydroxyethyl)-thiazolium chloride was prepared from du Pont 4-methyl-5-(2-hydroxyethyl)-thiazole and methyl iodide.[15] Eight g. of the resulting methiodide was dissolved in water and shaken for 1 hour with an excess of freshly precipitated silver chloride. The precipitated silver iodide was filtered off and the solution was concentrated in vacuum to a thick sirup. This was dissolved in 20 ml. of absolute alcohol, 15 ml. of ether was added and the methochloride was allowed to crystallize, then recrystallized from alcohol and ether; m.p. 136–138°. *Anal.* Calcd. for $C_7H_{12}ONSCl$: C, 43.4; H, 6.25; Cl, 18.3. Found: C, 43.4; H, 6.2; Cl, 18.4. 3-Benzyl-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride was prepared by the procedure of Livermore and Sealock[16]; m.p. 145–147°.

Dihydrothiamine was prepared by reduction of thiamine with trimethoxyborohydride according to the procedure of Bonvicino.[17]

The white sodium salt of the thiol form of thiamine (III), the yellow sodium salt of thiamine and thiamine monochloride were prepared according to the procedures of Zima and Williams.[6]

Reagent grade (Baker) absolute methanol was employed. Moisture was carefully excluded.

The Tricyclic (Dihydrothiachromine) Form of Thiamine.—Five grams (0.015 mole) of thiamine chloride hydrochloride was suspended in 20 ml. of cold absolute alcohol and 0.03 mole of sodium ethoxide in cold ethanol of total volume 12 ml. was added with stirring. The sodium chloride was quickly filtered off by suction on a sintered glass filter and the filtrate was allowed to stand for about 3 hours in the cold. The white crystalline solid was collected on a buchner funnel. Moisture was carefully excluded through use of a rubber dam to prevent yellowing of the product. After washing with cold alcohol and peroxide-free ether the compound was dried *in vacuo* at room temperature; yield 1.1 g., m.p. 128–129° dec. *Anal.* Calcd. for $C_{12}H_{16}ON_4S$: C, 54.5; H, 6.1; N, 21.2; neut. equiv., 264. Found: C, 54.6; H, 6.2; N, 21.3; neut. equiv., 267; molecular wt. by comparison of spectrum in 0.1 *N* HCl with that of thiamine hydrochloride, 267.

Spectral Measurements.—A Beckman DU spectrophotometer with the cell compartment thermostated at 25° in most cases and at 19.2° in some experiments was used for quantitative spectral measurements. A Cary model 12 recording spectrophotometer was employed for preliminary evaluation of spectra.

Spectra usually were measured on 1.42×10^{-4} *M* solutions of the thiazolium compounds prepared by dilution of a stock solution. Spectra in methanol were obtained by adding an aliquot of the stock solution to methanolic KOH of appropriate concentration, mixing and reading the spectrum within 5 minutes for qualitative studies and within 2–3 minutes for quantitative measurements. The absorbancy of the yellow form decreased by 1–3% during the first 6 minutes after mixing.

Spectra in aqueous solutions were measured in buffers, usually of 0.2 ionic strength and consisting of potassium phosphates at *p*H 6.8, of sodium bicarbonate and KOH from *p*H 9–10, of piperidine hydrochloride and KOH from *p*H 10 to 12, and of KOH and KCl above 12. When the spectra changed rapidly with time, 0.1 ml. of an appropriate stock solution was plunged into 3.0 ml. of buffer in the spectrophotometer cell by means of the adder-mixer of Boyer and Segal[18] which permits complete mixing within 2 seconds or less. Readings were obtained at intervals of 5 seconds beginning 10 seconds after mixing. Satisfactorily accurate readings and timing were obtained through the help of a second person in timing and recording. Some data were also collected using a Varian linear recorder to plot transmittancy *versus* time. Extrapolation of absorbancies to zero time using semi-log paper was usually precise.

Following each kinetic measurement, the temperature in the cell was measured and found to lie within the limits $19.2 \pm 0.2°$. The *p*H was then measured using a Beckman model G meter and the temperature of the sample in the *p*H meter noted. The *p*H was corrected to 19.2°. Although thiamine can undergo irreversible oxidative reactions in air, we have taken no precautions to exclude air. This is justified by the short time intervals involved and by the demonstrated complete reversibility of the phenomena described here when the elapsed time was kept small. It appears that in most cases, only a few per cent. of irreversible decomposition occurs in the spectrophotometer cell in periods up to two hours.

Calculations.—The methanolic KOH solutions were standardized and the basicity of the final diluted solutions expressed as the logarithm of the excess KOH concentration. When the KOH concentration was low, the amount of KOH which had reacted with the thiamine was subtracted from the total amount added to give the excess. From the spectrum the amounts of yellow form, intermediate and free base present were computed and the correction was made on the assumption that 3, 2 and 1 equivalents of base, respectively, were consumed in the production of these forms from the hydrochloride. Satisfactory accuracy in the log [KOH] values was obtainable except below log [KOH] = −4.

The curve in Fig. 3B which shows the molar absorbancy index, a_M for the neutral plus intermediate forms in methanol was computed using the following data. From Fig. 3A the limiting value for a_M at 349 mμ was taken as 7.75×10^3 and assumed to represent a_M for the yellow form (the corresponding a_M in aqueous solution is 6.52×10^3). Within experimental error, the same molar absorbancy indices were obtained for the yellow sodium salt described by Zima and Williams.[6] At 290.3 mμ the a_M for the yellow form is 3.38×10^3.

First-order rate constants for the fading of the yellow color in aqueous solutions were computed by plotting the logarithm of the absorbancy at 349 mμ against time and measuring the slope. A linear relationship was observed (except in samples of low initial absorbancies) as might be expected when the absorbancy falls to zero at equilibrium. Actually a_M did not fall to zero, but from initial values as high as 6.5×10^3 to 0.02 to 0.1×10^3. a_M would be expected to decrease to 0.03×10^3 representing the 0.5% of yellow form still present at equilibrium. The presence of impurities and slow side reactions and the difficulty of measuring very low absorbancies accurately may account for the variation in these values. In some samples, at the lower *p*H values, only a small amount of yellow color was present and initial absorbancies were low even when the thiamine concentration was 1.42×10^{-3} *M* (10 times the usual concentration). In such cases it was necessary to plot the logarithm of the absorbancy at 349 mμ minus the final absorbancy to which the solution decayed against time in order to obtain a straight line.

The rate of pseudo-base formation from the neutral form was obtained from measurements at 255 mμ. a_M changes from 7.36 to 11.66×10^3 during the course of the reaction. The difference, $\Delta = 11.66 \times 10^3 - a_M$ (observed) at a given time was assumed to be proportional to the amount of the neutral form remaining at that time. Plots of log Δ *versus* time were linear and permitted calculation of the rate constants.

Estimates of the pK_{av} for conversion to the thiol form were also obtained from data at 255 mμ for thiamine and two simi-

(15) E. R. Buchman, R. R. Williams and J. C. Keresztesy, THIS JOURNAL, **57**, 1849 (1935).

(16) A. H. Livermore and R. R. Sealock, *J. Biol. Chem.*, **167**, 699 (1947).

(17) G. E. Bonvicino, Ph.D. Thesis, Fordham University, 1952; see also P. Karrer and H. Krishna, *Helv. Chim. Acta*, **33**, 555 (1950).

(18) P. D. Boyer and H. L. Segal in "A Symposium on the Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Eds., Johns Hopkins Press, Baltimore, Md., 1954, p. 523.

pler thiazolium salts. In this case the final equilibrium absorbancy was measured. Because of the slow reaction rates in the neighborhood of pK_{av}, and slow secondary reactions, these particular pK estimates have an uncertainty of as much as ± 0.2 unit.

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Metabolite Analogs. VII. Preparation of Some Benzimidazolyl Analogs of Ethyl Pteroylglutamate

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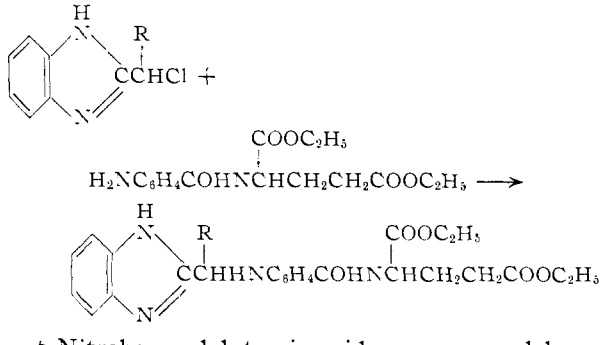
Some benzimidazolyl analogs of ethyl pteroylglutamate have been prepared. Substituents have been placed on the carbon atom of the methylene bridge and in the benzene ring of the benzimidazole.

Two benzimidazolyl analogs of pteroylglutamic acid have been reported previously, N-{4-[(2-benzimidazolyl)-methylamino]-benzoyl}-glutamic acid[1] and N{4-[(5-chloro-2-benzimidazolyl)-methylamino]-benzoyl}-glutamic acid. The first compound was reported to retain a certain degree of growth-promoting activity[1a] and also to be a weak growth antagonist.[1b] The 5-chloro compound was reported to be a stronger growth antagonist than the unsubstituted analog.[1b] It seemed to be desirable to extend this work, in several ways, in order to determine the possibility of obtaining strong anti-folic activity in this type of compound. In the present investigation, benzimidazolyl analogs of folic acid have been synthesized containing not only substituents in the benzene ring but also on the carbon atom of the methylene bridge. Substituents have been placed on the methylene bridge when there were no substituents on the ring and when there were substituents on the benzene ring. The choice of substituents in the benzene ring was influenced by the work of Hoover and Day.[2]

The syntheses of the benzimidazolyl analogs of pteroylglutamic acid involved four steps: (1) preparation of diethyl *p*-aminobenzoylglutamate; (2) preparation of 2-hydroxyalkylbenzimidazoles; (3) preparation of 2-chloroalkylbenzimidazoles; and (4) finally, the condensation of the chloroalkyl compound with the glutamate derivative.



p-Nitrobenzoylglutamic acid was prepared by a modified Schotten–Baumann procedure from *p*-nitrobenzoyl chloride and glutamic acid. The nitro group was reduced by catalytic hydrogenation over palladium and the corresponding aminobenzoylglutamic acid converted to its diethyl ester. Preliminary work had shown that the final products, the benzimidazolyl analogs of folic acid, were most readily isolated and purified in the form of their ethyl esters.

The 2-hydroxyalkylbenzimidazoles were prepared from the appropriate hydroxy acid and *o*-phenylenediamine by the Phillips method[3] or by fusing the reactants together. The hydroxy compounds were then converted to the corresponding chloro compounds by treatment with thionyl chloride.

In order to reduce side reactions to a minimum, the hydrochlorides of the chloro compounds were used for condensation with diethyl *p*-aminobenzoylglutamate. The condensations were carried out in dioxane solution in the presence of two equivalents of triethylamine. The main side-reaction which occurred to a greater or less extent, depending on the nature of the chloro compound, was self condensation. It had been shown earlier[4] that the 2α-chloroalkylbenzimidazoles do not always undergo normal anionic replacement reactions. They show a marked tendency to undergo self-condensation to form tetracyclic compounds. For example, 2-chloromethylbenzimidazole readily forms dibenzimidazo[1,2,-a,1′,2′,-d]piperazine. On several occasions during the course of the present work, high melting, crystalline compounds were isolated whose analyses indicated that they were tetracyclic. No attempt was made to purify these secondary products. Gummy materials also were separated from the reactions of the 2α-chloroalkylbenzimidazoles with ethyl *p*-aminobenzoylglutamate. They were assumed to be linear polymers formed from self-condensation of the chloro compounds.

For testing purposes, two benzimidazolyl analogs of the ethyl ester of pteroic acid were prepared also. They were prepared by condensing the chloroalkyl benzimidazoles with ethyl *p*-aminobenzoate.

All of the final products are being tested for physiological activity. The test results will be published elsewhere.

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(2) J. R. E. Hoover and A. R. Day, THIS JOURNAL, **77**, 4324 (1955); **77**, 5652 (1955); Progress Report, July 1955–January 1956, U.S.P.H.S. Grant C-2189, University of Pennsylvania.

(3) M. A. Phillips, *J. Chem. Soc.*, 2393 (1928).

(4) H. Skolnik, J. G. Miller and A. R. Day, THIS JOURNAL, **65**, 1854 (1943).