STRUCTURES OF THIAMINE IN ALKALINE SOLUTION AND STUDIES ON A THIAMINE ENZYME

by

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INTRODUCTION

The observation that an aqueous alkaline solution of thiamine initially has a yellow color which fades rapidly has prompted preliminary investigations in other laboratories. It seemed reasonable that a more complete investigation of the yellow form might lead to a better understanding of the chemistry of the vitamin with the possibility that the yellow form might have physiological importance. Since the yellow form appeared to have an ultraviolet absorption spectrum greatly different from that of thiamine and its hydrochloride, it seemed profitable to begin with a spectrophotometric investigation. It was hoped that such a method would lead to a better understanding of the structure and the manner of formation of the yellow material.

Initially it appeared that methanol tended to delay the fading and so a dual investigation in both aqueous and methanolic media was projected.

Since the yellow color formed in solutions of rather high alkalinity, we determined to investigate the effect of pH upon the transformation leading to the yellow substance. The question arose whether the yellow color exists at pH's too high to have physiological importance.

In the course of this investigation several unexpected features of thiamine chemistry were observed. A previously undescribed tricyclic form was found. It also became possible

to relate several alkaline transformations of the acid-stable vitamin.

Finally we decided to investigate spectrophotometrically a thiamine-requiring enzyme, carboxylase, in the hope that it might be possible to detect the bound form of the pyrophosphate of the vitamin. It was assumed that cocarboxylase would largely respond to pH changes as thiamine itself does. It seemed at least possible that the binding of the coenzyme to the enzyme might lead to the stabilization of a form of the former which is ordinarily found only at a higher pH.

A great deal of time was spent in purifying the enzyme since the one published purification scheme proved to be unreproducible and since the initial carboxylase activity of our wheat germ was low in comparison with that reported in this publication. In particular we wished to try enzymatic means to degrade the enzyme while maintaining its activity in the hope that the abbreviated enzyme would lend itself to more meaningful spectrophotometric measurements; the reported molecular weight for carboxylase from wheat germ indicated the possibility that the ultraviolet absorbancy of the enzyme might obscure spectrophotometric changes in the coenzyme.

LITERATURE SURVEY

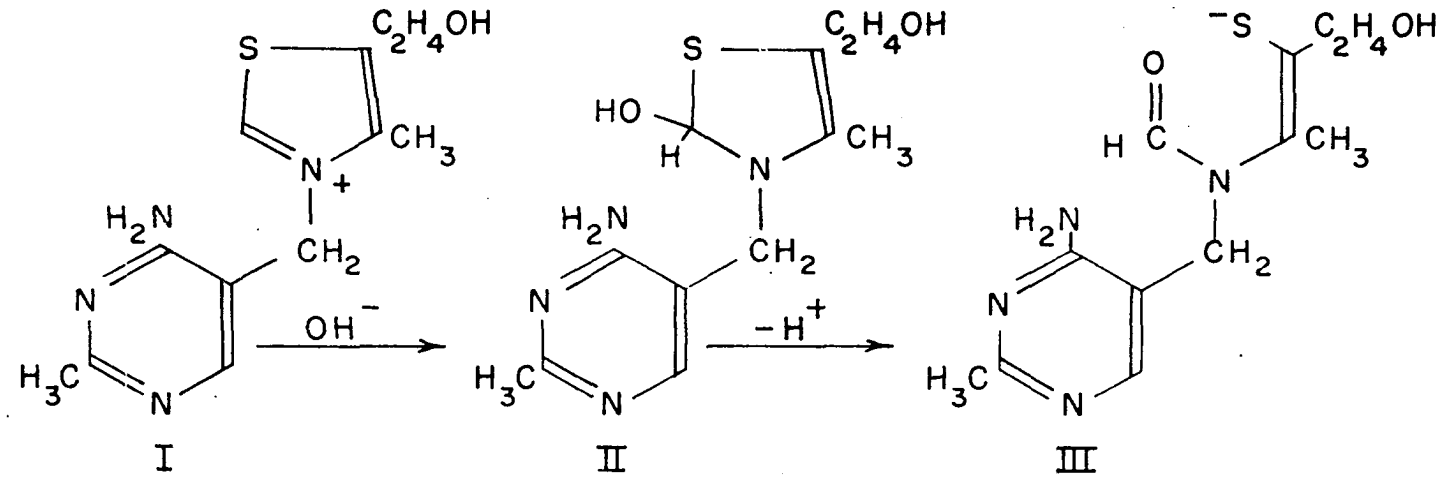
Structures of Thiamine

The structure of thiamine was elucidated by several groups of workers and was proved by the identical behavior of synthetic and natural material (1-7). The commercial form of the vitamin is the chloride hydrochloride or the mononitrate of the neutral form (Structure I, Figure 1). Lohmann and Schuster (8) established that cocarboxylase, the pyrophosphate ester of the thiazole β-ethylol group, is the physiologically active co-factor for yeast carboxylase. Subsequent investigations have established cocarboxylase as the common active form (9).

Very few modifications of the structure are permissible if the vitamin activity is to be maintained (9). The methyl groups on either the pyrimidine or the thiazole rings may be replaced by a few aliphatic analogues without complete loss of activity but the amino group, the hydrogen on the thiazole ring and the ring systems themselves are essential. Interestingly, in a non-enzymatic model system an analogue with a benzyl group replacing the pyrimidyl group has considerable thiamine activity (10).

There has been conjecture concerning the physiological role of thiamine disulfide (Structure IV, Figure 1). Some investigators have reported this form active (11) while others

Figure 1. Structures of thiamine and related compounds. Structure I represents the neutral form of thiamine; Structures II to VI, respectively, the pseudobase, the colorless thiol form, thiamine disulfide, the yellow form of thiamine as suggested by Zima and Williams (26), and thiochrome.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CH2
N
OH−
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HO
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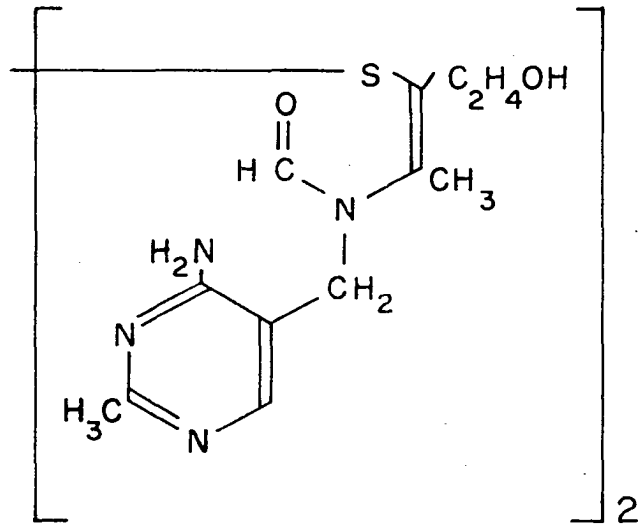
-S
O
H C
III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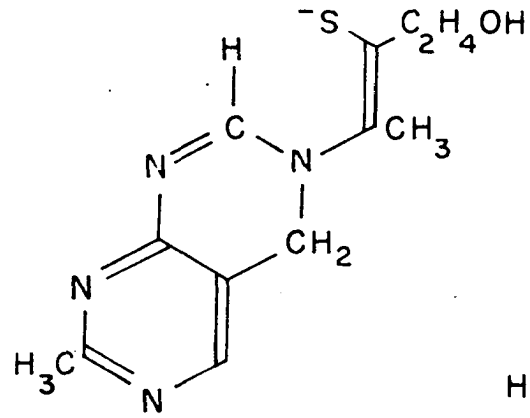
O
H C
N
CH3
H2N
CH2
N
H3C
N
2

IV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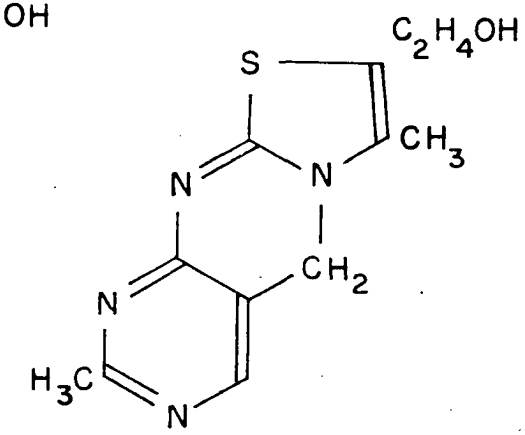
H
C
N
CH3
CH2
N
H3C
N

V


C2H4OH
S
CH3
N
N
CH2
N
H3C
N

VI

report it inactive with pyruvic oxidase and carboxylase (12, 13). While the disulfide may exist in nature (14, 15), its enzymatic activity most likely is due to prior reduction to the thiol form (16).

Mixed disulfides of thiamine and alkyl radicals are known from synthetic work (17-20). Some have been prepared merely by incubating thiamine with plant extracts (17, 18). The finding that the disulfide compound, α-lipoic acid, is a co-factor with cocarboxylase for pyruvic oxidase raises interesting speculations concerning a possible mixed disulfide between the co-factors in vivo.

Thiamine in alkaline solution undergoes several transformations. Electrometric titration of the neutral form with base gives a titration curve which indicates two moles of base taken up and whose shape indicates a diprotic dissociation with K_2 at least ten times greater than K_1 and with a pK average of 9.2 The titration must be performed slowly because equilibrium is not attained until 15 to 20 minutes after each increment of base. The titration is reversible if oxygen is excluded (21, 22). Clarke and Gurin (23) suggested that a pseudobase form (Structure II, Figure 1) is slowly formed from the neutral form by the uptake of a hydroxyl ion and that an open-ring ionized thiol form (Structure III, Figure 1) is formed by the removal of a proton from the pseudobase. At equilibrium little of the pseudobase is present since the keto analogue cannot be found after oxida-

tive treatment of the mixture (24). In addition, the shape of the titration curve precludes appreciable amounts of the pseudobase or of a protonated thiol form in equilibrium with it. Watanabe and Asahi (25) confirmed this by polarographic determination of the amount of ionized thiol form. The sodium salt of the thiol form has been prepared by Zima and Williams (26).

In addition, thiamine exhibits a transient yellow color in strong alkali (27). Zima and Williams (26) formed the sodium salt of the yellow form from thiamine hydrochloride and three equivalents of sodium ethoxide. Structure V, Figure 1, represents their suggestion for its structure.

Carboxylases

The non-oxidative degradation of pyruvic acid is catalyzed by carboxylases to yield carbon dioxide and a two-carbon fragment which may be combined with exogenous or endogenous acetaldehyde to yield acetoin. In some systems the two-carbon unit may react with another molecule of pyruvate to give α-acetolactate which may then be decarboxylated to give carbon dioxide plus acetoin. Acetaldehyde may serve as the sole substrate for acetoin synthesis for some carboxylases. In addition, acetaldehyde itself is often a major product.

The enzymatic activity has been studied in crude preparations from different sources. These differ largely in

respect to the initial substrate requirement and also in respect to the substrate which the two-carbon fragment accepts. Plant (28, 29) and porcine heart (30) preparations may form acetoin from acetaldehyde alone, whereas preparations from some bacteria use only pyruvate (31, 32). Yeast (33), wheat germ (28, 34) and porcine heart (30, 34) carboxylases using a mixed substrate of pyruvate and acetaldehyde produce acetoin in yields per unit time greater than yields from either substrate alone.

Carboxylase has been purified from yeast (35, 36), wheat germ (28) and Aerobacter aerogenes (37). Cocarboxylase and magnesium (or manganous) ion are co-factors and the pH optima are near 6.2. All decarboxylate several other α-keto acids at lower rates. Singer and Pensky (28) provided most of the information to follow concerning the wheat germ enzyme.

The wheat germ enzyme has been purified reportedly 2700-fold; yeast carboxylase, roughly 28-fold. Boffi et al. (38) report a greater purification for the yeast enzyme.

The bacterial "carboxylase" has been separated into two components, one of which catalyzes the formation of α-acetolactate and the other of which catalyzes the decarboxylation of this intermediate to yield acetoin. The yeast and porcine enzymes form α-acetolactate (39) but cannot decarboxylate it (40). The production of α-acetolactate as an intermediate in pyruvate catabolism has not as yet been reported for the wheat germ enzyme.

The ultraviolet absorption spectrum of the yeast enzyme shows no peaks attributable to thiamine but this may be due to the low degree of purification. Highly purified wheat germ enzyme shows small differences near 250 mμ between the spectrum of apoenzyme plus magnesium ion plus cocarboxylase and the sum of the spectra of the components. The absorbancies at all wave lengths of various mixtures of apoenzyme, its co-factors and acetaldehyde are slightly higher than the corresponding sums of the independent absorbancies according to Singer and Pensky (28). Measurements were made between 230 mμ and 300 mμ in phosphate buffer at pH 7 which is from 0.7 to 0.8 pH units removed from the pH optimum of the enzyme.

The wheat germ enzyme loses its cocarboxylase and magnesium ion early in purification without appreciable denaturation but the yeast enzyme binds the co-factors tightly. Exhaustive dialysis of the latter enzyme near pH 6 releases the co-factors with prior denaturation of the protein; however, they may be released at pH's near 8 and the activity may slowly regenerate at lower pH's.

The purification of the wheat germ enzyme requires defatting with acetone, aqueous extraction, isoelectric precipitation, alcohol precipitation, ammonium sulfate precipitation and dialysis. The decarboxylase activity assayed manometrically remains in the same ratio to the acetoin

activity at all stages of purification. Its molecular weight was estimated to be near one million from ultracentrifugal studies. Acetaldehyde and heavy metals inhibit the decarboxylase activity and p-chloromercuribenzoate inhibition indicates the presence of a sulfhydryl group in the active site. Eich and Cerecedo (41) report that pyrithiamine and oxythiamine pyrophosphate but not oxythiamine inhibit the enzyme if they are added to an apoenzyme preparation before cocarboxylase but not if added afterward.

Pyruvic Oxidases

Pyruvic oxidases are complex enzymes which also decarboxylate pyruvate. The decarboxylation is oxidative and in addition to cocarboxylase and magnesium ion, α-lipoic acid, Coenzyme A, FAD, and DPN are co-factors. Acetoin is not normally formed and the decarboxylation of pyruvate leads to acetyl Coenzyme A. However, Juni and Heym (40) describe pigeon breast muscle pyruvic oxidase which anaerobically forms acetoin from pyruvate with α-acetolactate as an intermediate. Apparently a portion of pyruvic oxidase is a "carboxylase".

Recently Escherichia coli pyruvate oxidase has been separated into three components, one of which is a cocarboxylase enzyme (42, 43). It is reported to have "carboxylase activity" and is, in fact, designated as

pyruvic carboxylase.

Pig heart α-ketoglutorate dehydrogenase and E. coli α-ketoglutarate dehydrogenase and pyruvic oxidase have been separated into two fractions, the first requiring cocarboxylase and α-lipoic acid and the second requiring FAD and DPN (43, 44).

The possibility that previously described carboxylases are portions of greater enzyme complexes remains open.

Mechanism of Thiamine Action

Thiamine in model systems catalyzes virtually the same reactions that the complete enzyme system does (45, 46). The model system, however, forms some α-acetolactate and little free acetaldehyde while enzyme systems yield little of the former and a great deal of the latter. Both decarboxylate pyruvate and form acetoin. That the model system alone can catalyze these reactions indicates an intimate role for the coenzyme in enzymatic catalysis and indeed implicates it as the site of catalytic action.

Breslow (47) has reviewed several mechanisms which have had some support in the past. Initially he proposed a mechanism which, while somewhat more elaborate, essentially involved the formation of an anion at the methylene bridge of thiamine (48). The anion then was presumed to attack nucleophilically the alpha carbon of pyruvate leading to decarboxylation and

to the formation of a α-hydroxyethyl adduct. The hypothesis continued with the formation of a keto group at the alpha carbon of the adduct, the breaking of the carbon-carbon bond linking the adduct to thiamine and the regeneration of thiamine.

The hypothesis was discarded, however, when Westheimer's group (49) and later Breslow (50) found that the hydrogens at the methylene bridge are not labile and do not exchange with deuterium oxide during catalysis. Instead it was found that the hydrogen at carbon 2 of the thiazole ring is labile and does exchange with deuterium oxide at neutral pH and room temperature (49, 51). Breslow (47, 51) then proposed a somewhat altered mechanism which nevertheless involved the important features of the earlier proposal and required the formation of the nucleophilic anion at the thiazole carbon 2. One intermediate in the degradation of pyruvate, after decarboxylation itself, would be thiamine substituted with a two-carbon anionic unit at the thiazole carbon 2. The charge, in fact, may not be localized since the dipolar ion intermediate may be stabilized by other resonance forms.

Implicit in Breslow's mechanism is the formation of an α-hydroxyethyl thiamine derivative by the addition of a proton to the dipolar ion.

Krampitz et al. (52) synthesized this derivative, and found it nearly as active as thiamine plus pyruvate with

alkaline washed yeast in yielding carbon dioxide. HET* was also a growth factor for L. fermenti or L. viridescens. Carlson and Brown (53) found that HETPP constitutes a large portion of the thiamine in yeast and other microorganisms. These authors isolated HETPP from a mixture of purified wheat germ carboxylase and pyruvate and also described methods for "bulk" enzymatic preparation of HETPP. Holzer and Beaucamp (54) reported similar findings for purified yeast carboxylase.

Both groups clearly established the identity of the substance isolated from enzymatic mixtures with synthetic material by use of paper chromatography. Both groups also established that HETPP labeled in the two-carbon adduct led to labeled acetoin thereby relating HETPP to the physiological process.

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*The abbreviation HET is used for α-hydroxyethyl thiamine; HETPP, for the pyrophosphate ester of the β-hydroxyl group of HET.

EXPERIMENTAL

Chemicals

The following chemicals were used with no further purification: cocarboxylase chloride (California Corporation for Biochemical Research, B grade), magnesium sulfate (Mallenkrodt, analytical grade), creatine (Nutritional Biochemical Corporation, c.p.), succinic acid (California Corporation for Biochemical Research, C grade), zinc sulfate (Baker), tris-(hydroxymethyl) aminomethane (Sigma 7-9), and reagent grade ethanol, acetone and methanol. Moisture was excluded from methanol during storage.

Alpha-naphthol was sublimed from crude material (Matheson) and was used within a week.

Thiamine chloride hydrochloride (Merck) was dried in a vacuum desiccator over magnesium perchlorate and was used without further treatment. Recrystallization of this material led to no change in its absorption spectrum. Pyrithiamine and oxythiamine were purchased from the California Corporation for Biochemical Research, Los Angeles, and 2-methyl-4-amino-5-(2-aminomethyl)-pyrimidine from Nutritional Biochemicals Corporation, Cleveland.

Both 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium chloride and 3-benzyl-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride were supplied by D. E. Metzler (55). Dihydrothiamine was

prepared by R. Yount according to the procedure of Bonvicino and Hennessy (56) and Karrer and Krishna (57).

The white sodium salt of the thiol form of thiamine, the yellow sodium salt of thiamine and thiamine monochloride were prepared according to the procedures of Zima and Williams (26).

Preparation of 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 filtered off by suction on a sintered glass filter for three 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 weight by comparison of spectrum in 0.1 M HCl with that of thiamine hydrochloride, 267.

Spectrophotometric Measurements

A Beckman DU spectrophotometer with the cell compartment thermostatted at 25° in most cases and at 19.2° in some experiments was used for quantitative spectral measurements. A Cary model 14 recording spectrophotometer was employed for preliminary evaluation of spectra.

Matched quartz or pyrex cuvettes (all one-cm. light path) were used.

Spectra of thiamine were measured on 1.42×10^{-4} M solutions prepared by dilution of stock solutions.

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 five minutes for qualitative studies and within three minutes for quantitative measurements. The absorbancy of the yellow form of thiamine decreased by 1-3 per cent during the first six minutes after mixing.

The complete U.V. spectra (1.42×10^{-4} M solutions) of the following compounds, structurally related to thiamine, were observed in 0.1 M and 0.4 M methanolic KOH: 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, oxythiamine (3-(4-hydroxy-

2-methyl-5-pyrimidyl-methyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride, and pyrithiamine (1-(4-amino-2-methyl-5-pyrimidylmethyl)-2-methyl-3-(2-hydroxyethyl)-pyridinium bromide hydrobromide).

Spectra of thiamine in aqueous solutions were measured in buffers, usually of 0.2 ionic strength and consisting of potassium phosphates at pH 6.8, of sodium bicarbonate and KOH from pH 9 to 10, of piperidine hydrochloride and KOH from pH 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 (58) which permits complete mixing within two seconds or less. Readings were obtained at intervals of five seconds beginning ten 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 $19.2 \pm 0.2^{\circ}$. The pH was then measured using a Beckman model G meter and the temperature of the sample in the pH meter noted. The pH was corrected to 19.2°. Although thiamine can

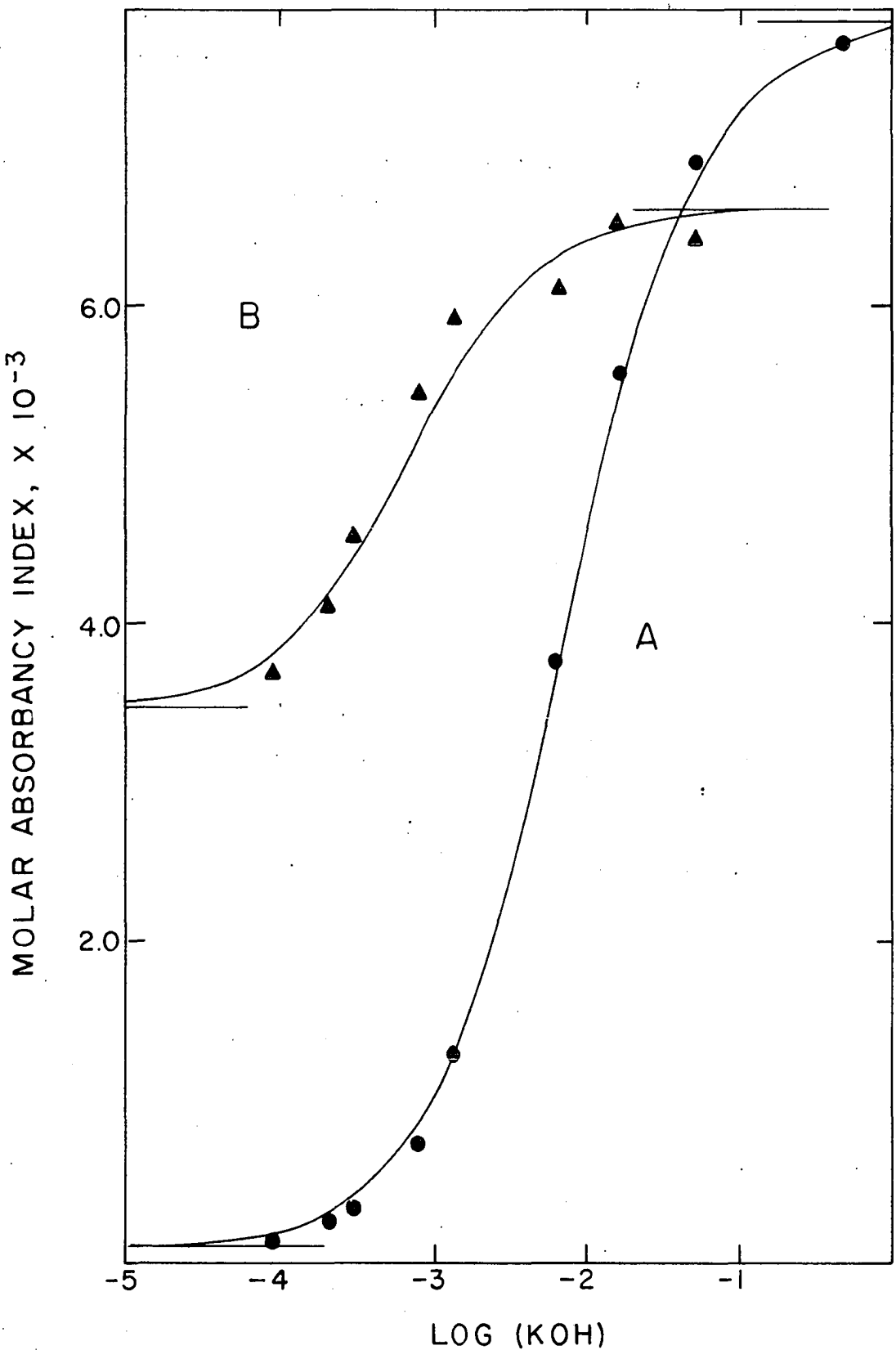
undergo irreversible oxidative reactions in air, we have taken no precautions to exclude air. This is justified by the short time intervals involved. It appears that in most cases, only a few per cent of irreversible decomposition occurs in the spectrophotometer cell in periods up to two hours.

Calculations of Spectrophotometric Data Involving Forms of Thiamine

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.

Curve B in Figure 2 which shows the molar absorbancy index a_M for the neutral plus any intermediate forms in methanol was computed using the following data. From Curve A, Figure 2, 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).

Figure 2. Ultraviolet absorbancy of thiamine versus the concentration of methanolic KOH. Curve A with solid circles represents the molar absorbancy index at 349 mμ; Curve B with solid triangles, the molar absorbancy index at 290.3 mμ after subtraction of the contribution of the yellow form. The horizontal lines represent limits used for the construction of theoretical curves.


MOLAR ABSORBANCY INDEX, X 10^{-3}
6.0
4.0
2.0
B
A
-5
-4
-3
-2
-1
LOG (KOH)

Within experimental error, the same molar absorbancy indices were obtained for the yellow sodium salt described by Zima and Williams (26). At 290.3 mμ the a_M for the yellow form is 3.38×10^3.

Spectrophotometric Studies with Carboxylase

The protein content of solutions containing pyruvic carboxylase was estimated from their absorbancy at 280 mμ using the approximation that one mg. of protein per ml. of solution will have an absorbancy of 1.000 in a one-cm. light path cuvette.

Complete spectra in the region 330 mμ-349 mμ were taken on carboxylase solutions at the third, fourth and fifth stages of purification without added co-factors.

Complete spectra of the apoenzyme at the final stage of purification were measured both in the presence and the absence of its co-factors. An apoenzyme preparation in 0.1 M, pH 6.80 imidazole buffer (1773-fold pure with a Purity index equal to 45.4) was used. In one experiment the spectrum of the preparation was observed diluted one to three with either 5×10^{-2} M, pH 6.2 succinate buffer or the same buffer containing appropriate amounts of co-factors to give final concentrations of 1.83×10^{-4} M cocarboxylase and 1×10^{-2} M magnesium sulfate.

In a second experiment the initial apoenzyme preparation

was diluted five to six with either 0.1 M, pH 6.80 imidazole buffer or the same buffer containing co-factors to give final concentrations of 4.7×10^{-5} M cocarboxylase and 7.5×10^{-3} M magnesium sulfate.

The spectra of the apoenzyme and its co-factors were constant during spectrophotometric measurement after the samples had stood in the cold overnight or for several days during which an almost imperceptible precipitate settled. However, the absorbancies increased directly after the preparation of the mixtures and a visible opalescence developed. The cloudiness was most noticeable in the cases of samples containing both co-factors and caused their absorbancy to be apparently greater than the arithmetic sum of the spectra of the separate components.

In these investigations the absorbancies of the slight precipitates formed upon standing were also measured. The samples were centrifuged briefly, the supernatants were removed and the residues were taken up in 6.0 ml. of the imidazole buffer. There were small differences in absorbancy; the precipitates from samples containing both apoenzyme and cocarboxylase absorbed more strongly after they were redissolved. In addition insoluble residues remained from all the samples.

Acetoin and Carbon Dioxide Assays for Carboxylase

The acetoin activity of pyruvic carboxylase was assayed by a procedure derived from that of Singer and Pensky (34). Aliquots of aqueous solutions of cocarboxylase chloride (1.95×10^{-4} M), magnesium sulfate (1×10^{-3} M), and succinate (pH = 6.0; 1×10^{-1} M), were added to an aliquot of the enzyme solution (usually 1.0 ml.) plus water to give a volume of 2.90 ml. The mixtures were thermally equilibrated at 25° in a water bath for approximately fifteen minutes, and 0.1 ml. of aqueous sodium pyruvate (3.08×10^{-2} M) was added to begin the sixty minute incubation period. All concentrations were expressed in terms of final concentration. A blank without substrate was run simultaneously.

At the final stage of purification the 1×10^{-3} M magnesium sulfate no longer saturated the enzyme. Optimal assay yields were achieved in the presence of a final concentration of 1×10^{-2} M magnesium sulfate. The usual cocarboxylase concentration was sufficient.

The incubates were deproteinized after the method of Somogyi (59). Three ml. of aqueous $ZnSo_4$ (10 per cent w/v) and 0.6 ml. of sodium hydroxide (2.5 N) in addition to 5.4 ml. of water were added in turn and the sample was thoroughly shaken. Substrate was then added to the blank. The material was centrifuged one-half hour at 1250 r.p.m. (International centrifuge, size 1 Model C50) after which 4-ml. aliquots were

withdrawn for the measurement of acetoin (60). One ml. each of creatine (0.5 per cent, w/v) and water were mixed with the aliquot containing acetoin and to these were added 1.0 ml. of α-naphthol in 2.5 N NaOH (0.5 g. per 10 ml.). The α-naphthol solution was prepared directly before use. The solutions were vigorously shaken and the optical density of the resultant red solution was observed at 525 mμ.

The color yield became optimal at approximately one hour after the addition of the color reagent. Several readings were made on each sample shortly before and after the one-hour period to insure observation of optimal color yield. The optical density at 525 mμ began to diminish at 1 1/4 to 1 1/2 hours after the addition of the color reagent in the case of several samples which used stock acetoin solution (5.95×10^{-4} M, final concentration) instead of enzyme solution in the incubate mixture and which were "deproteinized" as described above.

Acetaldehyde delays optimal color development. The optimum was not realized until 1 3/4 hours after beginning color development when the incubate was approximately 7.1×10^{-5} M in respect to acetaldehyde and 5.95×10^{-4} M in respect to acetoin. A lower acetaldehyde level (approximately 3.55×10^{-5}) did not delay the optimal color yield greatly.

The assays in most cases were run in triplicate and the precision was usually 5 per cent or less from the average of

replicates. A unit of enzyme was defined as the amount of enzyme which produces 0.1 μM of acetoin under the assay conditions. The Purity Index was the ratio of units per ml. to mg. of protein as measured by the absorbancy at 280 mμ. Aliquots of stock acetoin were taken so that the concentration of acetoin was 5.95×10^{-4} M or 5.66×10^{-4} M in the incubation mixture. These were treated according to the assay procedure without the one-hour incubation and gave an overall average optical density of 0.917 at 525 mμ per 1.0 μM of acetoin.

Several samples of α-carboxylase in early stages of purification were assayed by the manometric method of Singer and Pensky (28) and at the same time by the acetoin assay so that both assays might be correlated. The former assay employed aqueous cocarboxylase (1.95×10^{-5} M), sodium pyruvate (3.03×10^{-2} M) and magnesium sulfate (1×10^{-3} M) solutions in the side arm of the Warburg flask to initiate the reaction after approximately fifteen minutes of thermal equilibration. One mg. of bovine serum albumin, and succinate solution (pH 6.0; 1×10^{-1} M), in addition to enzyme solution and water were in the main chamber. Also in the main chamber was 0.6 ml. of stock succinate solution (pH 6.0; 1×10^{-1} M) saturated with dimedone. The total volume after mixing the side-arm and main chamber solutions was 3.0 ml. and all concentrations were expressed in terms of this final volume.

The incubation period was five minutes at 30°. Several determinations were allowed to proceed for longer periods of time and manometer readings were taken at intervals. In addition, the effect of enzyme concentration on carbon dioxide evolution was studied in two separate experiments.

Series of acetoin assays for which either the amount of enzyme or the length of incubation period was varied were run on the aqueous extract and the solution of the alcohol precipitate (see below) to determine the useful range of this assay.

Purification of Wheat Germ Carboxylase

Wheat germ sources

Carboxylase was purified by a modification of the procedure described by Singer and Pensky (28). Unheated, low starch wheat germ (Types 75 and A) was procured from General Mills, Inc., and aside from storage in the cold, was given no additional treatment before use. One sample which was desiccated *in vacuo* over magnesium perchlorate and sodium hydroxide in the cold for three weeks did not show atypical behavior. Types 75 and A are described by General Mills, Inc., as "essentially the same" as Type S-50 used by Singer and Pensky. In addition a commercially packaged, unheated material was procured from the Pavo Company, Minneapolis, Minnesota. The Pavo Company product was not used frequently.

Acetone powder

One kilogram of wheat germ was treated with portions of 20 liters of acetone (-15°) in a Waring Blendor at low speed. The total length of homogenization time was twenty minutes. The slurry was filtered by aspirator on a Buchner funnel. The powder was desiccated *in vacuo* over concentrated sulfuric acid in the cold. Before a period of standing and several times during that period, the desiccators were opened and the powder was stirred to assure complete removal of acetone. The powder was allowed to stand 12-24 hours in the desiccator. The yield was 700 g. of powder.

Aqueous extract

All purification was done *ca.* 3° unless otherwise stated. Distilled water was used.

One kilogram of acetone powder was treated with five liters of water for about one hour with stirring and was allowed to stand for from four to sixteen hours. The latter time was altered as convenient. The suspension was centrifuged at 2750 r.p.m. in the Servall "superspeed" centrifuge. The precipitate was discarded. Volume of supernatant was *ca.* 70 per cent of the original volume of water. This is in contrast to a volume 40 per cent of the original amount of water as reported by Singer and Pensky.

Isoelectric precipitate

All pH adjustments were made at $3^{\circ} \pm 1^{\circ}$ with cold 1 M or 0.1 M acetic acid. All were made quickly with rapid stirring.

An isoelectric precipitate was made from the aqueous extract by bringing its pH first to 5.35. After centrifugation at 4000 r.p.m. for 3/4 hour the first precipitate was discarded and the pH of the supernatant was then brought to 4.90. The precipitate was allowed to settle for twenty hours and was removed by centrifugation at 4000 r.p.m. for one hour.

The pH adjustments in the isoelectric precipitation must be carefully done. The pH of the initial precipitation was brought to a value different from 5.20 in several separate purifications and the best yield was found from a sample brought to 5.35.

Alcohol precipitate

The pH 4.90 precipitate was taken up in 675 ml. H_2O plus 225 ml. of 0.4 M succinate at pH 6.0 for four hours with thorough breaking up of the "clumps" and with a great deal of stirring. It appears that less time may result in incomplete dissolution and eighteen hours treatment results in some loss of activity. The suspension was centrifuged one hour at 4000 r.p.m. The supernatant was brought to pH 5.54. Cold (-15°) 95 per cent alcohol was added with stirring in the cold until enough was added to account for 15 per cent of the final

volume assuming additivity of volumes. The precipitate was allowed to settle for 1/2 hour and was removed by centrifugation at ≥600 r.p.m. for 1/2 hour. It was dried *in vacuo* in the cold over concentrated sulfuric acid. The yield was equal to 450 to 600 mg. of alcohol powder.

Ammonium sulfate precipitate

Two hundred mg. of alcohol powder was taken up in 50 ml. of 0.05 M, pH 7.65 tris buffer for approximately two hours. The suspension was centrifuged for one hour at 4000 r.p.m. The pH of the supernatant was brought to 8.0 and saturated (at 5°) ammonium sulfate solution was added. After the addition of 50 ml., the solution was allowed to stand 1/2 hour and was then centrifuged one hour at 4000 r.p.m.

The precipitate was saved and was taken up for several minutes in *ca.* 9 ml. of glass distilled water. It was dialysed against portions of 1½ liters of glass distilled water for ≥4-48 hours. A precipitate fell within the sac. Dialysis was carried on approximately twelve hours past this point. The precipitate was removed by centrifugation at 5000 r.p.m. for five minutes and was taken up in 25 ml. of 0.1 M, pH 6.80 imidazole buffer for one hour. The suspension was centrifuged one hour at 7000 r.p.m. and the precipitate was discarded. Centrifugations of 18,000 r.p.m. were used for samples prepared for spectrophotometric investigations.

Influence of co-factors upon yields during purification

To determine the influence of co-factors upon enzyme yields during purification, cocarboxylase chloride and magnesium sulfate were added either before the isoelectric precipitation or the alcohol precipitation, or before both. Typically the solutions were made 5.85×10^{-5} molar in respect to the former and 5×10^{-3} molar in respect to the latter, which concentrations represent three times the amount used to saturate the enzyme in the acetoin assay.

RESULTS

The Pseudo-Base and Colorless Thiol Forms of Thiamine

The ultraviolet absorption spectra in water of thiamine at pH 6.8, and at pH 10.4 ninety minutes after its addition to the alkaline buffer, are shown in Figure 3 by curves A and B, respectively. Curve A represents the well-known spectrum of the free base (61); it has two peaks at 233 mμ and 266 mμ and virtually no absorbancy beyond 310 mμ.

Curve A, Figure 4, is the similar spectrum of the free base form in methanol. The spectrum was taken from a solution of the hydrochloride to which an equivalent amount of KOH had been added.

Curve B, Figure 3, represents the spectrum of the colorless thiol form (Structure III, Figure 1), which forms slowly over a period of an hour at this pH. Its peak lies at 235 mμ with a shoulder in the region 250 mμ-270 mμ. The absorbancy of the colorless thiol is greater at all wave lengths than that of the neutral form; analogously, the addition of base to 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium chloride causes absorbancy increases at all wave lengths. Curve B also represents the spectrum in 0.02 M aqueous KOH of the crystalline sodium salt of the colorless thiol form prepared according to Zima and Williams (26). This spectrum also has been previously described (26).

Figure 3. Spectra of thiamine in aqueous solutions. Curve A represents the spectrum at pH 6.8; Curve B, the spectrum at pH 10.4 after standing ninety minutes, in 0.02 M potassium hydroxide after thirty minutes. Curve B also represents the spectrum of the white sodium salt of thiamine in 0.02 M potassium hydroxide. Curve C represents the spectrum of thiamine in 0.1 M sodium hydroxide at the time of mixing (from extrapolation).

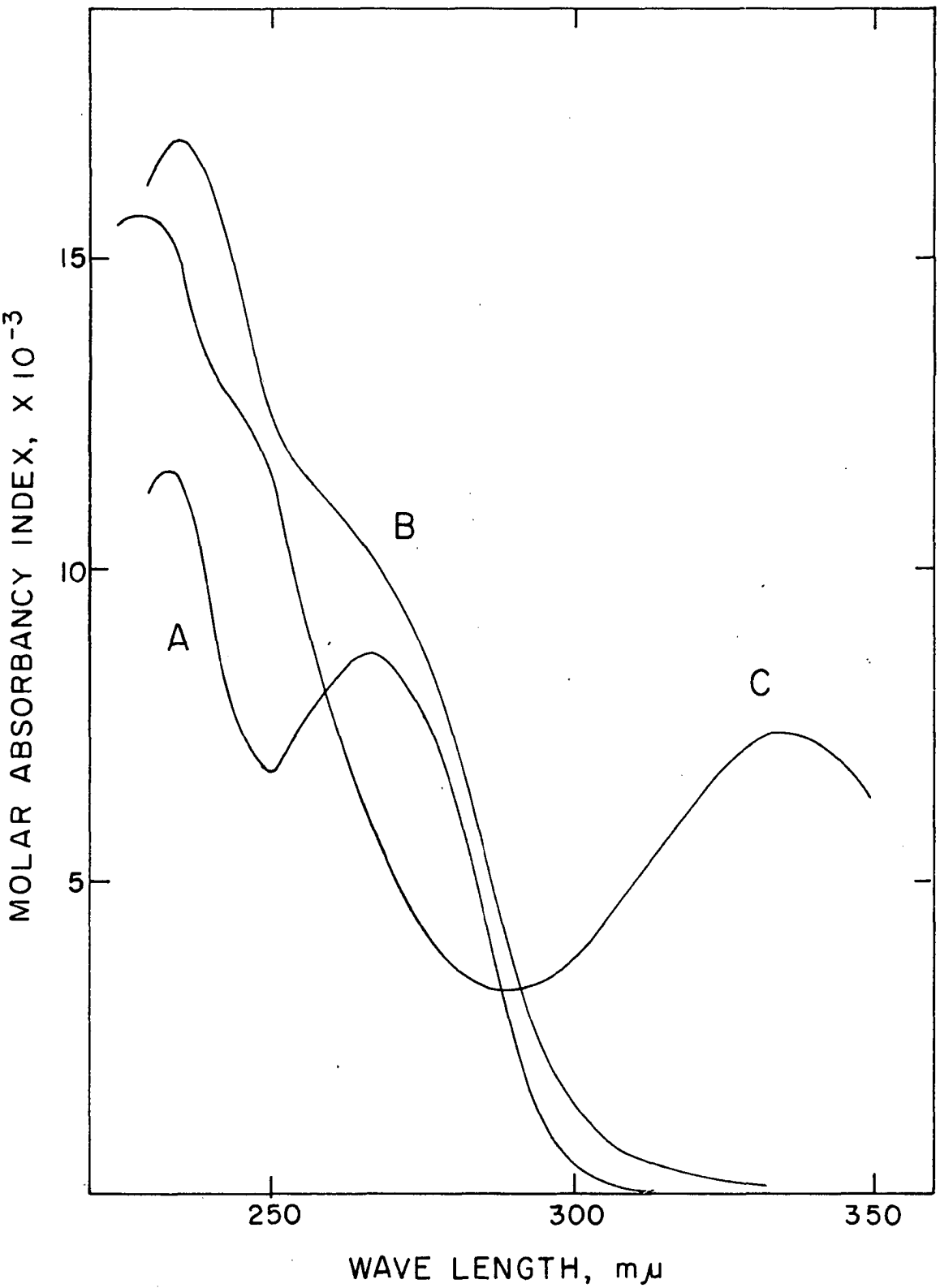
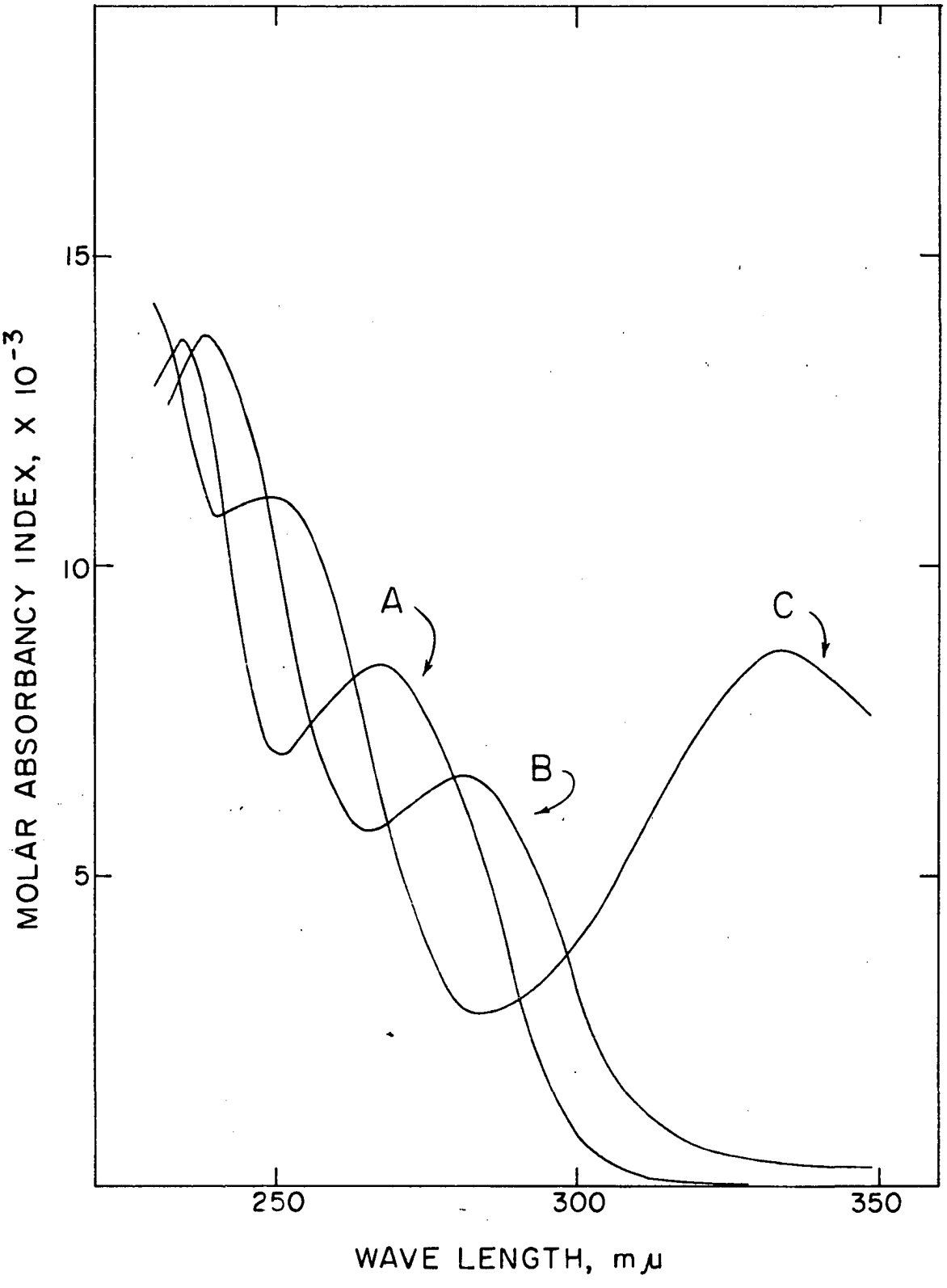

MOLAR ABSORBANCY INDEX, X 10⁻³
15
10
5
A
B
C
250
300
350
WAVE LENGTH, mμ

Figure 4. Spectra of forms of thiamine in methanolic solutions. Curve A represents the spectrum of the neutral form; Curve B, the calculated spectrum of the tricyclic intermediate; Curve C, the spectrum of the yellow form.


MOLAR ABSORBANCY INDEX, X 10^{-3}
15
10
5
A
B
C
250
300
350
WAVE LENGTH, mμ

The two reactions by which the neutral form of thiamine (see Figure 1) is converted to the colorless thiol form can be regarded as formally equivalent to the stepwise dissociation of a diprotic acid with characteristic acid dissociation constants.

It is evident from the shapes of the published titration curves that the conversion of thiamine to the colorless thiol form is similar to a situation wherein two protons from a diprotic acid dissociate simultaneously with no detectable amount of an intermediate singly dissociated form. The second dissociation constant in this event is much greater than the first. In thiamine solutions the pseudo-base form (Structure II, Figure 1) is never present in amounts great enough to affect the shape of titration curves (21, 25), nor can it be detected through oxidation to the corresponding keto compound (24).

Titrimetric measurements by Williams and Ruehle (21) give an average of the apparent pK's for the two steps of 9.0 and measurements by Watanabe and Asahi (25), of 9.33 at 25°. Our spectrophotometric titrations give a pK_{av} of 9.3 for thiamine and 10.3 for the simpler 3,4-dimethyl-5-(2-hydroxyethyl)-thiazolium chloride and 3-benzyl-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride.

The Yellow Form of Thiamine

The addition of thiamine to solutions of pH 11 and above leads to the instantaneous production of a yellow color, which fades rapidly in aqueous media (27), but which is clearly visible, depending on the basicity, for an hour or more in methanol.

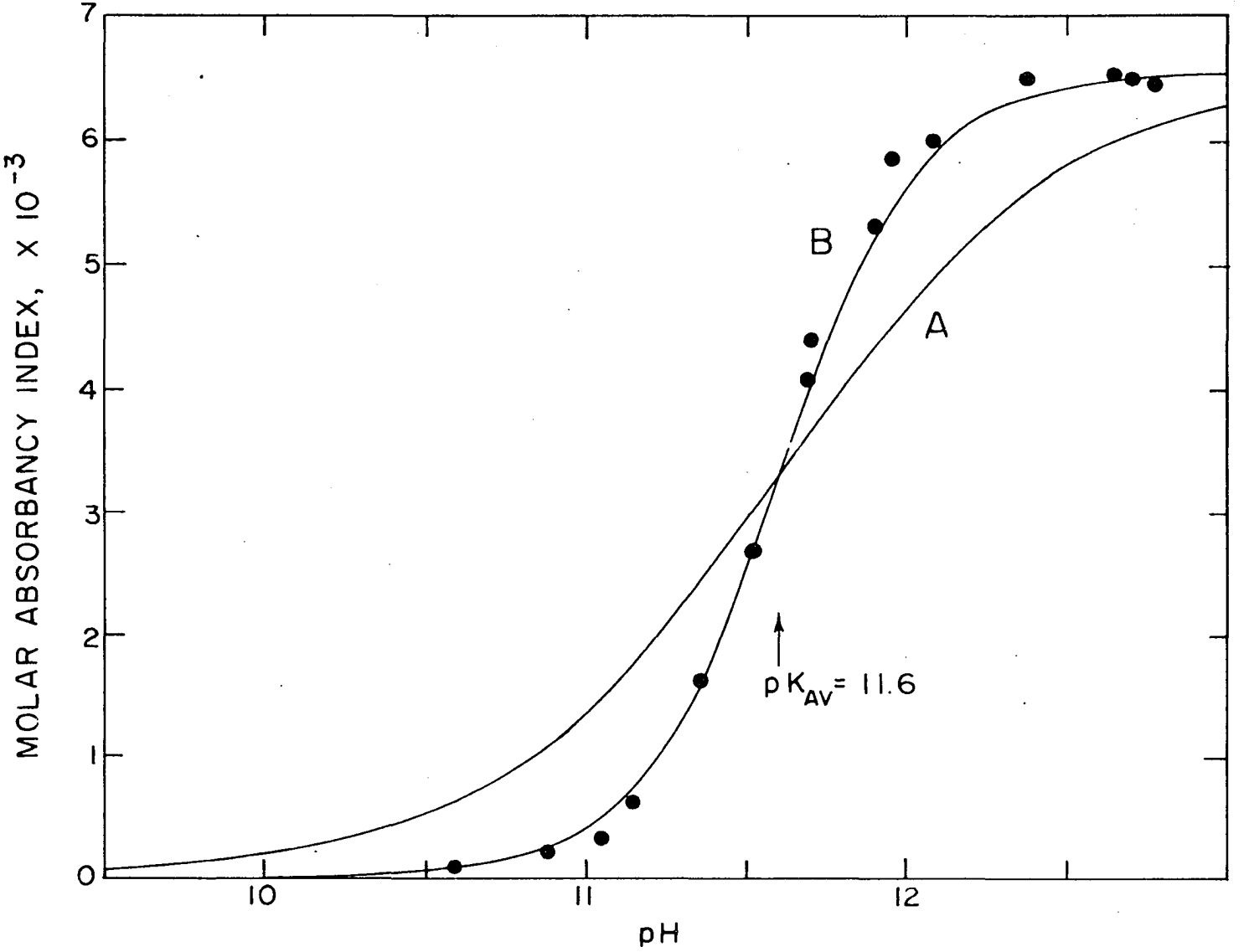
The spectrum of the yellow form is bathochromically shifted from those of other thiamine forms. Its peaks in water lie at 231 mμ and 335 mμ and in methanol, at 248 mμ and 334 mμ. The shorter wave length peak in methanol is represented by a shoulder in the region 245 mμ-255 mμ in aqueous media; the aqueous 231 mμ peak occurs in methanol at slightly lower wave lengths where measurement is difficult. Both spectra "tail" beyond 400 mμ, thereby giving an alkaline solution of thiamine a visible yellow color. Figure 3, Curve C shows the spectrum of thiamine in 0.1 M NaOH extrapolated to zero time. (See EXPERIMENTAL and Figure 4C, 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 known forms of thiamine absorb light above 325 mμ; hence the absorbancy at wave lengths above 325 mμ may be used to measure the concentration of the yellow form in mixtures. Commonly the absorbancy at 349 mμ was used for this purpose. In the event that other undescribed forms of thiamine occur they would be less likely

to interfere.

In water the yellow form decays within several minutes to a substance having a spectrum identical to that of the colorless thiol form (Structure III, Figure 1). 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 per cent or more of the original amount) by addition of more base.

Figure 5 shows the increase in zero time absorbancy at 349 mμ with increasing pH of an aqueous 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 has been constructed to pass through the experimentally observed mid-point at pH 11.6 and to approach the limiting absorbancies at the high and low pH ends; it has the shape theoretically expected for the dissociation of a monoprotic acid or a diprotic acid in which K_1 is much greater than 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

Figure 5. Ultraviolet absorbancy index of thiamine at 349 mμ versus pH. The data are extrapolations to the time of mixing. Curve A represents a theoretical curve constructed for a monoprotic acid; Curve B, the data fitted to a theoretical curve for a diprotic acid with no detectable singly dissociated intermediate.


MOLAR ABSORBANCY INDEX, X 10⁻³
7
6
5
4
3
2
1
0
10
11
12
pH
B
A
pK_AV = 11.6

the two protons dissociate simultaneously with no detectable amount of an intermediate singly dissociated form (K_1 is much smaller than 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 (62) and Schwarzenbach and Sulzberger (63). Also benzothiazole methiodide was reported to behave in the same way (64).

The formation of the yellow form of thiamine therefore is analogous to the previously described formation of the colorless thiol form. It appears 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 (26). 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.

The development of the yellow color in methanol shows a fundamental difference from that in water. In Figure 2, Curve A shows a plot of the absorbancy at 349 mμ against the logarithm of the excess unreacted KOH concentration. The solid line is a curve of the theroretical shape for a monoprotic acid and in this case fits the experimental points well. This suggests that in methanol a more normal situation pre-

vails in which K_1 is much greater than K_2 and that at intermediate basicities the thiamine should exist largely in the form of a singly dissociated intermediate.

The Tricyclic (Dihydrothiachromine)* Form of Thiamine

The spectra of the yellow and neutral forms of thiamine exhibit points of equal absorbancy near 263 mμ 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 per cent of the intermediate is ever present, that amount being present at pH 11.6.

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

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*A full justification for assuming a tricyclic structure for the intermediate to be described in this section will be given in DISCUSSION.

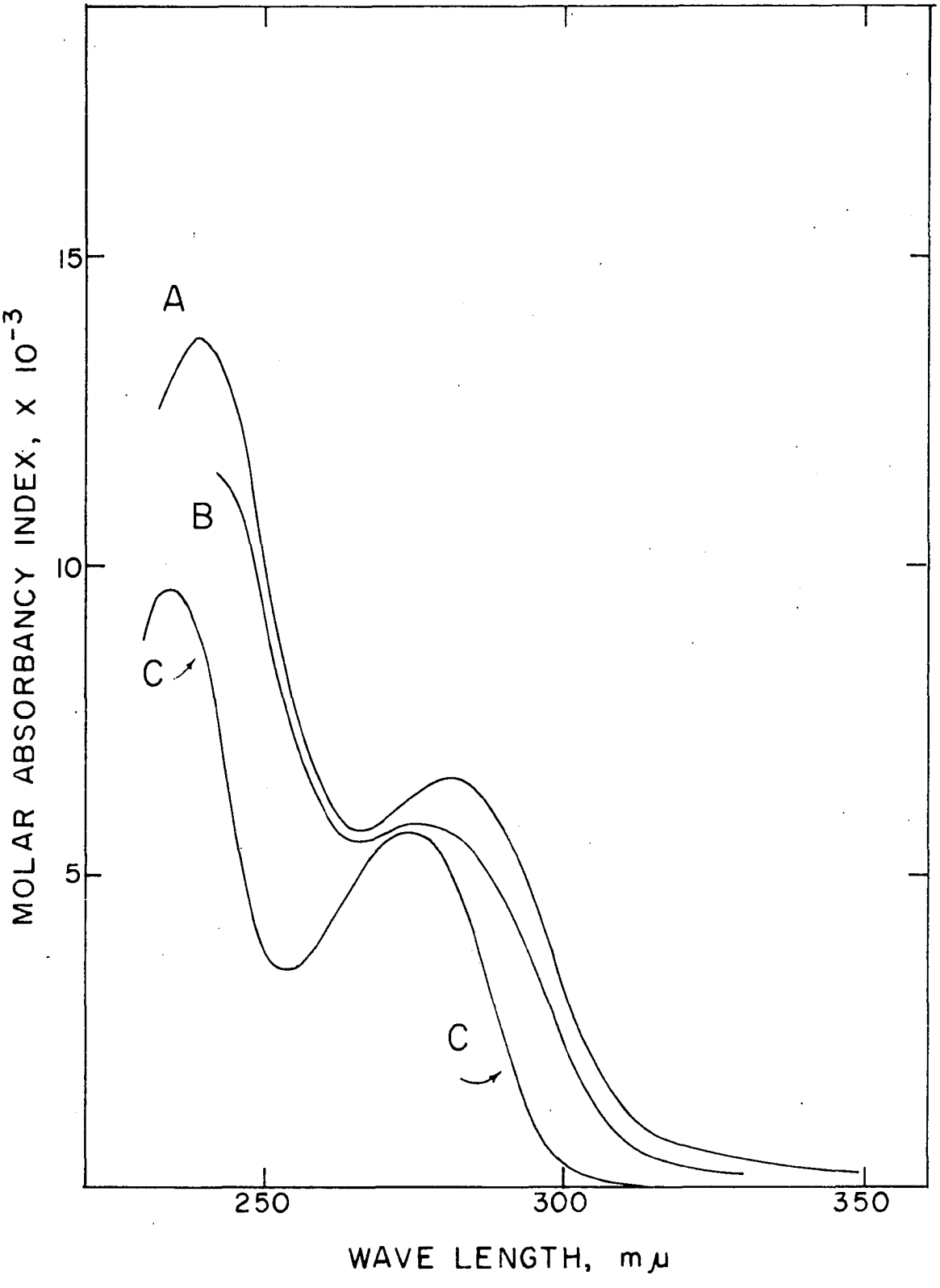
length and the difference used to compute the absorbancy index, a_M (neutral + intermediate), of the remaining mixture of neutral plus singly dissociated forms. The plot of a_M (neutral + intermediate) versus log KOH is in effect a titration curve for the production of the intermediate. Curve B, Figure 2, is a plot the apparent a_M (neutral + intermediate) at 290.3 mμ against the negative log of the unused KOH concentration. The solid line is again a theoretical curve for a monoprotic acid with a mid-point at log KOH = -3.2. From Figure 2A, the mid-point of the second dissociation step is estimated as log KOH = -2.15 and it follows that K_1 is about sixteen times K_2. These constants are sufficiently far apart that the lower points in Figure 2A 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 per cent 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) Figure 4B shows the spectrum of the intermediate form in methanol. The curve has two peaks, one at 239 mμ and the other at 279 mμ. While the spectrum "tails" slightly beyond 325 mμ, the molar absorbancy index of the intermediate is low and is nearly equal to zero at 349 mμ.

By treatment of thiamine hydrochloride suspended in ethanol with two equivalents of sodium ethoxide, the inter-

mediate form of thiamine was produced and isolated as a white crystalline solid free of both sodium and chloride ions. It dissolves in ethanol orbutanol 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 (Figure 4B) for the spectrum in methanol but is shifted about 3 mμ to shorter wave lengths. Curve B, Figure 6, is the spectrum of the isolated intermediate in butanol. The calculated spectrum of the intermediate is reproduced for convenience as Figure 6, Curve A.

The isolated intermediate is closely related to thiochrome (Structure VI, Figure 1) as evidenced by the development of an intense blue fluorescence similar to that of thiochrome in alcoholic solutions of the intermediate exposed to the air. Thiamine is readily oxidized to thiochrome by alkaline ferricyanide (65). 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 colorless thiol form. The thiol apparently is oxidized to thiamine disulfide instead of thiochrome. On the other hand, the yellow sodium salt and the intermediate in solution are

Figure 6. Spectra of tricyclic thiamine and of dihydrothiamine. Curve A represents the calculated spectrum of tricyclic thiamine in methanol; Curve B, the spectrum of the isolated tricyclic compound in butanol. Curve C represents the spectrum of dihydrothiamine in methanol.


MOLAR ABSORBANCY INDEX, X 10^{-3}
15
10
5
A
B
C
C
250
300
350
WAVE LENGTH, mμ

readily oxidized to thiochrome by alkaline ferricyanide.

Wostmann and Knight (66) have reported that thiamine conversion to thiochrome is nearly 100 per cent when methanol and isobutanol are included with the aqueous reagents. In a completely aqueous system the oxidation leads to about 65 per cent conversion. These findings lend additional support to the relationship between thiochrome and the tricyclic form since the latter is more stable in alcoholic media.

Spectrophotometric Studies on Thiamine Analogues

The following compounds which are structurally related to thiamine do not undergo spectral changes in 0.1 M or 5.4 M methanolic KOH corresponding to those changes 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). Pyrithiamine (1-(4-amino-2-methyl-5-pyrimidylmethyl)-2-methyl-3-(2-hydroxyethyl)-pyridinium bromide hydrobromide) gave no color in 0.1 M KOH but did give a very transient color with a maximum absorbancy at about 360 mμ-370 mμ in 5.4 M methanolic KOH. The extreme base concentration and experimental

difficulties make this behavior difficult to interpret.

Curve C, Figure 6, represents the spectrum in methanol of the free base for dihydrothiamine. This analogue is identical with the vitamin with the exception that the bond between atoms 2 and 3 in the thiazole ring is not unsaturated.

Assays for Carboxylase

Table 1 indicates the correlation of units of acetoin activity with units of activity measured by the Singer and Pensky (28) assay* for carbon dioxide evolution.

Table 1. Relationship between the carbon dioxide and acetoin assays of carboxylase

Nature of preparation	Units of carbon dioxide activity[a] per unit of acetoin activity[b]
Aqueous extract of acetone powder	6.40, 7.34, 7.87, ----
Isoelectric precipitate	7.35, 7.44, 9.11, ----
Alcohol precipitate	----, ----, 8.20, 6.27
Average	7.50

[a]One unit is defined by Singer and Pensky as 1 μl. of carbon dioxide per five minutes at 30° under standard assay conditions.

[b]One unit equals 0.1 μM of acetoin per one hour at 25° under standard conditions.

*A unit of activity is defined by these authors as the amount of enzyme which will catalyze the evolution of one μl of carbon dioxide under the standard conditions in five minutes.

Figure 7 shows the plot of carbon dioxide evolution versus time for the aqueous extract (Curve A) and for the alcohol precipitate in 0.05 M tris buffer pH 7.65 (Curve B). Both show a lag period and a linear portion. The abscissa intercept of the linear portion approaches zero as the enzyme is purified.

We have found that the plot of carbon dioxide evolution per five minutes incubation versus the amount of enzyme is similar to that of Singer and Pensky (28) who report that it is linear at low amounts of enzyme but falls off beyond ordinate values of 50 µl. of carbon dioxide. They also state that the plot is reproducible at all stages of enzyme purification and attribute the non-linearity to inhibition by endogenous acetaldehyde not trapped by dimedone and bovine serum albumin. These findings are in contrast to those in Figure 7 where the plot curves upward and where the amount of carbon dioxide evolved increases regularly well beyond 50 µl.

Figure 8 is a plot of the acetoin yield versus time of incubation of 1.0 ml. of the aqueous extract of an acetone powder (purification step I, 8.9 units per ml., 0.07 Purification Index, Curve A) and versus time of incubation of 0.5 ml. of the alcohol powder (purification step III, 5.3 units per ml., 2.43 Purification Index, Curve B) in 5 x 10^{-2} M tris buffer pH 7.65 brought to pH 6.0. Both demonstrate a

Figure 7. Plot of carbon dioxide evolution catalyzed by carboxylase versus incubation time at 25°. Curve A represents data from the aqueous extract (175 mg. of protein per assay); Curve B, data from a solution of alcohol powder (0.29 mg. of protein per assay; this sample is one-fifteenth as concentrated as those used for purification beyond this stage).

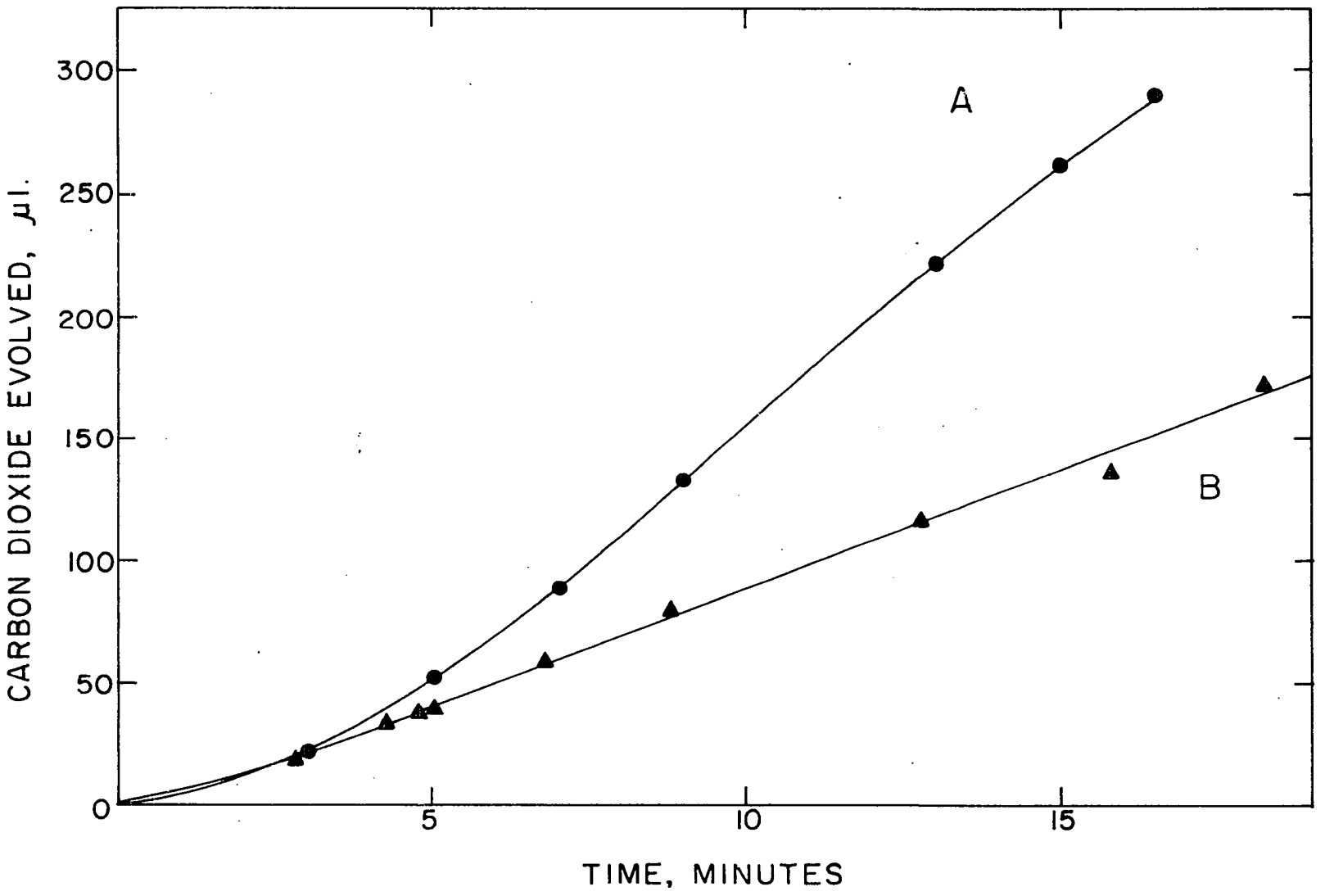
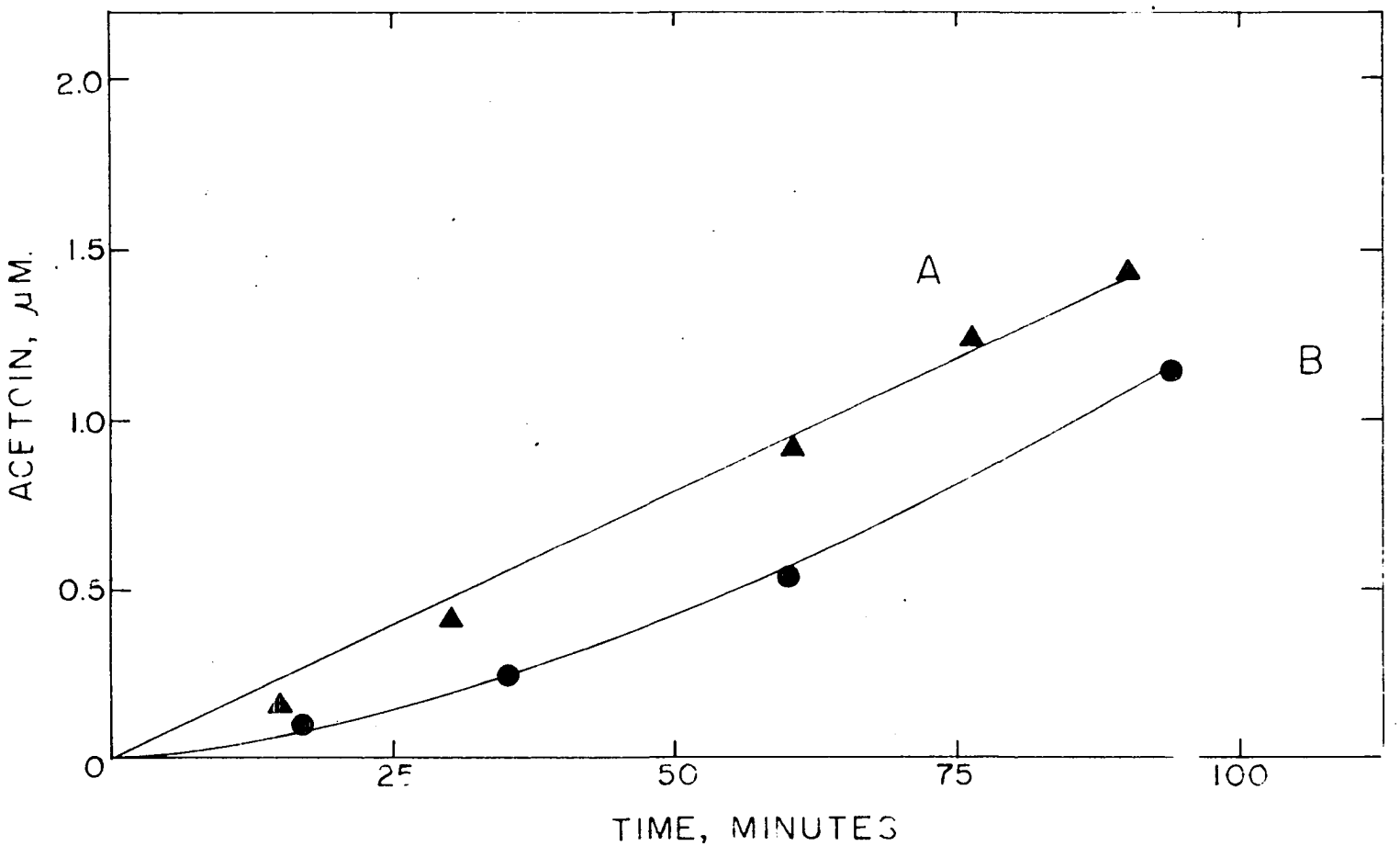

CARBON DIOXIDE EVOLVED, µl.
300
250
200
150
100
50
0
5
10
15
TIME, MINUTES
A
B

Figure 8. Plot of acetoin production catalyzed by carboxylase versus incubation time at 25°. Curve A, closed triangles, represents data from the aqueous extract (127.5 mg. of protein per assay); Curve B, closed circles, data from a solution of alcohol powder (0.66 mg. of protein per assay; this sample is one-seventh as concentrated as those used for purification beyond this stage).


2.0
1.5
1.0
0.5
0
ACETOIN, μM.
A
B
25
50
75
100
TIME, MINUTES

lag period which becomes more pronounced as the enzyme is purified.

Curve A, Figure 9, is a linear plot of the acetoin yield versus amount of enzyme as aqueous extract. Curve B, Figure 9, is a similar plot for a solution of alcohol powder. At amounts greater than 1.3 ml. of enzyme acetoin yields are greatly diminished from the amount expected from the initially linear plot. Most of these data are not represented in Figure 9B. Points are represented, however, that show an increased acetoin yield in this region with two- and three-fold increases in pyruvate concentration.

The linear or nearly linear portions of Figures 8 and 9 indicate ranges in which the acetoin assay can serve as an analytical tool in carboxylase purification.

Purification of Wheat Germ Carboxylase

The acetoin activities of wheat germ from several sources were determined on the aqueous extract of the acetone powder. Table 2 gives these values translated into units of carbon dioxide activity per gram of acetone powder. The factor relating the two activities (7.50 carbon dioxide units per 1 acetoin unit) is the average of values from Table 1.

Singer and Pensky's data based up the carbon dioxide alone clearly indicate at least four times the initial activity of the best preparation in this laboratory. In an effort

Figure 9. Plot of acetoin yield per hour versus the amount of carboxylase. The circles indicate assays with the standard pyruvate concentration; the triangle and the square indicate assays with pyruvate concentrations of two- and three-times, respectively, the usual amount. Curve A, open symbols, represents data from the aqueous extract (127.5 mg. of protein per ml. of enzyme solution); Curve B, closed symbols, data from a solution of alcohol powder (0.96 mg. protein per ml. of enzyme solution; this sample is one-fourth as concentrated as those used for purification beyond this stage).

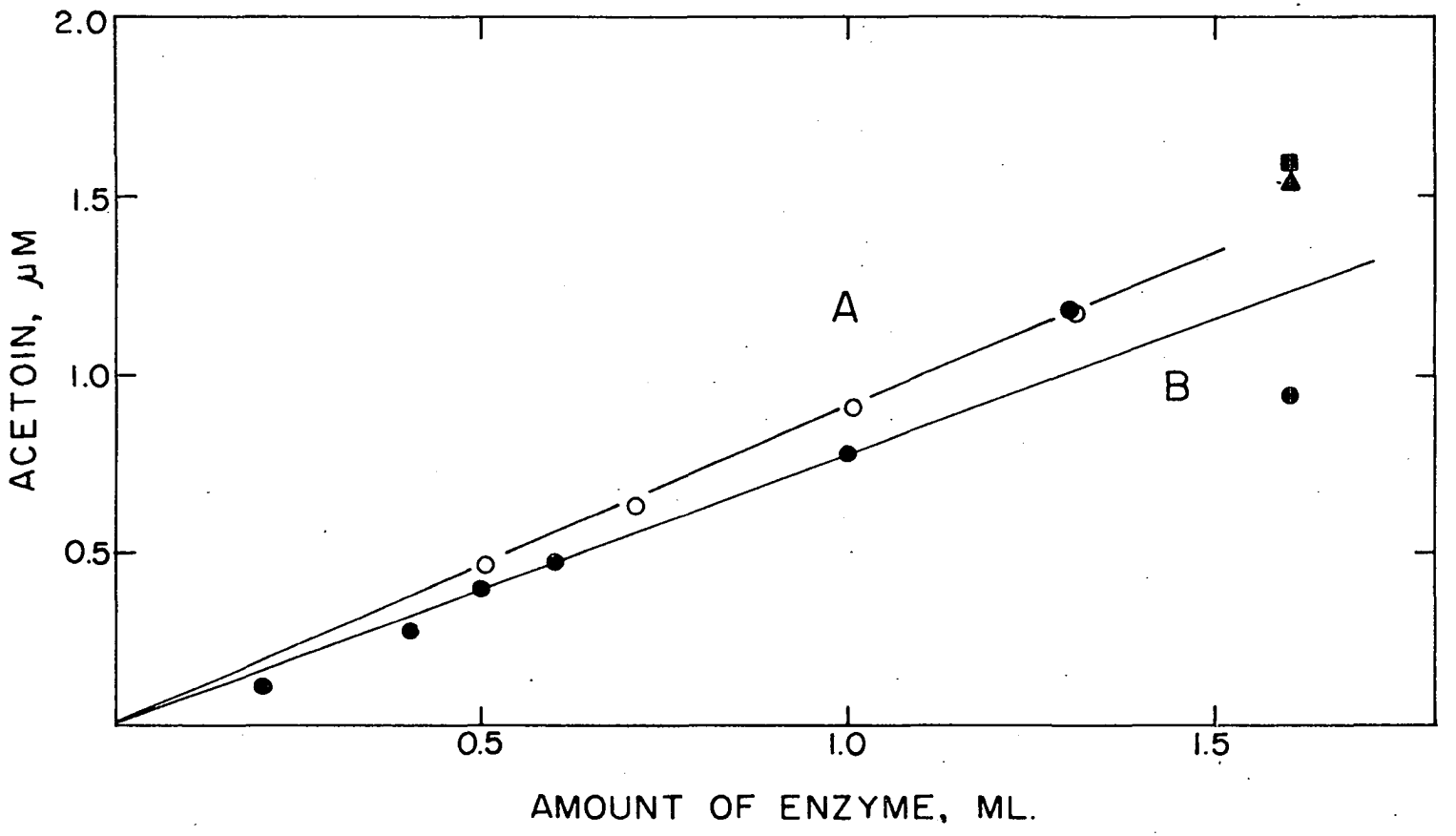

2.0
1.5
1.0
0.5
ACETOIN, μM
A
B
0.5
1.0
1.5
AMOUNT OF ENZYME, ML.

Table 2. Initial enzymatic activities from several types of wheat germ[a]

Type of wheat germ	Units of acetoin activity per gram of acetone powder	Units of carbon dioxide activity per gram of acetone powder[b]	Purity Index[c]
General Mills, Type 75[d]	25.4, 34.9	190.2, 262.7	0.35, 0.55
General Mills, Type A[d]	21.7, 26.2	163.3, 197.2	0.49, 0.29
Pavo Company, commercial package	10, 35.2	75.5, 265.7	0.21, ----
General Mills, Type S-50, Singer and Pensky's data	----	750-780[e]	ca. 2.2[e]

[a]Data from the aqueous extract of the acetone powder.

[b]Except for Singer and Pensky's data, these data were calculated using the conversion factor relating the acetoin and carbon dioxide assays in Table 1.

[c]Based upon the carbon dioxide assay.

[d]Described by the company as "essentially the same" as Type S-50.

[e]Data taken from Singer and Pensky (28).

to determine the influence of acetone treatment on subsequent activity two small samples of wheat germ which could be handled quickly were blended with cold acetone, one for three minutes and the other for thirty minutes. Both were filtered for several minutes and were desiccated as a thin layer of

powder over sulfuric acid *in vacuo* in the cold. The latter sample contained approximately 6/7 of the activity of the former.

In addition, an aqueous homogenate of Type A wheat germ and an aqueous extract of ball-milled Pavo wheat germ both had acetoin activities lower than comparable amounts of acetone powders. The aqueous homogenate (ten minutes homogenization) had approximately 50 per cent of the activity to be expected from the acetone powder; longer homogenization (45 minutes) increased the yield only a small amount.

The aqueous extracts of acetone powders produced in this laboratory had volumes 65-75 per cent of the original volume of water, whereas Singer and Pensky report aqueous extracts with volumes 40 per cent of the original. Efforts to increase the activity of the aqueous extract by using more acetone powder per volume of water (1:2, w:v, acetone powder:water instead of a 1:5 ratio) led to thick, difficult to prepare extracts which lost a great amount of activity upon further purification (*ca.* 20 per cent retention after the subsequent step).

The unaltered purification scheme of Singer and Pensky (28) led to great losses of total activity and to Purity Indices low in respect to the published work. In some instances, notably with the ammonium sulfate precipitation step, a subsequent step actually had a lower degree of

purification. Results similar to ours both for the amount of initial activity and for the total activity losses upon purification were found in another laboratory.* Data drawn from Eich and Cerecedo (41), who used wheat germ carboxylase but who did not explicitly discuss its purification, indicate final activity much lower than reported by Singer and Pensky. Eich and Cerecedo do not include data which will permit Purity Index calculations. Von Holt, et al. (16) report a preparation to the alcohol powder stage whose degree of purification is about one-half that reported by Singer and Pensky. We have been unable to find any other reports which may be used for purposes of comparison concerning wheat germ carboxylase.

The purification of carboxylase by modifications of the procedure (see EXPERIMENTAL) led to lesser losses of total activity and to better degrees of purification; however, we have been unable to equal either the degree of purification or the amount of retained total activity reported by Singer and Pensky. Table 3 gives the results of our purification step-by-step. Values are drawn for comparison from the work of Singer and Pensky (28) for purification with their original procedure.

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*G. M. Brown, Division of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts. Data concerning purification of wheat germ carboxylase. Private communication. 1960.

Table 3. The purification of carboxylase from wheat germ[a]

Purification step	Total acetoin units	Per cent yield	Purity Index	Relative purification
Aqueous extract	29,000-39,000[b] --[c]	100 100	0.060 --	1 1
Isoelectric precipitate	20,000-24,000 --	62-69 ca. 75	3.0-3.1 --	47-48 38-50
Alcohol precipitate	14,000-15,000 --	38-49 ca. 68	33.0-34.0 --	520-530 630-760
Ammonium sulfate fractionation and dialysis	5,200-6,800 --	17-18 ca. 63	94.0-100.0 --	1,500-1,600 2,000-2,800

[a]Data reported on the basis of 1 kilogram of acetone powder and rounded off to two significant figures.

[b]For each purification step the upper row of data represents General Mills Type A wheat germ.

[c]For each purification step the lower row of data is taken from Singer and Pensky (28).

The commercially packaged wheat germ from the Pavo Company was generally lower and more erratic in respect to initial activities. At intermediate stages of purification poor results were achieved compared with those from the General Mills product; however, during the final stage of purification, the Pavo Company material did not lose much activity and the final results were nearly the same as those from the General Mills material. The Pavo Company wheat germ ultimately led to carboxylase of a somewhat higher degree of

purification.

The addition of carboxylase co-factors in amounts large enough to saturate the enzyme before the isoelectric and alcohol precipitations had little effect on the yield; instead it was found that the pH adjustments in the isoelectric precipitation were the most sensitive factor. Faulty pH adjustment at this point was one of the major sources of activity loss in the entire purification scheme.

The greatest losses of activity accompanied the ammonium sulfate precipitation. By increasing the amount of added ammonium sulfate from the literature-recommended 33 per cent to 50 per cent, better yields were found but further increases had little effect.

Dialysis of the mother liquors against water after ammonium sulfate precipitation (33 per cent saturation) gave a preparation with extremely low acetoin activity. The assay of the precipitate preparation plus the assay of the mother liquor preparation accounted for only 36 per cent of the activity of the previous purification step. Assay of the combined preparation led to a small increase in the amount of retained activity.

Singer and Pensky reported good results at this step but added that occasionally low results were observed. These low results were not as low as reported here.

Chromatography of Carboxylase on DEAE-Cellulose

DEAE-cellulose (Cellex-D, California Corporation for Biochemical Research; 4.9 g.) was washed 1/2 hour in 1 M NaOH and filtered on a Buchner funnel. The material was washed twenty-five times with distilled water. The pH of a slurry of the material in water was adjusted with HCl to the pH of the first chromatographic buffer (either 0.05 M, pH 7.7 tris, or 0.1 M, pH 6.8 imidazole). The slurry was filtered and the DEAE-cellulose was washed seven times with the first buffer.

The washed material was put into a glass column (0.5 cm. diameter) and was allowed to settle under gravity. The initial buffer then was passed through tygon tubing from a reservoir to further pack the column under the influence of a hydrostatic head of approximately 32 inches. The column height was 34 cm.

Alcohol powder (16 mg.-69 mg.; ca 4 mg. per ml. of solvent) in 0.05 M, pH 7.7 tris buffer or in tris buffer brought to pH 6.8 was put on the column after centrifugation for one hour at 4,000 r.p.m. The first buffer, at the pH of the enzyme sample, was then permitted to pass through the column from the reservoir. The effluent waters were caught in test tubes mounted on a fraction collector which in turn was rotated by a timer set at intervals of from fifteen to sixty minutes as desired. The optical densities of tube samples were determined at 280 mμ. The flow rate through the column

was 10-16 ml. per hour.

No gradient elutions were used; fresh solvent merely was substituted for old solvent in the reservoir. Definite peaks were observed during the run of the first buffer but no enzymatic activity was found. No activity was found even after large volumes of initial solvent were passed giving eluates with low optical densities at 280 mμ.

The substitution of 0.1 M succinate buffer, pH 6.0 or pH 6.2, for the initial buffer led to the prompt appearance of two or three new peaks, the last of which contained enzymatic activity which accounted for an almost negligible amount of initial activity. Further elution with pH 6.0 succinate buffers, either 0.1 M or 3 M in respect to added sodium chloride led to no further activity.

Trypsin Treatment of Carboxylase

The effect of trypsin treatment upon carboxylase activity was studied with a very low-grade alcohol powder (ca. 80-fold pure). Two hundred and fifty ml. of 0.05 M, pH 7.65 tris buffer was stirred with 87.9 mg. of alcohol powder and then was centrifuged one hour at 4,000 r.p.m.; in the cold, the resultant solution was treated with enough trypsin in solution (14 mg. per 10 ml. of 10^{-3} M hydrochloric acid) to give a final trypsin to alcohol powder weight ratio of one to one hundred. The treatment was continued for twenty minutes,

an aliquot was withdrawn for an additional 140-minute incubation, and at the end of each incubation enough trypsin inhibitor (29.7 mg. per 20 ml. of 10^{-5} M sodium hydroxide) was added to be twice stoichiometric with trypsin. Then the pH was brought to 6.0 with 1 M acetic acid.

With this alcohol powder, despite the possibility of digestion of a large amount of impurities, trypsin treatment led to a loss of acetoin activity. After twenty minutes the activity diminished by 33 per cent; after 160 minutes, by 82 per cent.

These data indicated that it was not feasible to use trypsin as a purification tool or as a means of usefully diminishing the size of the carboxylase molecule.

The Treatment of Carboxylase with N-Bromosuccinimide

Witkop et al. (67-69) have reported the selective cleavage of tryptophanyl peptides with N-bromosuccinimide within several hours at pH's near 4. The reagent cleaves the tryptophan carboxyl peptide bond and leads to an increase in absorbancy at 310 mμ and to a decrease at 280 mμ. Later it was reported that tyrosinyl peptides may also be cleaved under similar conditions (70).

Fifty ml. of 0.05 M, pH 7.65 tris buffer was stirred with 20.8 mg. of alcohol powder and was centrifuged 1/2 hour at 4,000 r.p.m. The sample was treated with 2 mg. of

N-bromosuccinimide for 24 hours at 3°. After the treatment there were appropriate changes of absorbancy at the two wave lengths. The acetoin activity was completely abolished. After thorough dialysis an N-bromosuccinimide-treated sample also had no activity.

Spectrophotometric Studies on Carboxylase

The spectrum of the isoelectric precipitate in 0.1 M, pH 6.0 succinate has a smooth peak at 331 mμ which is near the maxima for the spectra of α-lipoic acid, DPNH and TPNH, reduced cytochrome C, etc. It is also near the peak of the yellow form of thiamine.

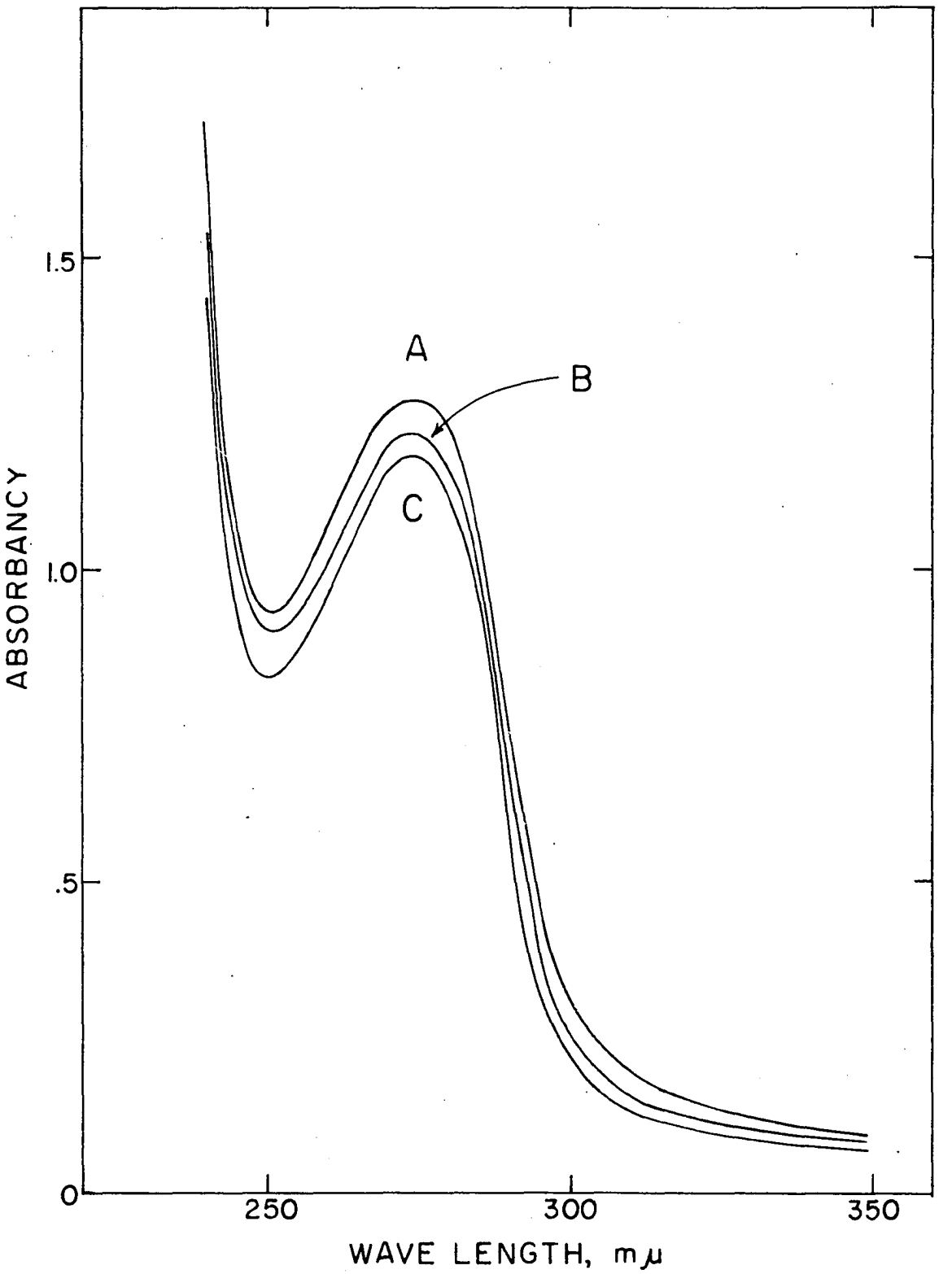
After 29 hours dialysis in the cold against succinate solution, the absorbancy at 330 mμ of the solution within the sac plus the absorbancy of the external solution equaled 82.6 per cent of the initial absorbancy at this wave length. After two weeks' dialysis against 0.1 M HCl in the cold the absorbancy at 330 mμ of the external solution of another sample nearly equaled the initial absorbancy times the dialysis dilution factor. If the yellow form of thiamine or the pyridine nucleotides accounted mainly for the absorbancy at 330 mμ, the acid treatment would have destroyed the absorbancy at this wave length. α-Lipoic acid may be implicated but the large absorbancy would indicate an unusually large amount of this vitamin. As the enzyme is purified the

absorbancy beyond 300 mμ diminishes greatly and there is no definite peak. A "tail", however, remains and this has a greater absorbancy than one would expect from ordinary protein absorbancy.

Figure 10 represents the spectra of apoenzyme (purified past the ammonium sulfate precipitation step) in the presence of cocarboxylase alone and also in the presence of cocarboxylase with magnesium ion; the figure also shows the arithmetic sum of the absorbancies of apoenzyme and cocarboxylase. All were measured at pH 6.8 after overnight standing in the cold. Magnesium sulfate does not alter the spectrum of the apoenzyme and so Curve A, which is the arithmetic sum may be properly compared with Curves B and C. Curve B is the spectrum of a mixture with the three components; Curve C, the spectrum of apoenzyme with carboxylase alone. Curves A, B, and C resemble each other in shape and the absorbancy of Curve A is greater at all wave lengths than that of Curve B which in turn is greater than that of Curve C.

In deriving difference spectra from Figure 10, the data were corrected for the absorbancies of the redissolved precipitates which had developed after the initial mixing of the samples. Directly after mixing, the samples demonstrate an apparent increasing absorbancy which seems to accompany a visible turbidity. These observations are most obvious in the solutions containing apoenzyme and both co-factors

Figure 10. Spectra of apoenzyme plus its co-factors. Curve A represents the sum of the separate absorbancies of apoenzyme and cocarboxylase; Curve B, the absorbancy of apoenzyme in the presence of both co-factors; Curve C, the absorbancy of apoenzyme in the presence of cocarboxylase.


1.5
1.0
.5
0
ABSORBANCY
A
B
C
250
300
350
WAVE LENGTH, mμ

especially if the cocarboxylase concentrations were greater than those described in EXPERIMENTAL. The turbidity appears to be more pronounced at pH 6.4 than at pH 6.8. After standing in the cold at least overnight, the solutions become clear with constant absorbancies and contain slight precipitates. Table 4 gives the absorbancies at several wave lengths of

Table 4. Absorbancies at several wave lengths of redissolved precipitates from samples used for Figure 10

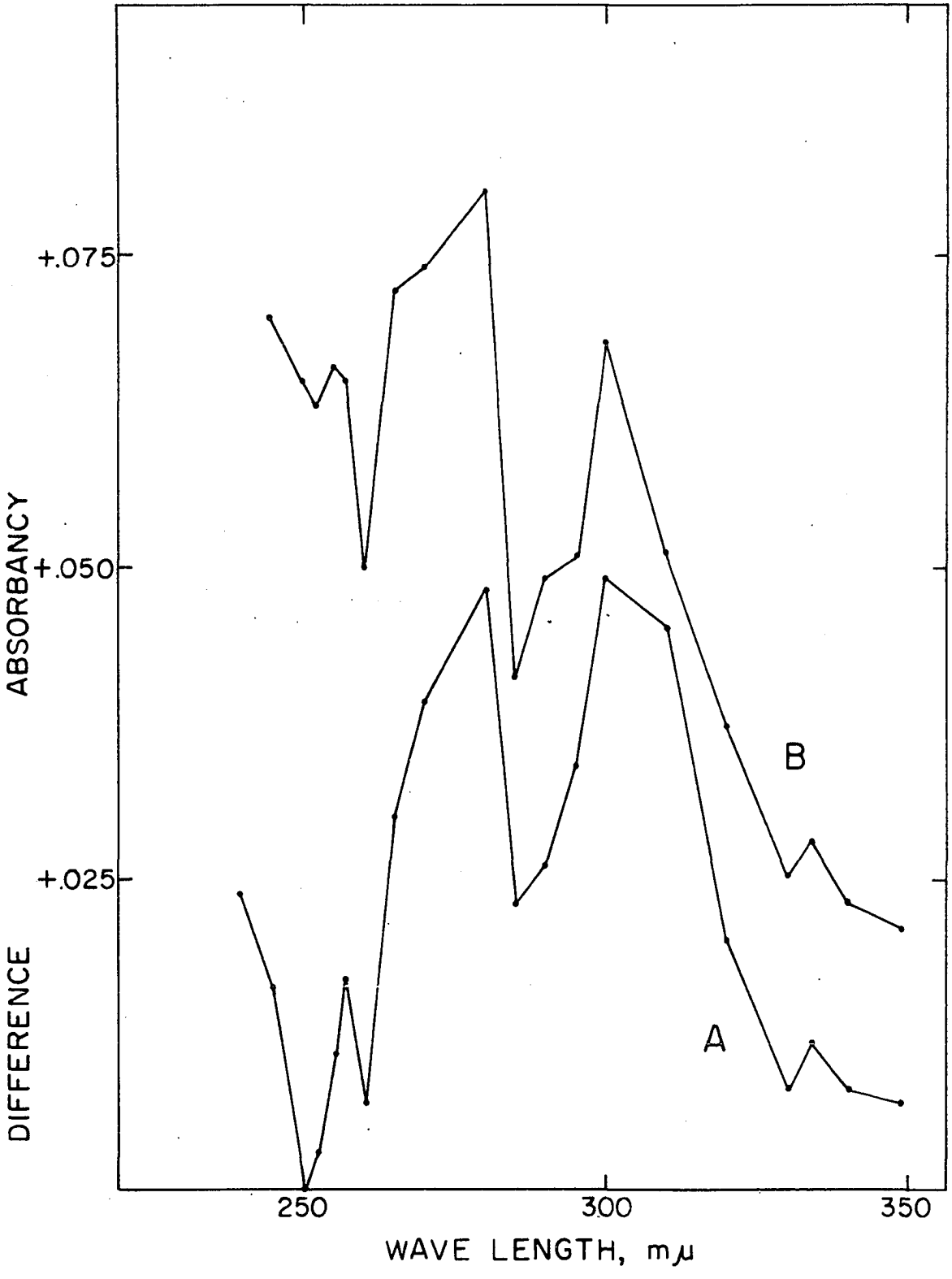
Wave length, mμ	Absorbancy, precipitate from apoenzyme sample	Absorbancy, precipitate from apoenzyme-cocarboxylase sample	Absorbancy, precipitate from apoenzyme-magnesium ion-cocarboxylase sample
240	0.040	0.096	0.087
250	.030	.068	.060
255	.034	.065	.059
260	.025	.069	.062
270	.038	.053	.056
280	.040	.051	.054
285	.035	.059	.055
290	.033	.060	.048
295	.019	.031	.034
300	.022	.024	.023
310	.013	.025	.018
320	.006	.017	.016
349	.005	.011	.011

these redissolved precipitates. The spectra of precipitate solutions from a sample of apoenzyme alone had small peaks at 280 mμ. The precipitate solutions from samples containing apoenzyme and cocarboxylase gave spectra with somewhat higher absorbancies and with small peaks near 260 mμ-270 mμ. The former spectra are typical of protein spectra; the latter appear to contain absorbancy due both to protein and cocarboxylase returned to solution after occlusion in the precipitates. The corrections were applied by adding them to the corresponding crude data.

Figure 11 represents the difference spectra at pH 6.8 of the spectra in Figure 10. The difference spectra are the sum of the separate absorbancies of apoenzyme and cocarboxylase minus the absorbancies of systems containing both apoenzyme and cocarboxylase with or without magnesium ion.

The differences are positive at all wave lengths and no marked peaks appear at wave lengths longer than 310 mμ. Curve A, Figure 11, represents data derived from a system including magnesium ion; Curve B, Figure 11, without magnesium ion. The difference spectra at pH 6.4 are approximately the same; however, the difference peak in the region 300 mμ-310 mμ is 40 per cent as great and the peak at 270 mμ-280 mμ is 50 per cent as great as the similar peaks in Curve A, Figure 11. The pH 6.4 experiments had 40 per cent as much protein content as did the pH 6.8 experiments.

Figure 11. Difference spectra of the sum of the absorbancies of apoenzyme and its co-factors all minus the absorbancies of apoenzyme in the presence of its co-factors. Curve A represents the summed absorbancies of apoenzyme, cocarboxylase and magnesium ion minus the absorbancy of a mixture of the components; Curve B represents data from a similar experiment from which magnesium ion was omitted. The solutions were buffered at pH 6.8. The apoenzyme preparation was at the final stage of purification (ca. 1800-fold pure; Purity Index approximately equal to 45).


+.075
+.050
+.025
ABSORBANCY
DIFFERENCE
B
A
250
300
350
WAVE LENGTH, mμ

Ultracentrifuge Studies

An enzyme preparation at the final stage of purification in 0.1 M, pH 6.8 imidazole buffer (five times more concentrated than the samples described in the purification scheme) was centrifuged ca. 8° at 59,780 r.p.m. in the Spinco Model E Ultracentrifuge. Photographs of the Schlieren patterns were taken at four or eight minute intervals.

Three peaks were observed: the fastest was barely visible. The intermediate peak was a minor component and the slowest peak represented most of the material. For the last a Svedberg constant of 7.77 was calculated by determining $\frac{d \log \text{distance}}{dt}$ from a plot of the log of the distance traveled versus time and by means of the equation, $S = \frac{.0383763}{\omega^2} \cdot \frac{d \log \text{distance}}{dt}$. The minor peaks did not travel a great deal faster. The value of 7.77 was not corrected for temperature or solvent viscosity. At this stage of purification Singer and Pensky (28) report three peaks from their ultracentrifugal studies, the major of which has enzymatic activity associated with it and has an S_{20} equal to 29. Their major peak comprised 93 per cent of the material.

After our centrifugation, when the two fast peaks had clearly sedimented, an aliquot was withdrawn from the centrifuge cell, subject to sampling errors, and the enzymatic activity and the optical density at 280 mμ were redetermined. These data indicate a purification of the enzymatic activity

and a probable association of the enzymatic activity with the slow-moving major peak. After centrifugation the enzymatic activity was 80 per cent and the optical density at 280 mμ was 60 per cent of the original values.

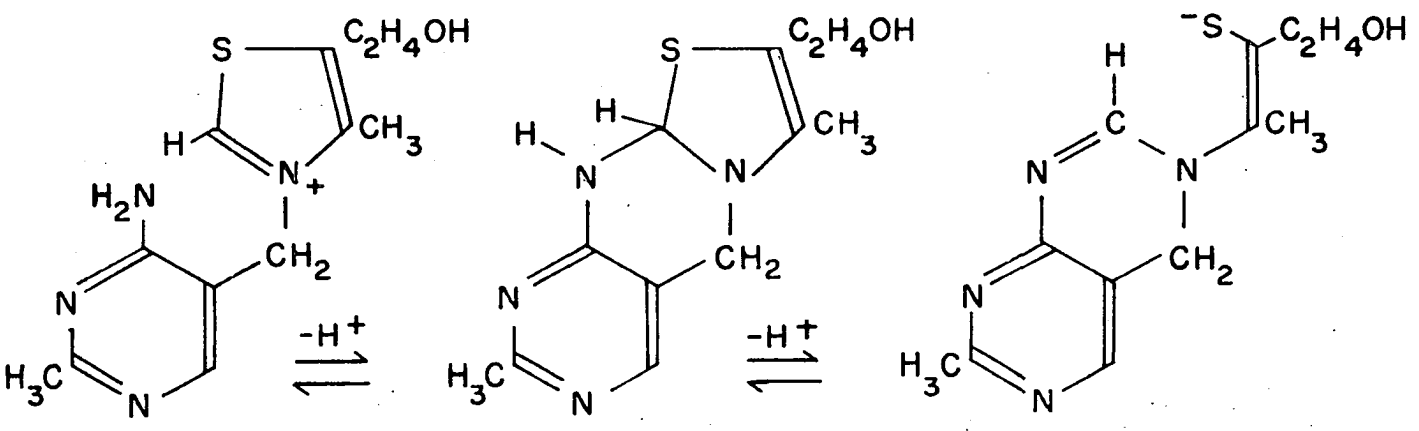
DISCUSSION

Structures of Thiamine in Alkaline Solution

The formation of the yellow form of thiamine requires the pyrimidine amino group, the methylene group linking the two rings, and the unsaturated carbon atom alpha to the quaternary thiazole nitrogen. In addition the sulfur atom appears to be necessary. Analogous compounds lacking one or more of these structural features do not become yellow with the addition of base. Pyrithiamine in which a pyridine group substitutes for the thiazole ring of thiamine does give a yellow color which fades rapidly even in extremely alkaline methanolic solution. Although the sulfur atom is absent, pyrithiamine nevertheless possesses a satisfactory structural grouping in that the pyridine ring is unsaturated and joins the pyrimidine ring via a quaternary nitrogen and a methylene bridge.

It seems likely that the yellow form of thiamine is a bicyclic compound with an opened thiazole ring (Structure III, Figure 12). The formation of this compound requires a nucleophilic attack by the pyrimidine amino group upon the unsaturated carbon 2 of the thiazole ring accompanied by the loss of two protons. The curve in Figure 5 is in effect a titration curve. It shows the absorbancy attributable to the yellow form at 349 mμ as a function of pH. The data fit a

Figure 12. Three forms of thiamine. Structure I represents neutral thiamine; Structures II and II, respectively, represent the tricyclic intermediate and the yellow forms.


S
C_2H_4OH
H
CH_3
N^+
H_2N
CH_2
N
H_3C
N
$-H^+$
H
H
N
N
CH_3
CH_2
$-H^+$
^-S
C_2H_4OH
H
C
CH_3
N
N
CH_2

I II III

theoretical curve whose shape indicates the neutralization of two moles of hydrogen ions per mole of thiamine with simultaneous removal of both hydrogens. The titrimetric data indicates that no intermediate formed by the neutralization of one hydrogen should exist in aqueous media. The existence of such an intermediate is also precluded by the absence of spectrophotometric changes at the isosbestic points of the yellow and neutral forms.

The thiazole carbon 2 appears to be an extremely sensitive position (see LITERATURE SURVEY).

The formation of the colorless open thiol form via a pseudobase (Structures I-III, Figure 1) from the free base indicates that this atom is sensitive to nucleophilic attack by hydroxyl ion and that these reactions are titrimetrically similar to the formation of the yellow structure.

Structure III, Figure 12, is supported by the elemental analyses of Zima and Williams (26) of the yellow crystalline material isolated from alkaline solution. These authors initially proposed the structure.

The spectrum of the yellow form is greatly shifted from the spectra of other forms (see Figure 3). An increase in the length of the conjugated chromophoric pathway usually is accompanied by a bathochromic shift. The proposed structure includes a conjugated system somewhat longer than that of thiamine itself.

The pyrimidine amino group can be extremely close to the thiazole ring by virtue of the methylene bridge. Thus the kinetically favored formation of the yellow form may be explained. The pK_{av} for the formation of it is considerably greater than the pK_{av} for the transformation of the free base to the open-ring thiol. Yet the yellow form occurs immediately upon the addition of base. In water the yellow form fades rapidly and is replaced by the open-ring thiol form.

Metzler (55) has related the fading to the appearance of the colorless form. The fading is first-order with respect to the yellow form at a given pH. First-order rate constants can be found by observing the change of absorbancy at 349 mμ. Similarly, the formation of the open-ring thiol form is first-order with respect to the free base at all pH's. Rate constants can be derived from the increase in absorbancy at 255 mμ. Since the yellow form does not exist appreciably below pH 10.6, the formation of the open-ring thiol can be measured at pH's in the region 10.0 to 10.8 without confusion. The logarithms of the first-order rate constants for either reaction neatly fit the same hyperbola-like curve which is described by the equation: $\log k_{pH} = \log k K_W - pH - \log (K_{av}^2 + H^{+2})$. Here k_{pH} is the first-order rate constant for either reaction, k, the second-order rate constant for the reaction of the free base with hydroxyl ion, K_W,

the ion-product of water, K_{av}, the average of the two equilibrium constants leading to the yellow form. Log k K_W is equal to -13.1 moles liter^{-1} second^{-1} and pK_{av}, 11.6 at 19.2°. The linear extensions of both branches of the plot meet at pH 11.6 which is near the pK_{av} for the formation of the yellow form.

The plot of the absorbancy of methanolic thiamine solutions at 349 mμ versus base concentration (Curve A, Figure 2) is dissimilar to the plot in Figure 5. The points in Curve A fit a theoretical curve which is typical of a monoprotic dissociation, indicating that in methanol the aqueous two-proton reaction is separable and that an intermediate between the free base and the yellow form can be found. Indeed as a function of intermediate base concentrations first increases in the absorbancy at 290 mμ and then subsequent decreases are found; at 263 mμ increasing the base concentration causes first a loss then a subsequent rise in absorbancy. Both wave lengths are isosbestic points for the neutral and yellow forms. The conversion of one form to another without an intermediate should lead to no change in absorbancy. Curve B, Figure 2, shows a plot of the increase of absorbancy at 290 mμ after subtraction of any absorbancy due to the yellow form versus the amount of base. These points also fit a theoretical monoprotic dissociation curve. Apparently methanol stabilizes the intermediate and it is possible to

detect the separate proton dissociations. This suggests that Structure II, Figure 12, represents the intermediate. It is a tricyclic structure created by nucleophilic attack on thiazole carbon 2 accompanied by monoprotic dissociation. It clearly is a logical intermediate between Structures I and III. Furthermore elemental analyses and the neutralization equivalent of the material prepared from one mole of thiamine hydrochloride and two moles of sodium ethoxide agree with theoretical values for Structure II. The isolated material has virtually the same spectrum in butanol as the spectrum of methanolic thiamine solutions at low alkalinity from which the absorbancy of other forms had been subtracted. The isolated substance disproportionates completely in water and partly in methanol to the yellow and free base forms. This agrees with the observation that in water virtually no intermediate ever exists and that in methanol no more than 58 per cent of the intermediate exists under optimal conditions.

Additional support for the tricyclic Structure II, Figure 12, comes from a comparison with spectra of other aminopyrimidines. The formation of Structure II amounts to an alkylation of the pyrimidine amino group with a disruption of a thiazole double bond. The absorption band of the tricyclic form near 280 mμ is shifted about 10 mμ to a longer wave length than that of the corresponding pyrimidine absorption band of dihydrothiamine, a thiamine analogue in which the

thiazole double bond on the nitrogen is reduced, and in which the amino group is not alkylated. Brown and Short (71) have shown that methylation of 4-aminopyrimidines leads to similar shifts of 8 mμ-9 mμ. However the intermediate absorbs more strongly around 240 mμ than does dihydrothiamine (see Figure 6). It is quite possible that in the intermediate the carbon-sulfur bond which breaks to form Structure III, Figure 12, is already weakened and that this polarization of the molecule results in a higher absorption around 240 mμ, similar to that observed by comparison of the spectrum of the colorless thiol with the spectrum of the neutral form.

The previously cited (see RESULTS) evidence for the role of the tricyclic form as a precursor in the oxidation of thiamine to thiochrome lends still further support to the proposed tricyclic structure.

Wheat Germ Carboxylase

The spectrum of wheat germ carboxylase in the presence of both co-factors is not greatly different from the sum of the separate absorbancies. Singer and Pensky reported a somewhat greater absorbancy at pH 7.0 at all wave lengths for the complete system over the absorbancy of the sum of the separate components. Our determinations indicate that after standing overnight in the cold the samples have slightly altered spectra. The system containing apoenzyme, magnesium

ion, and cocarboxylase absorbs less strongly at all wave lengths in comparison with the sum of the absorbancies of its members. The decrease in absorbancy is even more pronounced with the system containing only apoenzyme and cocarboxylase. The difference spectra (Figure 11) therefore are positive at all wave lengths when the absorbancy of a mixture is subtracted from the sums of separate absorbancies.

Direct measurement of the spectrum of bound cocarboxylase is rather difficult. Clearly the coenzyme is not bound with strongly enhanced or shifted absorption peaks. At first glance it would seem desirable to enhance the difference spectra in Figure 11; however, under the experimental conditions from which Figure 11 is derived, it would be possible to increase the protein concentration only by about 50 per cent. A further increase would lead to absorbancies beyond the range of reliable measurement. In addition, any increase would most likely lead to a greater tendency for protein precipitation and thereby further disturb the relationship between the spectra of the apoenzyme in the presence of its co-factors and those of the appropriate controls.

It would appear to be useful to diminish the size of the carboxylase molecule removing peptide chains containing large amounts of ultraviolet-absorbing amino acids while maintaining enzymatic activity. Thus the concentration of enzyme in abbreviated form could be increased without incurring diffi-

culties with spectrophotometric measurement. Unfortunately, the experiments with N-bromosuccinimide and also with trypsin indicate a destruction of enzymatic activity.* Pepsin was not used because its activity lies in a pH range where carboxylase is insoluble.

If cocarboxylase were bound in a configuration analogous to one of the known forms of thiamine, it must at best have only a slightly perturbed spectrum on the enzyme. The yellow form of thiamine has absorbancy at wave lengths where other forms do not absorb. If one assumes a molecular weight of 10^5** for carboxylase and also a mole-for-mole binding of the coenzyme, the optical density due to 10^{-5} molar presumably bound yellow form would be approximately 0.075 at 334 mμ. The imidazole solution at the final purification step contains ca. 10^{-5} M carboxylase using 10^5 as the molecular weight and assuming the absence of a large amount of impurities. Furthermore, due to our manner of deriving the difference spectra, the binding of the yellow form would lead to a negative absorbancy of 0.075 at 334 mμ. Based upon these assumptions it seems unlikely from Figure 11 that the yellow form is the

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*A preliminary experiment with carboxypeptidase indicated no loss of carboxylase activity. However, little ultraviolet absorbancy was removed by this enzyme.

**Singer and Pensky (28) report a rough estimate of 10^6 from ultra-centrifuge studies; Dr. Malcolm Rougvie similarly estimates a possible value of $1.5 \pm 0.5 \times 10^5$ by comparing the Svedberg constant of our slowest peak with constants of similar magnitude of proteins of known molecular weight.

bound form of cocarboxylase.

It seems likely, at least as far as these investigations go, that the neutral form of cocarboxylase is the bound form. The tricyclic and yellow forms all have isosbestic points with neutral thiamine. It follows therefore that if either the yellow or tricyclic form were bound to the enzyme their binding would diminish the concentration of supernatant cocarboxylase and a difference spectrum would have to be negative at some wave lengths.

If the yellow form were bound the difference spectra would have to be negative above 290 mμ, positive between 263 mμ and 290 mμ and negative below 263 mμ. If the tricyclic form were bound the difference spectra would be negative above 280 mμ, positive between 260 mμ and 280 mμ and negative below 263 mμ. The difference spectra indeed are positive and have maxima in the region 260 mμ-285 mμ but they merely go through minima at 285 mμ-295 mμ and rise again when they should change sign if a form other than the neutral were bound.

An interesting feature of Figure 11 is the fairly marked positive difference peaks at 270 mμ-280 mμ and at 300 mμ-310 mμ.

The 270 mμ-280 mμ peak perhaps may be explained on the basis of protein precipitates which did not return to the solutions which were used to measure correction factors. However, if this were so, the difference peak at 280 mμ in

Curve A, Figure 11, would represent an amount of insoluble protein more than the insoluble protein from the apoenzyme control. The amount would be 6 per cent of the original protein content. (The correction factors which were applied to the data at this wave length indicate that in the sample containing apoenzyme and co-factors 2 per cent of the original protein content precipitated in excess of the precipitate from the apoenzyme control.) The correction factor which was applied to the apoenzyme control (ca. 5 per cent of the total protein content) accounted for the difference in absorbancy at 280 mμ between the apoenzyme control and the undiluted stock enzyme solution.

It would appear that an unreasonably high protein precipitation would necessarily have to be invoked to explain the 270 mμ-280 mμ peak. Disparities in protein precipitation would not explain the 300 mμ-310 mμ peak.

Another possible explanation of both difference peaks is that there are hydrogen-bond differences between the samples and their controls (see Sela and Anfinsen (72) or Glazer et al. (73) for the magnitude and situation of absorbancy changes due to hydrogen-bond breaking in illustrative proteins like bovine serum albumin, ovalbumin, and ribonuclease).

The reported difference spectra derived by subtracting the absorbancy of a protein without hydrogen bonded tyrosines from the absorbancy of the protein with such bonds are posi-

tive and have maxima at 280 mμ and 288 mμ, a minimum at 283 mμ, and little difference absorbancy past 295 mμ. These difference peaks do not fit our data.

The difference peak at 300 mμ-310 mμ is difficult to explain. If cocarboxylase were bound as the neutral form and if the spectrum of the bound neutral form were lower in absorbancy, a change in this region might be expected (i.e., the absorbancy toward the red might "tail off" more abruptly). However, the difference absorbancy at 300 mμ would then represent a 56 per cent loss in absorbancy of all the cocarboxylase, bound and in the supernatant. This seems rather high. Based upon a molecular weight for carboxylase of 10^5 and a mole-for-mole binding of the co-factor, however, the total cocarboxylase loss of absorbancy at 300 mμ would be roughly 20 per cent if the bound cocarboxylase had no absorbancy at 300 mμ. It is significant that only the presence of cocarboxylase produces difference spectra; magnesium ion alone does not distort the absorbancy of the apoenzyme.

The difference absorbancy for the complete system with both co-factors and apoenzyme at 300 mμ at pH 6.4 is 40 per cent as great as that at pH 6.8; the 280 mμ absorbancy, 50 per cent. The amount of total protein used for the pH 6.4 experiments was 40 per cent of the amount used for the pH 6.8 experiments. Thus there appears to be a relationship between the amount of carboxylase and the difference spectra.

It seems likely that the binding of cocarboxylase takes place as the neutral form and leads to decreases in absorbancy of the bound form.

SUMMARY

The yellow color which thiamine initially exhibits in aqueous alkaline solution and which fades rapidly was investigated. Spectrophotometric titration at 349 mμ, a wave length unique to the yellow form, indicates, by the shape of the titration curve, a simultaneous loss of two protons during the transformation. The steps are inseparable in water and have a pKaverage of 11.6. The spectrum of the yellow form has maxima at 231 mμ and 335 mμ with a shoulder in the region 245 mμ-255 mμ. The molar absorbancy indices for these peaks are ca. 16, 7 and 11.5-13, all x 10^3, respectively.

Elemental analyses of the isolated yellow material, evaluation of the differences between the spectra of neutral and yellow thiamine, as well as the titrimetric data suggest an internal nucleophilic attack by the pyrimidine amino group upon the thiazole carbon 2. The nucleophile brings about a displacement of an electon pair to the quaternary nitrogen and leads to the opening of the thiazole ring by breaking the carbon 2-sulfur bond. The process is accompanied by simultaneous loss of two protons from the amino group.

Further support for nature of the transformation is provided by the observation that several compounds similar to thiamine but lacking one or more structural features involved in the above scheme do not exhibit analogous changes in their spectra at high pH's. The exception is pyrithiamine which has

an extremely transient yellow color in 5.4 M sodium hydroxide solution; the analogue has a pyridine ring substituted like the thiazole ring of thiamine.

In methanol the yellow form of thiamine is more stable and its color is clearly visible for about an hour. The spectrum in methanol peaks at 248 mμ and 334 mμ. The molar absorbancy indices are ca. 13 and 8, each x 10^3, respectively. In addition the methanolic spectrum has high absorbancy below 230 mμ, which is beyond the range of our measurements. Spectrophotometric titration at 349 mμ leads to data which fit a theoretical curve typical of a monoprotic dissociation. At the isosbestic points in the spectra of yellow and neutral thiamines there are changes in absorbancy at intermediate base concentrations. Such behavior indicates a detectable intermediate. The text gives calculations whereby it is possible to calculate a complete spectrum for the intermediate form when its concentration is maximal (56 per cent of all thiamine) and to derive a spectrophotometric titration curve at 290.3 mμ for its appearance. The titrimetric data also fit a theoretical monoprotic titration and indicate that the inseparable two-proton reaction in water is separable in methanol.

A procedure is given for the isolation of the intermediate which is increasingly stable in higher alcohols. The spectrum of this compound measured directly in butanol agrees

quite well with the calculated methanolic spectrum. The latter has maxima at 239 mμ and 279 mμ, with molar absorbancy indices of ca. 7 and 14, each x 10^3. Elemental analyses of the isolated intermediate, analyses of its spectrophotometric differences from the neutral form, as well as the observation that mixtures containing it are readily oxidized to the structurally related thiochrome, indicate that the intermediate is a tricyclic structure. It is formed by the nucleophilic attack of the pyrimidine amino group upon the thiazole carbon 2 accompanied by a loss of one proton from the amino group. A tricyclic structure fits neatly between the structure of neutral thiamine and the suggested structure for yellow thiamine.

A literature reference is quoted wherein the fading of the yellow color is related to the advent of the well-known and more stable colorless open-ring thiol form.

A variation of the published Singer and Pensky scheme for the purification of wheat germ carboxylase is presented. Use of the original scheme lead to great losses of activity and low degrees of purification. The altered scheme requires defatting with acetone, preparation of an aqueous extract, isoelectric precipitation between pH 5.35 and pH 4.90, alcohol precipitation between 0 and 15 per cent alcohol, ammonium sulfate precipitation between 0 and 50 per cent saturation and dialysis. The enzyme was assayed by colorimetrically

measuring acetoin yields from a pyruvate substrate. The revised procedure lead to 18 per cent yields of enzyme with 1500 to 1600-fold purifications. These are low in comparison with the published report as are the Purity Indices and initial activities.

Trypsin and N-bromosuccinimide damage the enzymatic activity of carboxylase.

Spectrophotometric investigations of carboxylase in the presence of either magnesium ion or cocarboxylase or both indicate that the inorganic ion does not perturb the spectrum of the apoenzyme but that the cocarboxylase does. At pH 6.4 or 6.8 apoenzyme with cocarboxylase has lower absorbancy at all wave lengths than does apoenzyme with cocarboxylase and magnesium ion. The latter has lower absorbancy than the arithmetic sum of the separate absorbancies of the components. The difference spectra in both cases are positive and have peaks in the regions 270 mμ-280 mμ and also 300 mμ-310 mμ. The data suggest that thiamine is bound to the enzyme as the neutral form with somewhat diminished absorbancy. Experimental difficulties in deriving these spectra are discussed.

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