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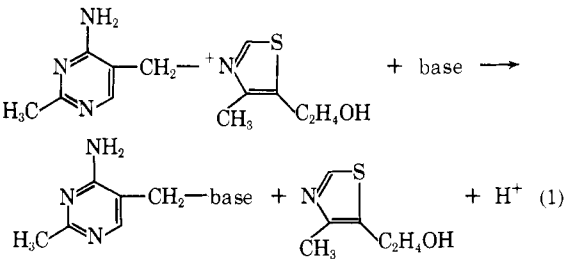
The Extracellular Thiaminase I of *Bacillus Thiaminolyticus*. I. Purification and Physicochemical Properties*

James L. Wittliff† and R. L. Airth‡

ABSTRACT: Thiaminase I catalyzes the decomposition of vitamin B_1 by means of a base-exchange reaction. The enzyme was purified 200-fold from culture filtrates of *Bacillus thiaminolyticus* using a combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. Homogeneity of the enzyme preparations was determined by ultracentrifugation, polyacrylamide gel electrophoresis, and immunodiffusion in agar gel. The absorption spectrum of thiaminase I is that of a simple protein with a maximum at 277 mμ and a minimum at 252 mμ. The enzyme has an $s_{20,w}$ = 3.1 S and a $D_{20,w}$ = 6.6 × 10^{-7} cm^2/sec which suggest a molecular weight of 44,000. Thiaminase I migrates as an anion at pH 9.5 when subjected to disc electrophoresis and displays maximum enzymatic activity over a broad pH range of 5.8–6.8. The K_m values of thiaminase I at pH 5.8 and 25° are 8.7 × 10^{-6} M for thiamine and 2.9 × 10^{-3} M for aniline. The temperature optimum of the enzyme activity is 37° and Q_{10}(10–20°) = 1.93 and Q_{10}(20–30°) = 1.33. Energies of activation of 9800 cal/mole and 2700 cal/mole were determined for the thiaminase I reaction. The change in the value of the activation energy of the reaction is thought to be due to a reversible inactivation of the enzyme. Thiaminase I has a temperature inactivation coefficient (T_i) of 63.5°.

Various thiamine-decomposing enzymes have been found in fish, mollusks, arthropods, ferns, and bacteria (see reviews by Fujita, 1954; Metzler, 1960; Kimura, 1965; Murata, 1965). Two mechanisms of vitamin B_1 degradation by thiaminases from several sources have been studied (Fujita, 1954; Metzler, 1960). One reaction was shown to require a basic substance as a cosubstrate and was catalyzed by an enzyme subsequently named thiaminase I (thiamine:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2). The reaction is represented in eq 1. Two bacteria, *Bacillus thiaminolyticus* Matsukawa et Misawa and *Clostridium thiaminolyticum* Kimura et Liao, which have been



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isolated from human feces, are known to produce thiaminase I. A second type of enzyme, thiaminase II (thiamine hydrolase, EC 3.5.99.2), has been isolated from *Bacillus aneurinolyticus* Kimura et Aoyama (Kimura, 1965).

Recently Douthit and Airth (1966) demonstrated that the thiaminase I from *B. thiaminolyticus* is an extracellular enzyme. Furthermore, the bacterium exhibited a single vitamin requirement for thiamine (the primary substrate for thiaminase I) when grown on a defined medium. Wang and Airth (1967) have suggested that thiamine may play a regulatory role as a repressor of thiaminase I synthesis. As part of an ensuing study of the control of synthesis and release of bacterial thiaminases (I and II) it was necessary to purify the enzymes, to compare them physicochemically and serologically, and to measure the rates of enzyme synthesis. This and a subsequent paper will concentrate on the purification and properties of thiaminase I.

Materials and Methods

Chemicals and Reagents. All chemicals were reagent grade unless otherwise specified. Glass-redistilled water was used in all experiments. Thiamine–HCl was purchased from Mann. Sodium phosphates (Mallinckrodt) and Tris (Sigma) were used for column buffers. Crystalline bovine serum albumin (Armour) was used as a standard in protein estimations. Aniline was redistilled (bp 183–184°) for use in enzyme assays. All of the cross-linked dextran gels (Sephadex G-25, G-100, and DEAE Sephadex A-50) were purchased from Pharmacia Fine Chemicals.

Enzyme Assay. The spectrophotometric assay of thiaminase I (Douthit and Airth, 1966) was used with the following modifications: 0.1 M sodium phosphate buffer (pH 5.8) and thiamine:aniline molar ratio = 1:29. Activity was measured at 25° with a Beckman DU spectrophotometer (λ 248 mμ) with a Gilford 2000 recorder. An enzyme unit (U) is defined as that amount of enzyme which catalyzes the formation of 1 μmole of product in 1 min at 25°.

Protein Estimations. Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. The method of Warburg and Christian (1941) was used for protein estimation on fractions from the Sephadex columns.

Production of Enyzme. The organism used as a source of thiaminase I was *Bacillus thiaminolyticus* strain M. The defined medium of Douthit and Airth (1966) was selected for the culture of this bacterium. Erlenmeyer flasks (2 l.) containing 1 l. of inoculated medium each were incubated at 37° for 28–30 hr in a Gyrotory shaker (New Brunswick).

Purification of the Enzyme. All operations were performed at 0–4° unless otherwise noted. Step 1: 28–30-hr old cultures of *B. thiaminolyticus* grown in defined medium were pooled and centrifuged in a continuous-flow Sorvall centrifuge (30,000*g*, 4°). The flow rate was approximately 100 ml/min. Step 2: solid ammonium sulfate (516 g/l., 75% saturation) was added slowly with stirring to the clear, yellow supernatant from step 1. The pH of the supernatant before precipitation was 6.6–6.8 and after salt addition 6.0–6.5. The mixture was left standing 14–16 hr at 4°. The precipitate was removed by centrifugation in the continuous-flow centrifuge (30,000*g*, 4°) at a flow rate of 40–50 ml/min and stored in a liquid nitrogen freezer (Linde Co.). Step 3: the precipitate from step 2 was suspended in cold, glass-redistilled water (1.5 ml/g wet wt) using a Ten-Broeck homogenizer. The mixture was centrifuged at 30,000*g*, 0°, for 15 min and the reddish-tan supernatant was removed. Step 4: the supernatant was desalted by passing it through a Sephadex G-25 column (2.5 × 38 cm). Fractions containing protein were eluted from the G-25 column with glass-redistilled water. Occasionally, the elution of protein was followed at 280 and 260 mμ with a flow-through cell (Gilford Instruments). The fractions containing thiaminase I activity were pooled. Step 5: solid ammonium sulfate (313 g/l., 50% saturation) was added slowly with stirring to the G-25 fractions. After stirring for about 1 hr, the suspension was centrifuged for 15 min at 30,000*g*. The precipitate was discarded. Step 6: the supernatant from step 5 was brought to a final saturation of 70% (159 g/l.) with solid ammonium sulfate and the suspension was stirred for 1 hr, then centrifuged at 30,000*g* for 15 min. The supernatant was discarded and the precipitate was frozen at −20°. Step 7: the precipitate from the 50–70% saturated ammonium sulfate step was dissolved in a minimal amount of 0.05 M sodium phosphate buffer (pH 7.0) and dialyzed overnight against two 4-l. portions of the same buffer. After dialysis, the fraction was centrifuged at 30,000*g* for 15 min to remove a small amount of insoluble material. Step 8: the dialyzed material from step 7 was applied to a Sephadex G-100 column (2.5 × 39 cm) equilibrated with 0.05 M sodium phosphate (pH 7.0). The protein was eluted with the same buffer. Fractions containing the thiaminase I activity were pooled. Step 9: the enzyme from the Sephadex G-100 step was dialyzed overnight against two 4-l. portions of 0.1 M Tris–HCl buffer (pH 8.2). The contents of the dialysis tubing were centrifuged at 30,000*g* for 15 min to remove any insoluble material which might have formed during this step. Step 10: after centrifugation, the dialyzed material was applied to a DEAE-Sephadex column (2.5 × 38 cm) previously equilibrated with the same buffer as in step 9. When all of the material was loaded onto the column, the gel was washed with two bed volumes of the Tris–HCl buffer to remove all material not bound to the anion exchanger. The remaining proteins bound to the gel were eluted using 0.1 M Tris–HCl buffer (pH 8.2) and a gradient of 0.0–1.0 M sodium chloride. The fractions containing thiaminase I activity were pooled and frozen at −20°.

The elution patterns from the various chromatography steps are presented in Figure 1. Chromatography of the enzyme on Sephadex G-25 was effective not only as a desalting step but served to remove any color in the precipitate from the ammonium sulfate

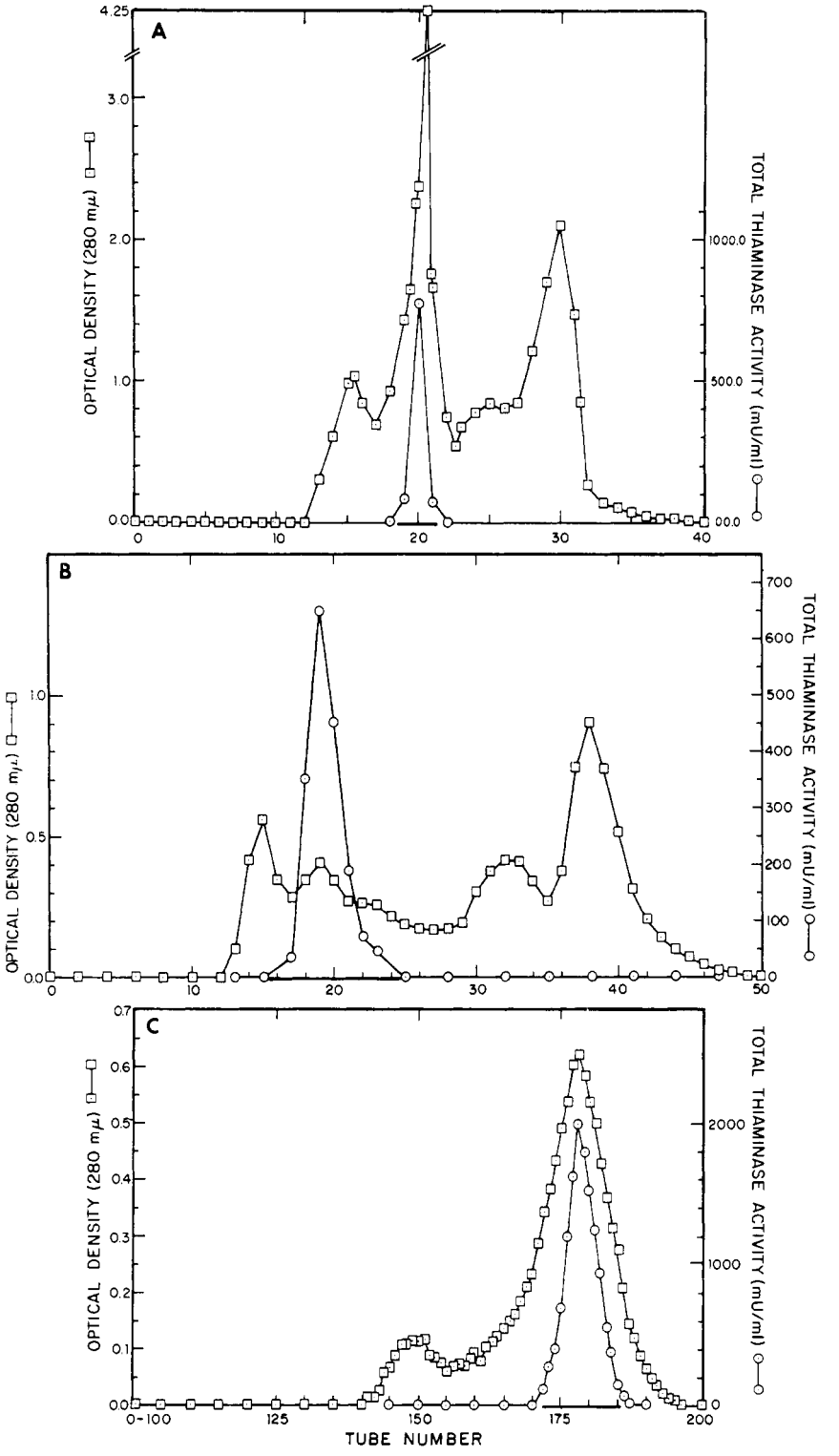

FIGURE 1: Elution patterns. (A) As measured by optical density at 280 mμ, from a Sephadex G-25 column. The redissolved precipitate from the 0–75% saturated ammonium sulfate step was placed on the column and eluted with glass-redistilled water (4°) at a flow rate of 0.85 ml/min (8.5-ml fractions/tube). (B) As measured by optical density at 280 mμ, from a Sephadex G-100 column. The redissolved and dialyzed sample from the 50–70% saturated ammonium sulfate step was applied to the column and eluted with 0.05 M phosphate buffer (pH 7.0). The flow rate was 0.3 ml/min (3.0-ml fractions/tube). (C) As measured by optical density at 280 mμ, from a DEAE-Sephadex A-50 column which was equilibrated with 0.1 M Tris–HCl buffer (pH 8.2). After sample application, the column was washed with two bed volumes of the Tris–HCl buffer and the bound proteins were eluted with 0.1 M Tris–HCl buffer (pH 8.2) containing a gradient of 0.0–1.0 M NaCl at a flow rate of approximately 0.44 ml/min (1.5-ml fractions/tube).

TABLE I: The Purification of Extracellular Thiaminase I.[a]

Step	Total Protein (mg)	Total Act (mU)	Sp Act. (mU/mg of protein)	% of Total	Purificn
30-kg supernatant	3470.0	114,000	32.8	100.0	1
0–75% $(NH_4)_2SO_4$	136.0	35,540	261.3	31.2	8
Sephadex G-25	80.0	30,500	378.0	26.8	11
50–70% $(NH_4)_2SO_4$	38.7	15,850	410.0	13.9	12
Sephadex G-100	9.5	12,250	1287.0	10.8	39
DEAE Sephadex	1.7	11,200	6007.0	9.8	183

[a] For details, see text.

step. The enzyme resolved as a single peak after ion-exchange chromatography and could be frozen at −20° for 6 months without an appreciable loss in activity. Both the gel filtration and ion-exchange chromatography steps greatly improved the specific activity of thiaminase I (Table I).

Criteria of Purity. Preliminary ultracentrifugal analyses of the purified enzyme were performed with a Spinco Model E ultracentrifuge equipped with a phase-plate schlieren optical system and a temperature control. The centrifugations were conducted at 20° with a rotor speed of 59,780 rpm. The method of Raymond and Weintraub (1959) for vertical polyacrylamide gel electrophoresis (E. C. Apparatus Corp.) was employed as another test of homogeneity.

Double diffusion in agar gel (Oudin, 1952) was employed to check the purity of the thiaminase I preparations using antisera prepared against the purified enzyme (unpublished data). The gels containing the enzyme and antibody preparations were allowed to incubate overnight at room temperature in a humid desiccator.

Ultraviolet Absorption Spectra. The ultraviolet absorption spectrum of the enzyme was determined with a Beckman DU spectrophotometer using quartz cuvets.

Density Gradient Centrifugation. Density gradient centrifugation was performed in a Model L Spinco preparative ultracentrifuge with a temperature setting of −8°. An SW-39 L swinging-bucket rotor was used at a speed of 39,000 rpm. Centrifugation time was 20 hr. Linear gradients were obtained using 5 and 20% (w/v) sucrose solutions in 0.05 M sodium phosphate buffer (pH 7.0).

Sedimentation Studies. A Spinco Model E analytical ultracentrifuge equipped with a phase-plate schlieren optical system and temperature control was used. Sedimentation–velocity experiments were conducted generally at 59,780 rpm (20°) using conventional 12-mm cells or synthetic boundary cells (rubber valve type). The boundary displacements were measured with a Nikon microcomparator (10× objective). Sedimentation coefficients were computed from plots of log x (x being the boundary distance to rotation axis in millimeters) *vs.* t (time in seconds) (Schachman, 1959). These were corrected to the density of water at 20° using the equation of Svedberg and Pederson (1940).

Diffusion Studies. Owing to the small amount of material available, diffusion measurements were conducted in the Model E analytical centrifuge at 13,410 rpm (20°) in a synthetic boundary cell (rubber valve type) according to the method of Ehrenberg (1957). The enzyme concentration was approximately 1 mg/ml in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1 M potassium chloride.

Disc Electrophoresis. The method of Ornstein (1964) using a Canalco Model 6 system (Canal Industrial Corp.) was selected. The acrylamide gels were prepared in 0.05 M Tris–glycine buffer (pH 8.9) and electrophoresis was conducted at room temperature at pH 9.5 in columns (0.6 × 7.0 cm) for 1 hr at 5 ma/column. The gels were stained with aniline black (1 g/200 ml of 7% acetic acid) and electrolytically destained.

Effect of pH. The optimal pH of the base-exchange reaction was determined at 25° over a pH range of 4.0–8.5 using sodium acetate, McIlvaine's citrate–phosphate, sodium phosphate, and Tris–HCl buffers (all buffers were 0.1 M final concentration in the cuvet). Because the assay was of a spectrophotometric nature, the absorbancy of each of the reactants and products (properly dissolved in each buffer at various pH values) was determined at 248 mμ. These readings were used to calculate the molar absorbancies required to generate new conversion factors for each pH as given in the thiaminase assay equation of Douthit and Airth (1966). The conversion factors ranged from 0.230 to 0.385.

Effect of Substrate Concentration. The effect of thiamine concentration from zero to 99 × 10^{-6} M on the initial rate of the base-exchange reaction was measured in 0.1 M sodium phosphate buffer (pH 5.8). The aniline concentration (11.5 × 10^{-4} M) was held constant. The effect of aniline concentration on the initial rate of reaction was measured also over a range of 0–30 × 10^{-4} M. The thiamine concentration (39.6 × 10^{-6} M) was held constant in this experiment. Molarities are cuvet concentrations.

Effect of Protein Concentration. The effect of enzyme concentration on the initial rate of the reaction was measured over a range of 0–8 μg of thiaminase I/assay.

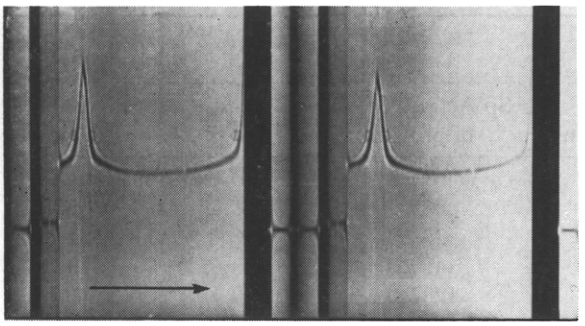

FIGURE 2: Schlieren pattern of a solution of purified thiaminase I (1.76 mg/ml) in 0.1 M Tris–HCl (pH 8.2) at 20°, during sedimentation at 59,780 rpm. Photographs were taken at a bar angle of 55° at 64 min and 80 min after reaching top speed.

All reactions were conducted with standard assay conditions in 0.1 M sodium phosphate buffer (pH 5.8) as given by Douthit and Airth (1966).

Effect of Temperature. The effect of temperature on the initial rate of the base-exchange reaction was determined over a range of 10–55° in 0.1 M sodium phosphate buffer (pH 5.8). Temperature was controlled by adjusting the thermostat of a variable-temperature apparatus (Polyscience Corp. and Brinkmann Instruments) which circulated water through the thermospacers.

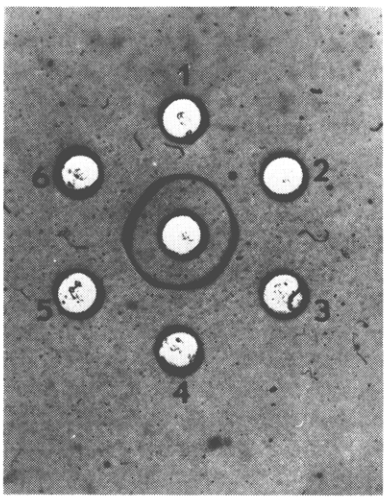

FIGURE 3: Double diffusion in 1% Ionagar gel at 25°. Approximately 8 μl of the undiluted antithiaminase I was placed in the center well. Various fractions from different steps of the purification procedure were placed in the surrounding wells: 1, DEAE-Sephadex chromatography; 2, 30,000*g* supernatant; 3, redissolved 0–75% saturated ammonium sulfate precipitate; 4, Sephadex G-25 chromatography; 5, redissolved 50–70% saturated ammonium sulfate precipitate; and 6, Sephadex G-100 gel filtration.

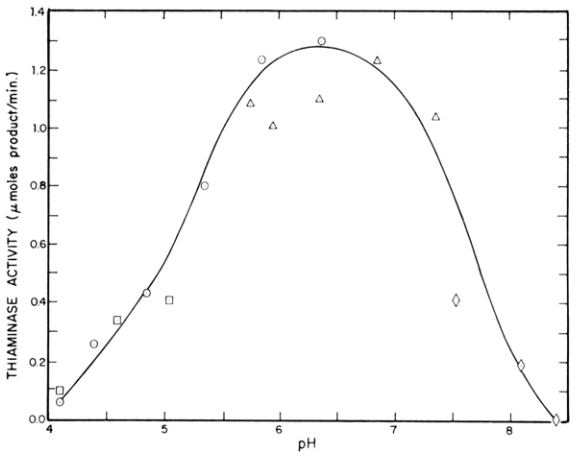

FIGURE 4: Effect of pH and buffer type on the activity of thiaminase I from *B. thiaminolyticus*. All buffers were 0.1 M final concentration in the cuvet. The activities at 25° have been corrected for absorbancy changes of the reactants and products at the various pH values. See text for details. (⊡) Sodium acetate, (⊙) citrate phosphate, (△) sodium phosphate, and (◇) Tris–HCl.

Thermostability Studies. The effect of temperature on the stability of thiaminase I was measured in the following fashion. A constant amount of enzyme in 0.5 M sodium phosphate buffer (pH 5.8) was incubated for 20 min at various temperatures (25–75°) in a controlled-temperature water bath (Precision Scientific Co.). After incubation, the enzyme solutions were cooled immediately in an ice bath and assayed for residual thiaminase I activity.

Results

Purity of the Enzyme. A sample of thiaminase I (from the DEAE-Sephadex column), which had been dialyzed against 4 l. of 0.1 M Tris–HCl (pH 8.2), displayed a single symmetrical peak when subjected to ultracentrifugal analysis (Figure 2). Gels (17 × 12.5 cm) were prepared with 5% cyanogum in 0.1 M Tris–borate buffer (pH 8.9) containing EDTA (5.6 g/l.). The enzyme when subjected to electrophoresis (4° for 1–3 hr at 400 v) migrated as a single band at pH 8.9 in polyacrylamide gel.

Double diffusion in agar gel yielded one major precipitin band and two faint, secondary bands when the purified enzyme which had been used as antigen was treated with rabbit antithiaminase I. However, thiaminase I obtained from subsequent purifications gave only one band when treated with antithiaminase I (Figure 3). Each fraction from earlier steps in the purification procedure also formed a precipitin line which was continuous with the precipitin band deposited by treating purified thiaminase I with antithiaminase I (Figure 3).

Ultraviolet Absorption Spectra. The ultraviolet absorption spectrum of a sample (0.37 mg/ml) of purified

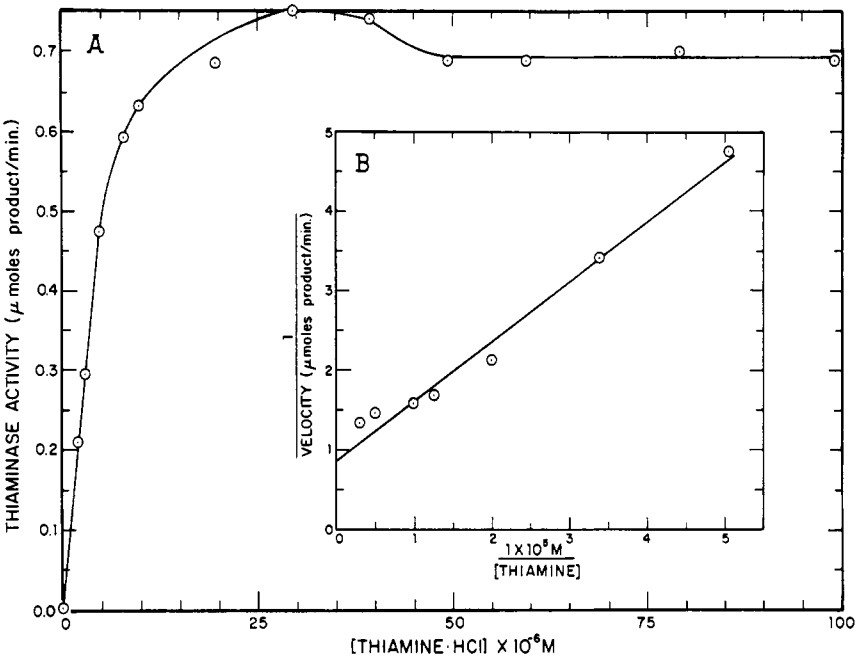

FIGURE 5: Thiamine concentration studies. (A) The effect of thiamine concentration on the initial rate of the base-exchange reaction of thiaminase I from *B. thiaminolyticus*. Aniline concentration was held constant at 11.5×10^{-4} M. The velocities were measured at 25° in 0.1 M sodium phosphate buffer (pH 5.8). (B) A double-reciprocal plot of the data in A according to the method of Lineweaver and Burk (1934). The K_m value for thiamine was 8.7×10^{-6} M.

thiaminase I in 0.1 M Tris–HCl buffer (pH 8.2) containing 1.0 M NaCl was that of a simple protein. An $OD_{280\,m\mu}/OD_{260\,m\mu}$ ratio of 1.6 was obtained with an absorption maximum at 277 mμ and a minimum at 252 mμ.

Density Gradient Centrifugation. Thiaminase I separated as a single, symmetrical peak in sucrose density gradients with bovine plasma albumin added as a reference protein. Bovine plasma albumin (1 mg) was added to a solution of thiaminase I (1000-mU total) and the mixture of the two proteins (total volume, 0.5 ml) was layered on top of a 4.0-ml sucrose gradient. On the average, 20 fractions of 16 drops each (70 drops/ml) were collected from each gradient and assayed for thiaminase I activity and optical density at 280 mμ. A sedimentation coefficient was estimated using the method of Fontaine and Condliffe (1963). In separate but identical gradients in the same rotor, the enzyme and bovine plasma albumin were sedimented independently to determine protein distribution. No interaction between thiaminase I and bovine plasma albumin was observed; each sedimented to the same sucrose density when mixed as when centrifuged independently. Over 90% of the thiaminase I activity placed on the gradient was recovered. Using a value of 4.4 S for the sedimentation coefficient of bovine plasma albumin (Ehrenberg, 1957), an approximate sedimentation coefficient of 3.7 S was calculated for thiaminase I.

Sedimentation and Diffusion Studies. Thiaminase I also sedimented as a single, symmetrical peak in the ultracentrifuge (Figure 2). In a preliminary experiment to determine the effects of protein concentration on the sedimentation velocity coefficient of thiaminase I, three concentrations (2.2, 1.7, and 1.0 mg/ml) were used; each gave an $s_{20,w} = 3.1$ S at pH 7.0 in 0.1 M sodium phosphate buffer containing 0.1 M KCl. The diffusion coefficient was 6.6×10^{-7} cm²/sec using the "maximum ordinate–area" method of Ehrenberg (1957). Schlieren patterns were enlarged on a Nikon microcomparator (10× objective) and the traced areas were measured with a planimeter. The diffusion coefficient (cm²/sec) was calculated from plots of $(A/H)^2/4\pi$ *vs.* t (A being the area under the curve, and H, the height of the peak) (Ehrenberg, 1957). The value was corrected to the density of water at 20°.

Since the partial specific volumes of most proteins range between 0.70 and 0.75 ml/g (Mahler and Cordes, 1966), a partial specific volume of 0.73 ml/g was assumed. Using this assumption, the molecular weight of extracellular thiaminase I from *B. thiaminolyticus* was calculated as 44,000.

Disc Electrophoresis. Zone electrophoresis was carried out on a sample of thiaminase I (approximately 12-μg total) in polyacrylamide gel columns. Human serum, subjected to electrophoresis in another column, served as a reference. Thiaminase I migrated as an anion with a rate of migration greater than that of human serum albumin.

pH Optimum. The pH–activity curve at 25° for thiaminase I is given in Figure 4. The enzyme activity has a broad pH optimum of 5.8–6.8.

Effect of Substrate Concentration. The thiamine

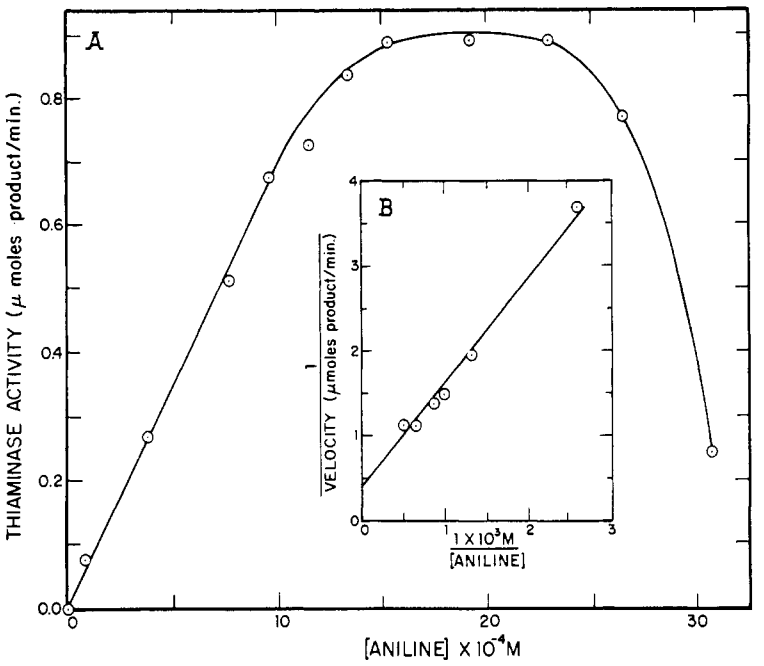

FIGURE 6: Aniline concentration studies. (A) The effect of aniline concentration on the initial rate of the base-exchange reaction of thiaminase I from *B. thiaminolyticus*. Thiamine concentration was held constant at 39.6×10^{-6} M. The velocities were measured at 25° in 0.1 M sodium phosphate buffer, pH 5.8. (B) A double-reciprocal plot of the data in A, excluding the inhibitory concentrations, according to the method of Lineweaver and Burk (1934). The K_m value for aniline was 2.9×10^{-3} M.

saturation curve for thiaminase I activity at 25° and pH 5.8 is shown in Figure 5A. The saturation curve does not follow strict Michaelis–Menten kinetics (Mahler and Cordes, 1966). The K_m value calculated from a double-reciprocal plot (Figure 5B) according to the method of Lineweaver and Burk (1934) was 8.7×10^{-6} M. Only data from the initial linear portion of the saturation curve were used in this plot.

The aniline saturation curve is presented in Figure 6A. At concentrations above 24×10^{-4} M, aniline was highly inhibitory in the base-exchange reaction. The K_m value calculated from a double-reciprocal plot (Figure 6B) was 2.9×10^{-3} M. Only data from the initial linear portion of the saturation curve were used for this plot. The maximum velocity (V_m) was 19.0 U/mg of protein.

Effect of Protein Concentration. A linear relationship exists between product formation and the amount of thiaminase I per assay in the range measured (1–8 μg of enzyme). The concentrations of both substrates were at saturation.

Temperature Optimum. The enzyme activity had a temperature optimum of 37°. The temperature coefficient, Q_{10}(10–20°), was 1.93 and Q_{10}(20–30°) was 1.33. An Arrhenius plot of the data is presented in Figure 7. The curves of the plot gave activation energies of 9800 cal/mole at the lower temperatures and 2700 cal/mole at the higher temperatures.

Thermostability Studies. More than 90% of the activity of thiaminase I remained after heating 20 min at 60° in 0.5 M sodium phosphate buffer (pH 5.8) (Figure 8). However, heating for 20 min at 65° completely destroyed the thiaminase I activity. The temperature inactivation coefficient (T_i, temperature at which 50% of the original activity remains after heating 20 min: Wilson *et al.*, 1964) was 63.5°.

Discussion

Several attempts have been made to purify bacterial thiaminase I (Kuratani: *cf.* Murata, 1965; Nose and Okada, 1958; Yokota, 1959a,b). In 1961, Ebata and Murata succeeded in purifying a thiaminase from the filtrate of a 5-day-old broth culture of *B. thiaminolyticus* (strain unknown). These workers have consistently used the filtrates from old cultures grown in undefined media as starting material for the purification of thiaminase I. Since the over-all objectives of this laboratory are to study extracellular enzyme synthesis and release and to compare the physicochemical and serological properties of thiaminase I and thiaminase II, purification of thiaminase I of known extracellular origin was a necessity. Additionally it was important that the enzyme be produced by bacteria grown in a defined medium.

Homogeneity of the thiaminase I preparations was shown using three different criteria. The enzyme preparation from the DEAE-Sephadex chromatography step (180–200-fold total purification) appeared as a single, symmetrical peak in the ultracentrifuge and as

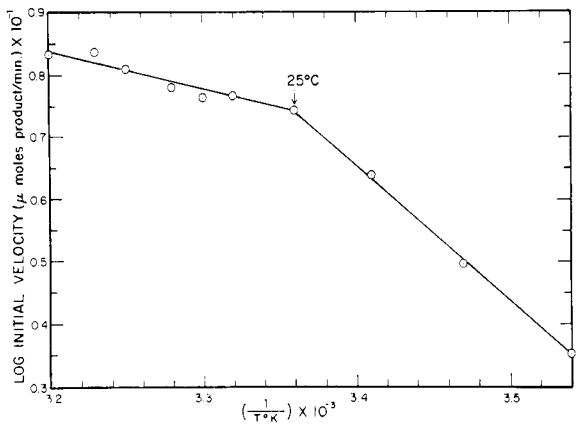

FIGURE 7: Arrhenius plot showing the influence of temperature on treation velocity. Activation energies computed from the two curves are 9800 and 2700 cal/mole.

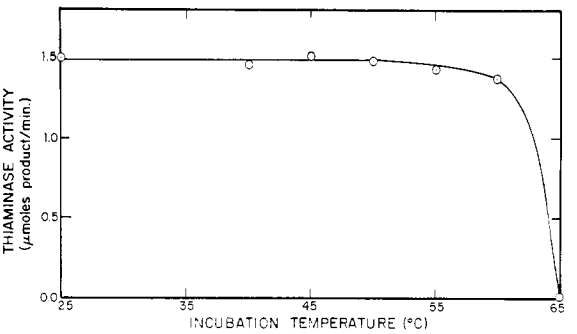

FIGURE 8: The effect of incubating thiaminase I at various temperatures on the initial velocity of the base-exchange reaction. Samples of the enzyme in 0.5 M sodium phosphate buffer (pH 5.8) were incubated for 20 min at different temperatures ranging from 25 to 65°. After incubation the samples were cooled immediately in an ice bath and assayed for residual thiaminase I activity. The temperature inactivation coefficient (T_i) is 63.5°.

one band when subjected to electrophoresis in polyacrylamide gel. Additionally, the enzyme preparation precipitated as a single band when treated with rabbit antithiaminase I using the technique of double-diffusion in agar gel.

The ultraviolet absorption spectrum of extracellular thiaminase I from *B. thiaminolyticus* is that of a simple protein with an $OD_{280\,m\mu}/OD_{260\,m\mu}$ ratio of 1.6. The spectrum of the enzyme has an optical density maximum at 277 mμ and a minimum at 252 mμ.

The purified enzyme has a sedimentation velocity coefficient of 3.7 S calculated from sucrose density gradient experiments and 3.1 S calculated from sedimentations in the analytical centrifuge. Both the sedimentation coefficient determined from a sucrose gradient experiment and that determined from sedimentation in the analytical centrifuge are higher than the value (2.0 S) reported by Ebata and Murata (1961) for thiaminase I. The diffusion coefficient was 6.6×10^{-7} cm^2/sec compared to 4.7×10^{-7} cm^2/sec as given by Ebata and Murata (1961). These workers reported a molecular weight of approximately 40,000 for thiaminase I while the data obtained in this laboratory suggest a molecular weight of 44,000. Although the molecular weight reported by Ebata and Murata (1961) is similar to that reported here, the sedimentation and diffusion coefficients determined in the two laboratories do not agree. It would appear that this similarity in the molecular weight of thiaminase I is coincidental. Thiaminase I has a molecular weight which is in the range of molecular weights reported for most bacterial exoenzymes (Pollock, 1962).

Nose and Okada (1958) reported that a partially purified preparation of thiaminase I migrated toward the anode in alkaline conditions. The thiaminase I purified by Ebata and Murata (1961) also migrated as an anion at pH 7.7. Our results are consistent with those given by these workers.

Maximum enzyme activity was displayed over a broad pH range of 5.8–6.8. Tashiro (*cf.* Fujita, 1954) reported the pH optimum of thiaminase I from *B. thiaminolyticus* to be 5.0. Yokota (1959c) found a pH optimum of 6.0 while working with a partially purified enzyme from *B. thiaminolyticus*.

The K_m values of thiaminase I at pH 5.8 and 25° are 8.7×10^{-6} M for thiamine and 2.9×10^{-3} M for aniline. Product formation of the base-exchange reaction is proportional to thiamine concentration to approximately 10×10^{-6} M. The thiamine saturation curve indicates that this substrate was slightly inhibitory at concentrations above 35×10^{-6} M. Although the initial rate does not follow strict Michaelis–Menten kinetics, such a curve has been reported by Warner and Finamore (1965) working with a type of pyrophosphohydrolase from brine shrimp eggs. The aniline saturation curve illustrates that this substrate was highly inhibitory at concentrations above 24×10^{-4} M. However, a dependence of the initial rate of the reaction on aniline concentration was observed to approximately 15×10^{-4} M. This inhibition may be due to molecular blocking of the active site(s) on the enzyme, substrate aggregation, or some other as yet unknown effect. Ebata and Murata (1961) reported that the K_m value for thiamine was 0.9×10^{-3} M and for pyridine was 1.0×10^{-3} M at pH 6.5 and 30° using thiaminase I purified from *B. thiaminolyticus*. Although the K_m value which we report for aniline agrees reasonably with that reported for pyridine, the K_m values for thiamine differ by a factor of 100. Kuratani (*cf.* Murata, 1965) published a K_m value of 0.37×10^{-6} M for thiamine. Ebata and Murata (1961) also reported a V_m of 30.5 mg of thiamine/min per mg of protein expressed as the amount of thiamine decomposed. The maximum velocity (V_m) of the thiaminase I reaction as measured in this laboratory was somewhat lower, *i.e.*, 6.4 mg of thiamine/min per mg of protein. The differences between the results reported by Ebata

and Murata and those presented here may reflect the possibility that the two thiaminases are not identical. Also the possibility exists that these differences may be attributed to variations in the assay conditions.

The temperature optimum of the enzyme activity was 37°. Yokota (1959c) published a temperature optimum of 50° for the activity of a partially purified preparation of thiaminase I while Tashiro (*cf.* Fujita, 1954) reported a temperature optimum of 30°. The Q_{10}(10–20°) of the thiaminase I purified in this laboratory was 1.93 and the Q_{10}(20–30°) was 1.33. Fujita (1954) reports that Q_{10}(20–30°) = 1.3 for thiaminase I from *B. thiaminolyticus* and that the activity definitely decreased above 30°.

An Arrhenius plot of the temperature *vs.* activity data gave two straight lines, the slopes of which change at 25°. These plots were concave downward (Koshland, 1959) and gave energies of activation of 9800 cal/mole at the lower temperatures and 2700 cal/mole at the higher temperatures. Mazrimas *et al.* (1963), working with thiaminase I isolated from carp viscera, calculated an energy of activation of 9600 cal/mole. This enzyme also had a temperature–activity optimum of 40°. Curves that show a change in activation energy have been discussed (Koshland, 1959), and a number of different explanations have been proposed (Dixon and Webb, 1964). Several possible explanations for Arrhenius plots which are concave downward are summarized as follows: (1) an over-all process involving two successive reactions with different temperature coefficients, (2) the enzyme exists in two forms having different activation energies, and (3) a reversible inactivation of the enzyme. Mazrimas *et al.* (1963) suggest that the reaction catalyzed by thiaminase I isolated from carp is not just a simple displacement, but is at least a two-step reaction. This suggestion was made after investigating the enzymatic rates as a function of the basicity of a series of substituted anilines.

An indication that the inactivation of the enzyme at temperatures greater than 45° is, at least, partially reversible is suggested by the following thermostability data. The activity of the enzyme when assayed at 55° was only 51% of that measured at 25°. However, when the enzyme was incubated at 55° for 20 min, immediately cooled to ice-bath temperature, and then assayed in the usual manner at 25°, the residual activity was 95% of the activity of the 25° treated enzyme. These data suggest that the break in the Arrhenius plot is a reflection of the reversible inactivation of thiaminase I at temperatures above 45° but not exceeding 65°. Thiaminase I has a temperature inactivation coefficient (T_i) of 63.5°.

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