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The mechanism of thiamine action

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THE MECHANISM OF THIAMINE ACTION

by

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TABLE OF CONTENTS

I. INTRODUCTION

The concept of growth factors necessary for proper nutrition of various living organisms, in addition to the usual proteins, carbohydrates and fats, had undoubtedly crossed the minds of many men[1] before Casimir Funk but it remained for him to grasp the universality of this concept and to put it into words. Funk was a Polish biochemist who was working at the Lister Institute in London when he wrote (2): "The deficient substances, which are of the nature of organic bases, we will call 'vitamines' and we will speak of a beriberi or scurvy vitamine, which means a substance preventing the special disease." This unique name, though later shown to be a misnomer since most vitamins are not amines, nevertheless dramatized the issue and brought the attention of the scientific world to focus on this important problem. Funk himself wrote in 1922 (3): "The word 'Vitamine' served as a catchword which meant something even to the uninitiated, and it was not by mere accident that just at that time, research developed so markedly in this direction."

Other people before him, as R. R. Williams wrote (4), either had "lesser vision or lesser courage" and failed to

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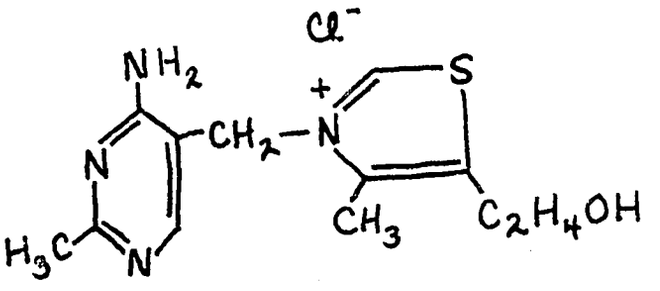
[1] Harris interestingly traces the history of the idea of "growth factors" in his little book "Vitamins and Vitamin Deficiencies" (1).

relate their findings to a more universal concept. Thus the thiamine containing anti-beriberi preparations of Suzuki (5) from rice (Oryza sativa) bore the name "oryzanin", while preparations of Edie, et al. (6) from yeast (Torula) bore the name "torulin". Needless to say, neither name caught on.

It is perhaps unfortunate that the first vitamin discovered, thiamine, also turned out to be one of the most unstable. Thus, although the Dutch physician, Eijkman, first showed in 1897 that beriberi could be cured by feeding rice polishings, it was some 30 years before the vitamin was isolated as a pure crystalline substance. This long delay no doubt reflects a great deal the lability of thiamine. Looking back on some of the stringent methods used in isolation, for example, precipitation with phospho-tungstic acid, silver nitrate, or elution with barium hydroxide, it seems remarkable that any of the thiamine survived at all. And in fact it took still several more years before there was sufficient vitamin of known purity on which to do structural studies. The lead in this work was taken by R. R. Williams and coworkers in association with Merck Laboratories and in 1937 they published (7) the first successful synthesis of thiamine (I).



(I)

This synthesis was rapidly followed by others (8, 9) and synthetic methods of preparation soon replaced the more tedious isolation procedures.[1]

It is pertinent now to ask where and how does thiamine function. The earliest clues involved the observation (11) that carbohydrates aggravated the symptoms associated with thiamine deficiences. While working on the isolation of thiamine, R. A. Peters and his group at Oxford became concerned with the metabolism of vitamin B_1 (thiamine) deficient organisms. Working with brain preparations from thiamine deficient pigeons, Peters and Kinnersly (12) showed that lactic acid accumulated. Later, Peters and Thompson (13) with similar preparations showed that both pyruvic and lactic acids accumulated in B_1 deficient brains but not in

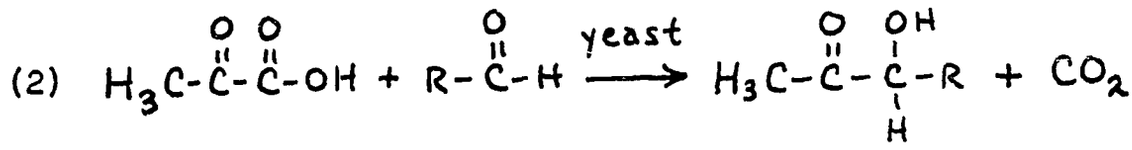
[1] An interesting account from the American viewpoint of the race to decipher the structure and synthesize thiamine has been given by Williams in his booklet (10) explaining the Williams-Waterman Fund.

normal brains. This indicated that somehow a lack of thiamine blocked their utilization. Then Peters (14) made the important discovery that normal pigeon brain slices had a higher oxygen uptake than avitaminous brain slices when glucose, pyruvate or lactate were used as substrates. However, when thiamine was added the oxygen uptake of the deficient brain slices was raised to normal. Thus it could be deduced that thiamine was directly involved in the oxidation of pyruvic acid and the response is so specific that it actually can be used as an assay for thiamine (catatorulin effect). Once pyruvic acid was removed lactic acid no longer accumulated (15).

Still another decarboxylating system existed in yeast and had been studied in particular by Neuberg and his group since its discovery in 1912. The enzyme involved was christened "carboxylase" and it was found to split α-keto acids into carbon dioxide and the next lower aldehyde (equation 1).

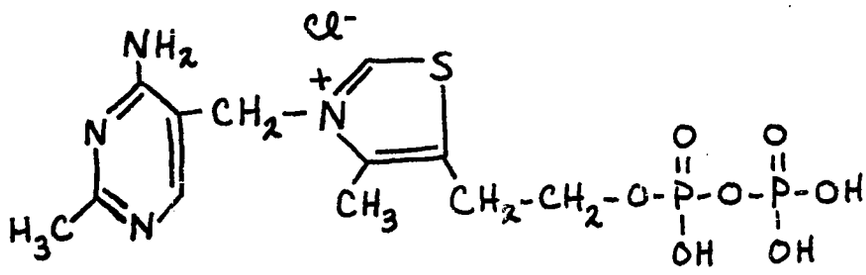
(1) $$R-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-OH \longrightarrow R-\overset{O}{\overset{\|}{C}}-H + CO_2$$

Neuberg and his coworkers also discovered that fermenting yeast had the ability to condense a variety of added aldehydes with the two carbon fragment arising from pyruvate to yield variously substituted ketols (equation 2).



Neuberg named this two carbon fragment "nascent acetaldehyde" since it always yields acetaldehyde or a condensation product of acetaldehyde. The results of these studies have been reviewed by Neuberg (16).

Twenty years later, Auhagen (17) split yeast carboxylase into two factors, both needed for activity. One was a protein and the other was a thermostable factor which was named cocarboxylase. In 1937 Lohman and Schuster (18) isolated cocarboxylase from yeast and showed it to be the diphosphate ester of thiamine (II).



(II)

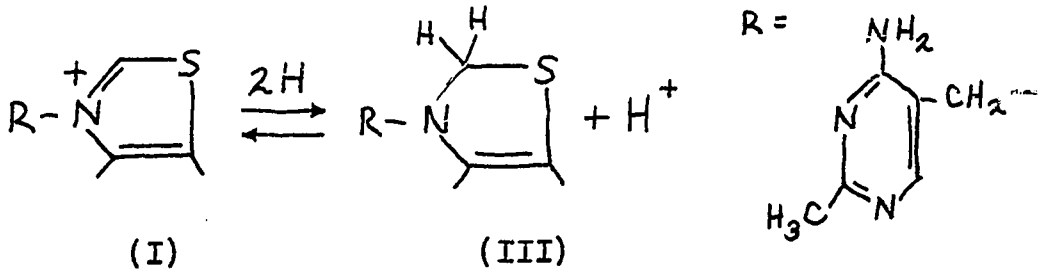
It was quickly shown that diphosphothiamine was also the coenzyme involved in pyruvic acid oxidation in bacteria (19) and in pigeon brain preparations of Peters.

In subsequent years, using mainly bacterial systems, pyruvate was shown to be not only the precursor of acetaldehyde and acetic acid, but also of molecular hydrogen, lactic acid, acetyl phosphate, formic acid, acetoin, α-acetolactic acid, all the members of the citric acid cycle, acetoacetic acid, carbon dioxide and water. The experimental work which led to the identification of these products is given in an excellent review by Ochoa (20). All the above reactions are mediated in some respect by thiamine diphosphate. Despite the seeming diversity of these substances, it seemed clear almost from the start that the function of thiamine was somehow to produce an activated two carbon fragment from pyruvate. This could then condense with different electrophilic reagents to give the wide variety of products observed.

There was, however, some question as to the oxidation state of the activated two carbon fragment. Yeast carboxylase always simply decarboxylated pyruvate to acetaldehyde, but all animal tissues and many bacterial systems gave either acetic acid or carbon dioxide and water as final products. This apparent oxidizing ability of thiamine led to two proposals of redox systems, both involving forms of the thiazolium ring. Since neither of them are seriously considered now, they will be mentioned only briefly.

Lipmann (21) proposed the first redox system in analogy with the newly found oxidation-reduction of the nicotinamide

co-enzymes. That is, the double bond between the quaternary nitrogen and the number two carbon atom of the thiazolium ring (equation 3) would be reduced and oxidized much as the quaternary nitrogen of the pyridinium ring was thought to undergo reduction and oxidation.

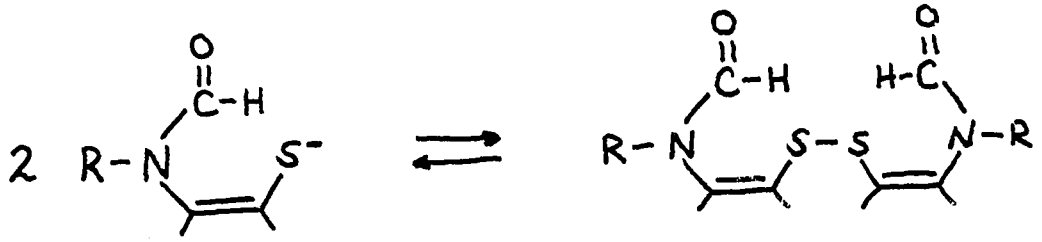
(3) 

(I) (III)

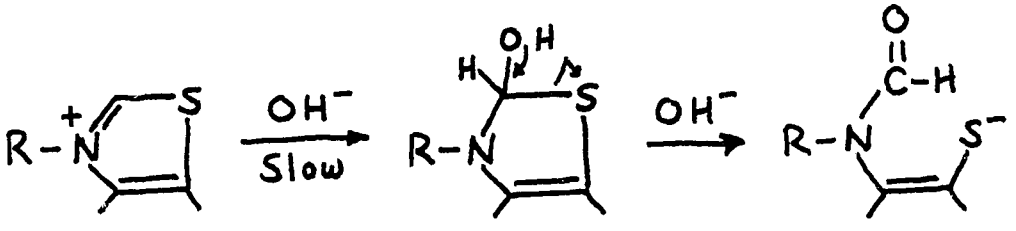
Lipmann and Perlman (22) prepared what was thought to be dihydrothiamine (III) by reducing thiamine with sodium dithionite and hydrogen on platinum black. They were unable to isolate a product from either reaction mixture. Later it was shown that the dithionite had cleaved thiamine into its pyrimidyl and thiazole halves (23). Using lithium aluminum hydride, Karrer and Krishna (24) were able to prepare authentic dihydrothiamine. Their product was only 7% as active as thiamine when tested in thiamine deficient rats. This low activity undoubtedly rules out any reversible oxidation-reduction of the thiazolium ring as being physiologically significant.

The second redox scheme was proposed by Zima and Williams (25) and involves the oxidation of the thiol form of thiamine (IV) to the disulfide form V (equation 4). The

thiol form is formed by the action of two moles of base on the thiazolium ring of thiamine (equation 5) and goes by way of an intermediate pseudo base form (VI).

(4) 

(IV) (V)

(5) 

(I) (VI) (IV)

Thiamine disulfide (V) may be prepared by the action of dilute hydrogen peroxide on thiamine at pH 7.5 and is reduced back either by cysteine or glutathione (26). When tested on animals thiamine disulfide is some 60-70% as

active as thiamine. However, tests with thiamine deficient pigeon brain preparations showed that some reducing compound, such as cysteine, had to be present before oxygen was taken up (27). Thus before thiamine disulfide is active it must undoubtedly be reduced back to the thiol form.

The discovery that lipoic acid (VII) (also called thioctic acid) was necessary for pyruvic acid oxidation removed the requirement for thiamine to act as a redox catalyst. The two carbon fragment of decarboxylated pyruvic acid could now be transferred to one of the sulfurs of lipoic acid. This

(VII)

would in essence oxidize the two carbon fragment to an acetyl grouping and reduce the disulfide linkage to two thiol groups A schematic mechanism modified somewhat from Gunsalus (28) is shown in Figure 1.

All the steps in Figure 1 have been essentially verified and the acetyl dihydrolipoate, dihydrolipoate, and acetyl CoA are all known to react in stoichiometric amounts (28). The reactions of the acetyl co-enzyme A are now well known and need not be elaborated in detail. It is sufficient to point out that acetyl co-enzyme A is the common intermediate

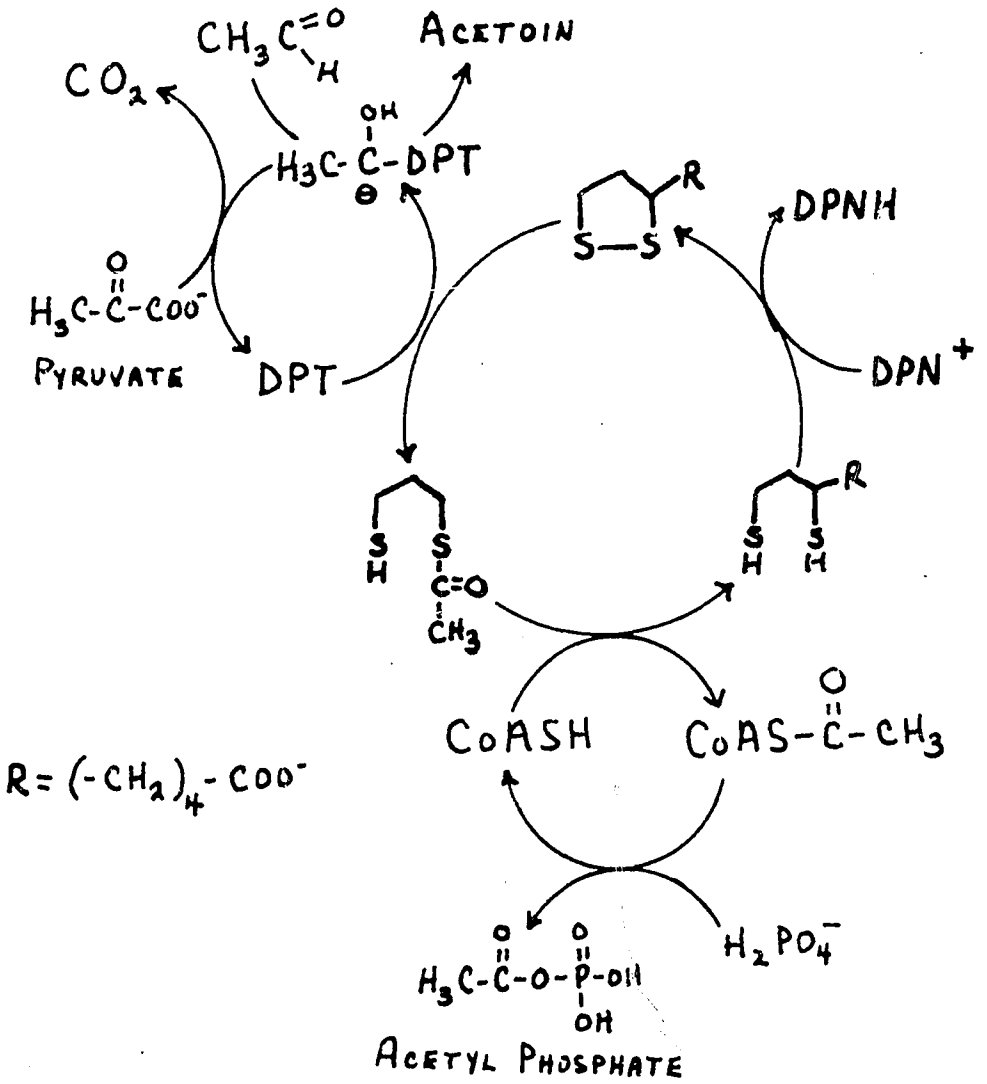


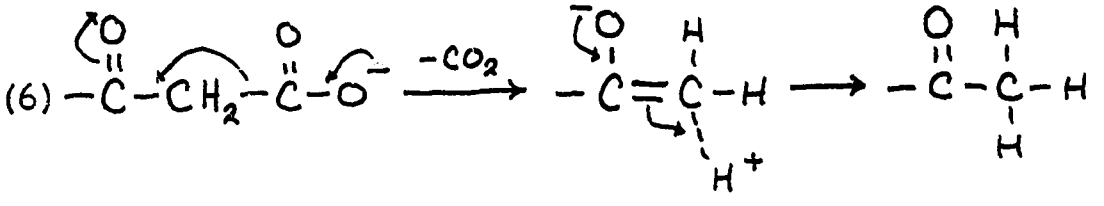
Figure 1. Relationship of lipoic acid to pyruvate metabolism

Abbreviations used: DPT = diphosphothiamine; DPN^+ and DPNH = oxidized and reduced diphosphopyridine nucleotides; CoASH = co-enzyme A.

between fat and carbohydrate metabolism and its discovery explains why thiamine deficient animals could not convert carbohydrate to fat. It also explains why fat spares thiamine deficiencies since the acetyl co-enzyme A formed from fats, unlike that from carbohydrates, does not need diphosphothiamine. The acetyl co-enzyme A may then condense with oxaloacetate to give citric acid and subsequently be metabolized through the citric acid cycle, the main energy source of animals. The formation of acetyl co-enzyme A also explains many of the products known to originate from pyruvic acid. Thus it can be seen that the two carbon fragment from pyruvic acid may condense at either of two oxidation states, the acetaldehyde level as Neuberg showed or at the "active acetate" level of acetyl co-enzyme A. The DPNH formed on oxidizing dihydrolipoate back to lipoic acid can be used to reduce pyruvate to lactic acid or to reduce carbon dioxide to formic acid. Thus the origin of two acids known to occur from bacterial dismutations of pyruvic acid can be rationalized.

While the discovery of lipoic acid and co-enzyme A helped clarify pyruvate oxidation a great deal, there still remained the question which had been the subject of innumerable speculations since the role of thiamine in α-keto decarboxylations became known. That is, what part (or parts) of the thiamine molecule are necessary for catalysis and what is the nature of the reaction sequence during a

catalytic cycle. It is this question which will be the subject of this thesis. It may be stated at the outset that the part of thiamine used as the attacking agent must be a nucleophile. That is, it must carry either a partial or a whole negative charge since the initial attack on pyruvate will be at the partially-positive carbon of the carbonyl group. The function of thiamine will then be to attract the pair of electrons forming the carbon-carbon bond between the carbonyl group and the carboxyl group and to stabilize the anion which results when carbon dioxide is lost. In β -keto acids this function is carried out by the β -keto group itself and as a consequence these acids tend to decarboxylate spontaneously. This is illustrated in equation 6.

(6) 

However, there is no such activating group in α-keto acids and it is precisely this function which thiamine must fulfill. A highly analogous situation has been studied by Metzler *et al.* (30) in which the decarboxylation of α-amino acids is facilitated by combination with the vitamin co-enzyme pyridoxal phosphate.

The mechanisms proposed up to the time of the beginning of this thesis may be grouped into four classes, each one

differing as to the part of thiamine used as the attacking agent. The first mechanism, interestingly enough, was suggested even before the chemical nature of thiamine was known. When the German chemist Langenbeck heard that an additional organic molecule, co-carboxylase, was needed for carboxylase activity, he correctly predicted (31) that it would contain a free amino group. He based his prediction on the fact that various primary amines would act catalytically to decarboxylate α-keto acids when heated in phenol-cresol solvent. Langenbeck pictured the catalytic cycle as involving first the formation of a Schiff-base between the 4-amino group on the pyrimidine ring and the carbonyl group of the α-keto acid, followed by decarboxylation and the shift of a proton to the α-carbon atom. The resulting aldimine then may be hydrolyzed to an aldehyde plus catalyst, or as in the case of his anhydrous model systems, cleaved with another molecule of substrate to yield an aldehyde plus a new aldimine. Studying pyruvic acid decarboxylation, Langenbeck also showed that it was possible for the aldimine intermediate to condense with another acetaldehyde to give acetoin. This same type of acyloin condensation had been shown to occur in yeast by Neuberg and Simon in 1921 (32). Stern and Melnick (33) criticized Langenbeck's mechanism on the basis that the 4-amino group on the pyrimidine was extraordinarily unreactive. For example, ketene, which will ordinarily acetylate amines before it will acetylate hydroxyl groups, reacts

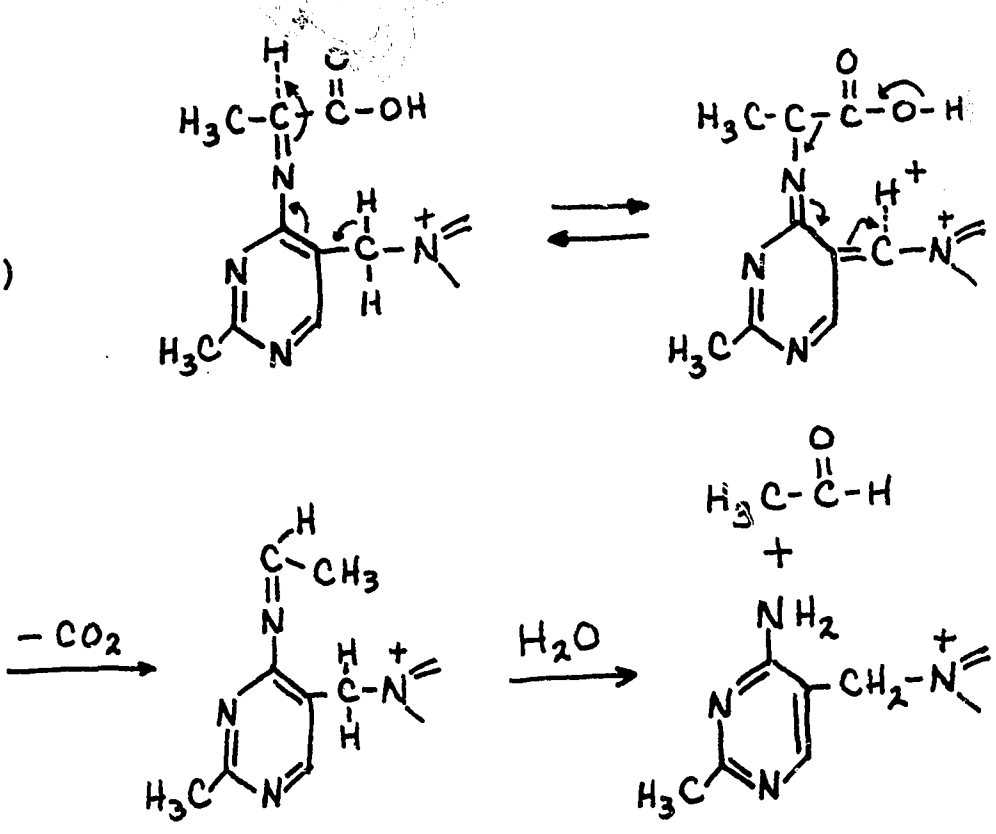
preferentially with the primary alcohol group of thiamine. In addition, nitrous acid reacts but very slowly to deaminate thiamine. Thiamine also was inactive as a catalyst under Langenbeck's test conditions.

Despite this objection, Weil-Malherbe (34) postulated a role for the intermediate carbinol amine. In this case, as the intermediate decarboxylated, the hydrogen of the amino group would be transferred as a hydride ion to C-2 of the thiazole ring in an internal oxidation-reduction. The return of the hydride ion to the 4-amino nitrogen reductively cleaves the N-acetyl bond to give acetaldehyde and free catalyst. If the N-acetyl bond is simply hydrolyzed, acetic acid plus dihydrothiamine (III) results, the dihydrothiamine presumably being oxidized back to thiamine by some other agent. This scheme gained a certain amount of support because it explained both the oxidative and non-oxidative decarboxylation of pyruvate.

Once the role that lipoic acid played in the oxidation of pyruvate was clear, Wiesner and Valenta (35) extended Langenbeck's scheme somewhat to make it more in line with modern organic mechanistic views. To rationalize the decarboxylation they tautomerized the imine form using a proton from the methylene bridge. The positive charge of the thiazolium nitrogen should help labilize this hydrogen. This provides a nicely conjugated system which can stabilize the resulting anion, once decarboxylation has taken place.

Hydrolysis of the newly formed aldimine yields acetaldehyde and regenerates the catalyst. An abbreviated mechanism starting with the pyruvate-thiamine imine is given in equation 7.

(7)



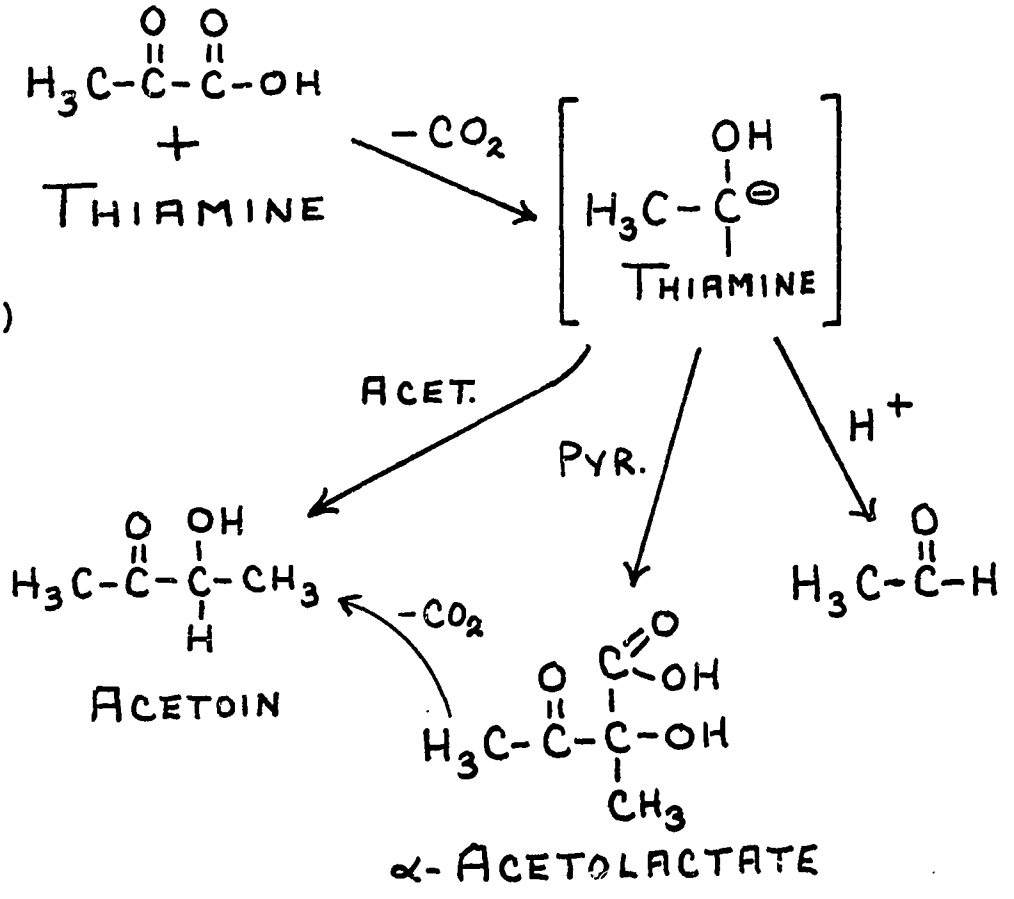
A second mechanism involving the thiol form of thiamine (IV) as the attacking agent was hinted at by Karrer (36) and was given in detail by Johnson (37). As Johnson visualized it, the thioketol of pyruvate would be formed which could then decarboxylate by expanding the octet of sulfur. However, cysteine, thioglycollic acid, and thiophenol are inactive as catalysts for the formation of acetoin under conditions which thiamine is known to work (38).

The prospects for solving the riddle of thiamine's active site brightened considerably when Mizuhara and co-workers (39, 40) found that thiamine alone in a slightly basic medium would decarboxylate pyruvate, and in the presence of acetaldehyde form small amounts of acetoin. Mizuhara based his system on the accidental discovery of Ugai et al. (41) that certain thiazolium salts, including thiamine, in mildly basic solutions would catalyze both benzoin and furoin condensations from benzaldehyde and furfuraldehyde, respectively. Mizuhara's model system offered an excellent opportunity to test thiamine analogs for catalytic activity. While innumerable analogs had been tested in biological systems, it could never be determined with any certainty whether inactivity was due to the lack of a necessary grouping or whether it was simply a reflection of the stereochemical demands of the enzyme for its co-enzyme. Also, the entire problem of phosphorylated analogs vs. unphosphorylated analogs could be forgotten. Studies of the pH dependence of the reaction could also be informing. Thus Mizuhara found that on adding one, two or three equivalents of base per equivalent of thiamine chloride hydrochloride that the two to one ratio gave the best yield by far. Since the pseudo-base form of thiamine (VI) is in equilibrium with the quaternary amine form of thiamine (free base) at this pH, Mizuhara envisioned the attack on pyruvate to come from the free pair of electrons on the

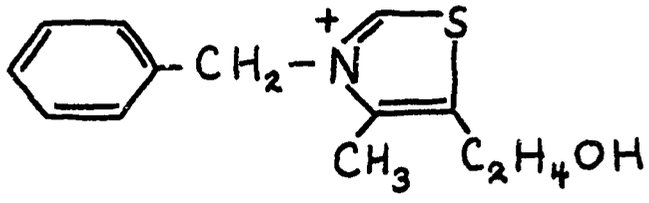
tertiary amine of the thiazole ring. A quaternary amine addition product would result and the positive charge on the nitrogen should help stabilize the resulting anion once decarboxylation occurred. The carbanion was then free to pick up a proton to give acetaldehyde or to condense with acetaldehyde to give acetoin.

In the presence of pyruvate alone as substrate, Koffler and Krampitz (42) reported that α-acetolactate was the main product. The addition of acetaldehyde stimulated acetoin production but not α-acetolactate formation. A generalized view of these reactions may be pictured as in equation 8.

(8)



In 1956 Breslow (43) discovered that 3-benzyl-4-methyl thiazolium chloride would catalyze acetoin formation. This was confirmed shortly thereafter by Metzler and Manzo (44) using a similar compound (VIII) which had in addition a 2-hydroxy ethyl side chain on C-5 of the thiazole ring.[1]



(VIII)

Thus any mechanism involving the 4-amino group of the pyrimidine ring could be ruled out conclusively. Breslow, reasoning from analogy to pyridinium salts, postulated that the attacking agent was an "ylid" carbanion formed by loss of a proton from the methylene bridge carbon. It could condense with the carbonyl group of pyruvate with the loss of a water molecule. Addition of a hydroxyl group, as in pseudo base formation, leads to a rearrangement whereby the condensed pyruvate (or other α-keto compound) either picks up a proton or another carbonyl compound. Decarboxylation then can

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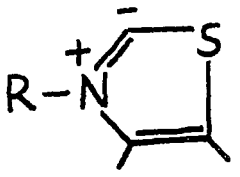
[1]This compound will be called simply the "benzyl analog of thiamine" in future discussions.

occur simply by a reversal of the rearrangement and loss of hydroxide ion. The final step is just the reverse of the condensation.

Shortly thereafter, Ingraham and Westheimer published a note (45) challenging Breslow's mechanism on the basis of deuterium isotope exchange studies. They had run the model system in deuterium oxide, split the thiamine into two halves with bisulfite ion and looked for the deuterium in the pyrimidinyl methyl sulfonic acid which had precipitated. There was none. If Breslow's mechanism was correct then deuterium should have been incorporated into the thiamine at the methylene bridge carbon. However, there was still some question as to whether or not the deuterium might have been incorporated into thiamine and then subsequently lost by exchange during the work-up of the sulfonic acid.

It was our thought that synthesizing and testing a series of thiamine analogs should help decide conclusively what part of the thiamine molecule was essential for catalytic activity. Later, when kinetic studies revealed the presence of an intermediate, this intermediate was studied by its reactivity with carbonyl compounds and stability at different pHs. During the course of these studies Breslow (46) made the remarkable discovery that the hydrogen on C-2 of the thiazolium ring was extremely labile even at near neutrality. Thus a thiazolium zwitter-ion (IX) could easily be formed.



(IX)

Assuming this is the catalytically active form, a concise mechanism (47, 48) may be written explaining all the known reactions catalyzed by thiamine. This is illustrated in Figure 2, for the decarboxylation of pyruvic acid and the production of either acetaldehyde or an α-ketol. Since there seems little doubt as to the validity of these findings, the results of this thesis will be discussed in light of this proposed mechanism.

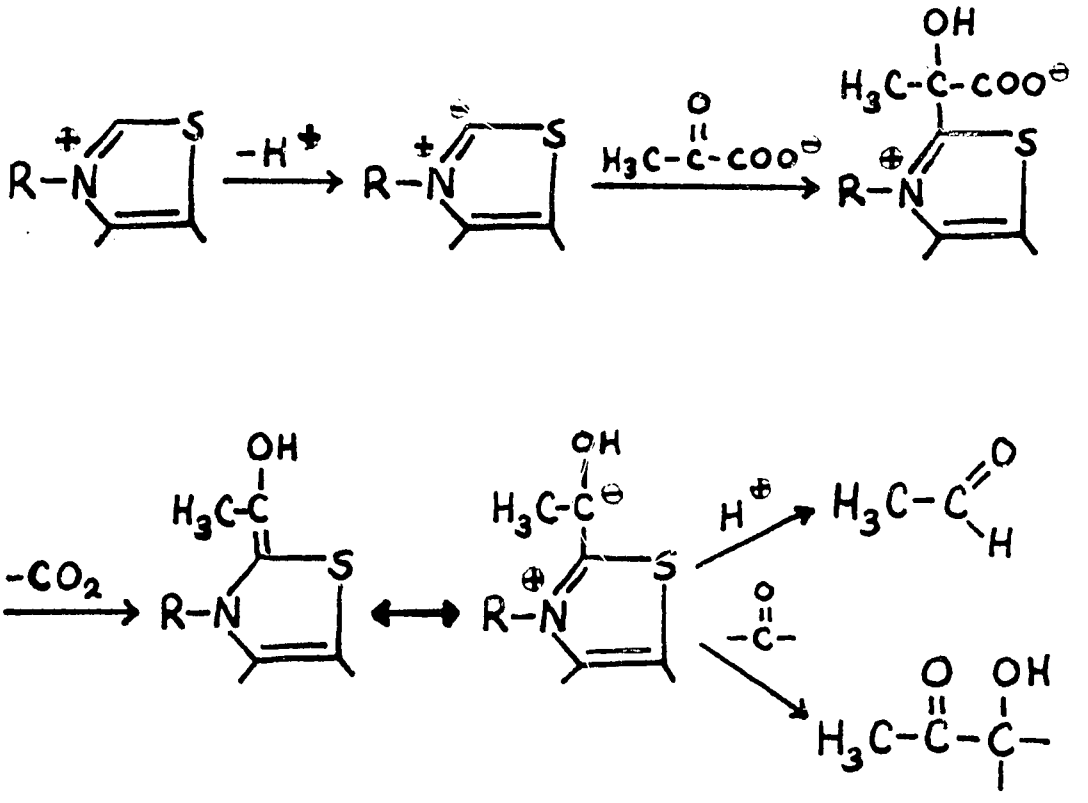


Figure 2. Breslow's zwitter-ion mechanism for thiamine action (48)

II. METHODS

A. Chemicals and Reagents

Sodium pyruvate was either made from redistilled pyruvic acid by the method of Price and Levintow (49) or was a commercial product recrystallized from water and absolute alcohol after treatment with charcoal. Nutritional Biochemicals Corp. was found to be the best commercial source. Their preparations contained the least polymeric contaminants and proved the easiest to purify. However, even after double recrystallizations there remained a small amount of carbonyl contaminant as shown by paper chromatography. In a water saturated n-butanol, 90% formic acid (45 to 5) system (50) the contaminent had an Rf of 0.1 and pyruvic acid of approximately 0.7 when detected with 0.05% 2,4-dinitrophenylhydrazine spray reagent. However, this contamination was only visible on chromatograms which had been heavily overloaded with the pyruvate solution and was therefore neglected.

Thiamine chloride hydrochloride was used as received from Merck and Co. except for that used in the measurement of the equilibrium constant of the ring opening reaction. Then a portion was recrystallized until it gave the theoretical neutral equivalent.

Acetoin was redistilled under nitrogen and allowed to form the crystalline dimer by standing in the cold. The dimer was filtered, washed with ether and used without further purification.

Acetaldehyde was redistilled from a commercial product or was made by depolymerizing redistilled paraldehyde by heating in the presence of a small amount of sulfuric acid (51). The purified product was stored in a desiccator at -20° to hinder oxidation.

Paraldehyde - Commercial paraldehyde was redistilled (25°/15 mm) through a small column and stored at 2°.

α-Naphthol was a commercial product redistilled under nitrogen and stored at -20° to prevent oxidation.

B. Stock Solutions

Standard Paraldehyde Solutions were prepared according to Stotz (52).

Stock Solution - One ml of paraldehyde (1.00 gm) was diluted to one liter with water to give a concentration of one mg per ml.

Working Standard - One ml of stock solution was diluted to 500 ml to give a 2.0 μgm per ml solution.

Acetaldehyde Solutions - 1 M acetaldehyde solutions were made in the following manner: A 10 ml volumetric flask was partially filled with water, weighed and chilled

at -20°. Using a previously cooled pipet, 0.55 ml of cold acetaldehyde (-20°) was added to the volumetric flask and the flask reweighed. This method generally sufficed to give 440 mg ± 10 mg of acetaldehyde which on dilution to 10 ml gave a 1.0 M ± 0.02 M solution. Fresh solutions were made daily as needed. Less concentrated solutions were made from dilutions of these 1 M solutions.

Approximately 10^{-4} M solutions were used as standards for acetaldehyde determinations by aeration. These solutions could be conveniently standardized by comparing their color yields (without aeration) with those of the standard paraldehyde solutions.

Standard Acetoin Solution - Stock solutions (2.5×10^{-3} M) were made by dissolving 0.550 gm of acetoin dimer in 250 ml of water. These solutions were stable up to three months stored in the cold. Working standards (0.1 μMole per ml) were 1 in 25 dilutions of the stock solutions.

α-Acetolactate Solutions were prepared in the following manner: Approximately 0.2 ml of α-methyl-α-acetoxy ethyl acetoacetate (53) was added to a tared 100 ml volumetric flask and weighed. This gives about one mMole of compound. Thirty ml of water was added and the flask cooled to 0° in an ice bath. Two equivalents of base were added slowly with cooling and the flask swirled until all the α-methyl-α-acetoxy ethyl acetoacetate dissolved. The solution was allowed to warm to room temperature and was diluted to

volume. Incubation of aliquots of this solution at pH 2 or below at 40° for one hour was sufficient to quantitatively decarboxylate the α-acetolactate to acetoin. Acetoin then could be measured colorimetrically. Samples treated this way generally gave greater than 90% of the expected yield.

Thiamine and Sodium Pyruvate Solutions were either 1 M or 0.5 M and were kept at 2° and used for periods up to one month.

Buffer Solutions - Triethanol amine buffers were made from recrystallized triethanol amine hydrochloride and standard base. Borate buffers were made from recrystallized borax and hydrochloric acid. All other buffers were made with C.P. chemicals.

p-Hydroxy biphenyl reagent - A 1.5% solution was made by dissolving 0.375 gm of recrystallized p-hydroxy biphenyl in 25 ml of 0.5% sodium hydroxide. This solution is stable for about two months at room temperature.

5% α-Naphthol - 1 gm of powdered, white α-naphthol was dissolved in 20 ml of 2.5 N sodium hydroxide. It must be prepared fresh with each set of determinations.

0.5% Creatine - 1 gm of creatine (C.P., Nutritional Biochemicals Corp.) was dissolved in 200 ml of water. This is stable indefinitely at room temperature.

0.05% 2,4-Dinitrophenylhydrazine Spray Reagent - 0.125 gm of 2,4-dinitrophenylhydrazine (recrystallized from n-butanol) was dissolved in 250 ml of 2 N hydrochloric acid.

C. Analytical Methods

Acetoin was determined by the colorimetric method of Westerfeld (54). The color reaction is unknown but it has been shown to involve the action of oxygen, creatine and strongly basic α-naphthol on acetoin to give a red product in alkaline solutions (55). The reaction is fairly specific for acetoin and its oxidation product biacetyl. Propionyl methyl carbinol and propionoin, both very similar in structure, give purple and olive-green products, respectively (56). Benzil and benzoin give colors some thousand times weaker than biacetyl and acetoin. Glyoxal gives no color and methyl glyoxal about 1% the color of an equivalent amount of biacetyl. Pyruvate and acetaldehyde do not give any color and α-acetolactate gives at most only 4-6% the color of acetoin.

Procedure: One ml of 0.5% creatine was added to 5 ml of solution containing 0.1 to 0.5 μmoles of acetoin. Then one ml of a 5% basic α-naphthol solution was added. To insure complete oxidation the tubes were shaken for the first third of a 60 min. incubation period in a neoprene covered test tube rack attached to a rocker arm shaker. Readings were taken at 525 mμ with a Beckman Model B Spectrophotometer. Creatine and α-naphthol reagent blanks were used to zero the instrument. Standard acetoin solutions were run periodically to check the reagents. Agreement with Beer's law and the calculation of the calibration constant are given in Table 1.

Table 1. Acetoin concentrations versus absorbancy at 525 mμ

Acetoin concentration	Absorbancy (525 mμ)	Calibration Constant $K = \frac{\mu\text{Moles acetoin}}{\text{Absorbancy (525 m}\mu\text{)}}$
0.1	0.295	0.340
0.2	0.580	0.345
0.25	0.715	0.350
0.4	1.170	0.342
0.5	1.430	0.349
		Ave. = 0.345

Inhibition: Thiazolium salts, in general, and acetaldehyde were found to interfere with normal color development of acetoin. Since thiol compounds are known to inhibit this reaction (57), it is assumed that it is the open ring thiol forms of the thiazolium salts which are diminishing the color. For example, when 20 micromoles of thiamine and 200 of pyruvate were present, only 77% of the expected color formed. However, for the most part the thiamine content never exceeded 4 micromoles and the color yield was at least 96% of maximum.

The color test is atypical when acetaldehyde is present. Color development is inhibited and does not become maximal until after 24 hours. This interference was avoided by

aerating out the excess acetaldehyde from aliquots of reaction mixtures (usually 0.5 ml) over a period of three hours at room temperature. Air was passed into the samples using 5 inch stainless steel syringe needles. To stop acetolactate formation during the aeration, the pH of the solutions was lowered to about 6 by addition of hydrochloric acid or phosphate buffers.

Determination of Acetaldehyde - A modification of the direct aeration procedure of Neidig and Hess (58) was used. Pairs of 2.0 x 17.5 cm test tubes containing alternately 3 to 7 ml of sample and 7 ml of color reagent were set up in an aeration train. The color reagent was prepared by adding cautiously with cooling 6 ml of concentrated sulfuric acid to 1 ml of water and one drop of 5% copper acetate solution. After cooling, 0.2 ml of the p-hydroxy biphenyl reagent was layered on and mixed. The receiving tubes were cooled in an ice bath to keep the blanks at a minimum. Air, washed both with sulfuric acid and distilled water, was passed through vigorously for one hour. At the end of this time, the receiving tubes were incubated for 15 min. at 40° to allow color development to take place and for one minute at 100° to dissolve the excess p-hydroxy biphenyl. The tubes were cooled in cold tap water and read at 575 mμ on a Beckman Model B spectrophotometer.

Standard acetaldehyde solutions, both with and without aeration, and blanks, were run with each set of tubes.

Unaerated standards were prepared by substituting one ml of acetaldehyde or paraldehyde solutions for the one ml of water in the color reagent mixture. However, it was found that standards prepared this way were always approximately two times higher than comparable aerated standards. This disagreement could be resolved only if the standards in which the acetaldehyde (or paraldehyde) had been added directly were treated in exactly the same manner as the aerated samples. That is, they were included in the aeration train paired with dummy tubes containing only water. In this case, the two standards were identical. This is shown in Table 2.

Table 2. Comparison of directly added versus aerated acetaldehyde standards

	Ml of 10^{-4} acetaldehyde	Ml H_2O	Direct addition		Aerated	
			Absorbance 575 mμ	μgm acetaldehyde (calc)	Absorbance 575 mμ	μgm acetaldehyde (calc)
1.)	0.2	0.8	0.105	1.6	0.100	1.5
2.)	0.5	0.5	0.215	3.3	0.210	3.2
3.)	0.7	0.3	0.295	4.45	0.280	4.3
4.)	1.0	0.0	0.470	7.2	0.450	6.9
5.)	paraldehyde (4 μgm)		0.260	-	-	-

$$K = \frac{\text{μgm paraldehyde}}{\text{Absorbance 575 mμ}} = 15.4$$

One possible explanation for this lower color product is given in Table 3. This shows the effect of dilution by water on the color yield on a series of unaerated samples. Thus, if in an actual run 0.3 ml of water was aerated over with the acetaldehyde, the color was only 78% of that expected.

Table 3. Effect of dilution on acetaldehyde color yields

No.	Total ml H_2O/tube	Absorbance 575 mμ	% Color (based on 1.0 ml H_2O)
1.)	0.9	0.48	106
2.)	1.0	0.45	100
3.)	1.1	0.415	92
4.)	1.2	0.385	86
5.)	1.3	0.350	78

All tubes contained the same amount of acetaldehyde solution, one drop of 5% cupric acetate, 6 ml of concentrated sulfuric acid, and 0.2 ml of 1.5% p-hydroxy biphenyl.

Some further comment is due on these difficult to reproduce determinations. Certainly, since the color reaction is known, other factors leading to lower yields very possibly enter in. Therefore it is very important to keep conditions as standard as possible. A worthwhile addition to these determinations would be an air flowmeter, so that the aeration rates could be kept the same in all experiments. The

use of nitrogen in place of air does not offer any advantages, nor does it seem to affect the color yield. The 15 min. incubation at 40° helps the reproducibility. Other procedures described in which there is no time allowed for color development before heating at 100° led to lower and erratic results.

Halide Determinations were done by the Volhard method in which an excess of standard silver nitrate is added to a known weight of halide and the excess silver ion titrated with standard potassium thiocyanate. Run on 0.1 mMole this method suffices to give an accuracy of about one part in a hundred.

pH Measurements and Titration Curves were made utilizing the Beckman Model G pH meter. Titrations of long duration, because of the slow establishment of equilibrium, were done under an atmosphere of nitrogen. Compounds before titration were routinely dried at 78°/1 mm to remove any traces of water or solvent.

Spectral Measurements were made with a Beckman Model DU spectrophotometer using matched silica cells. Infrared spectra of the thiazolium salts were made on KBr pellets.

Measurement of the Temperature Dependence of pK_{av} of the thiazolium ring opening reaction (equation 5) was done in the following manner: Approximately 0.2 mMoles of the thiazolium salt was weighed into a shortened test tube (18 x 100 mm) and dissolved in exactly two (one equiv. for the benzyl analog of

thiamine) equivalents of 0.1 N NaOH. The solution was equilibrated for at least five minutes at the appropriate temperature. Nitrogen was bubbled through the solutions by means of 5 inch syringe needles. pH measurements were made using long probe electrodes (Beckman #39166) with the pH meter standardized at each temperature using Beckman pH 10 buffer. Two separate sets of two samples were run for each compound and values at each temperature agreed with themselves for the most part within ± 0.03 pH units.

D. Experimental Technique for Model System Studies

Three different types of reaction vessels were used; Thunberg tubes which were evacuated for 5-15 minutes by a water pump, Warburg flasks under nitrogen, or special reaction flasks also under nitrogen. All runs were anaerobic because of the finding of Mizuhara, et al. (39), that acetoin yields were greatly diminished in the presence of air.

The special reaction flask is shown in Figure 3. The center neck is stoppered with a 16 mm rubber serum cap (Aloe Scientific Co.) which permits sampling at various time intervals by means of a hypodermic syringe needle. Nitrogen is bubbled through the main chamber for 15 min. at 40° by the right hand side arm and is allowed to escape through a syringe needle in the rubber cap. At the end of this time the nitrogen is shut off by the glass stopcock, the syringe

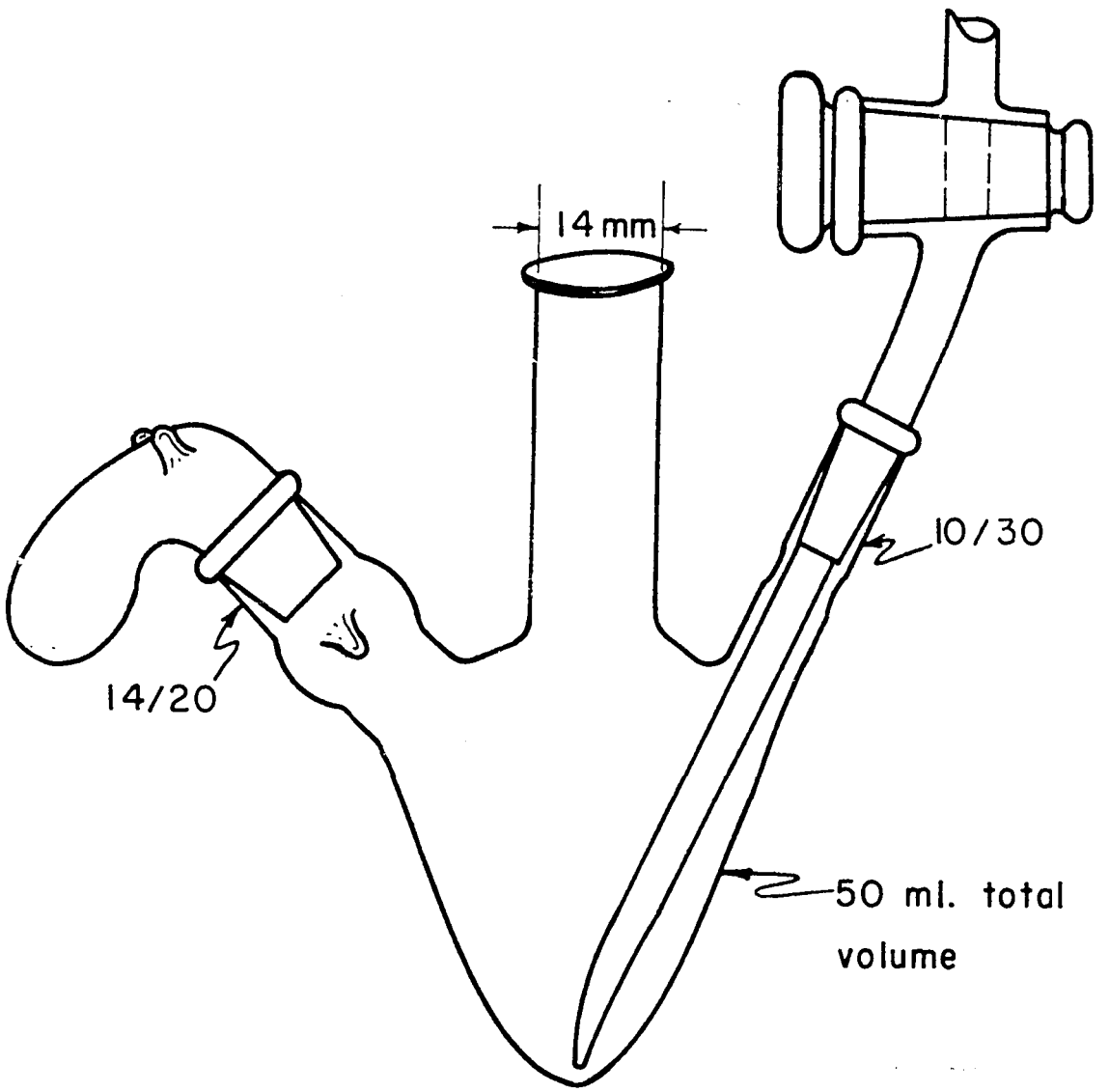


Figure 3. Special reaction flask for kinetic studies (drawn to scale)

attached to the needle, and the side-arm tipped to start the reaction. This side-arm (or the Thunberg cap) contained 0.1-0.5 ml of thiamine hydrochloride solution. In the majority of the cases the main chambers contained buffer, sodium pyruvate, water and sufficient sodium hydroxide to bring thiamine added to the desired pH. It was this latter sodium hydroxide which caused untold trouble. Attempts to duplicate in Thunberg tubes the acetoin yields obtained in triethanolamine buffer with the Warburg vessels always gave lower results. However, with borate buffers the yields in both vessels were identical. This anomaly was traced to the surprising lability of the pyruvate in mild excess of base. Triethanolamine buffers (pK = 7.9) in contrast to borate buffers (pK = 9.2) had insufficient capacity to prevent the pH of the pyruvate-buffer-sodium hydroxide solution from rising to about 12 before the thiamine hydrochloride was tipped in. As shown in Table 4, in triethanolamine buffers close to 30% of the pyruvate was destroyed during the 30 minutes required for preparation, evacuation and preincubation of the samples.

All tubes contained 0.5 mM of pyruvate, 1 ml of pH 9.0, 0.25 M buffer and enough water to give a final volume of 2.4 ml. Tubes were evacuated for 15 min. during a 30 min. period at room temperature, incubated for 5 min. at 40°, cooled, the pH measured and the relative amounts of

Table 4. Effect of high pH on pyruvate stability

No.	Buffer	Millimoles NaOH	pH	Absorbancy 520 mμ	% Loss
1.)	triethanolamine	0.1	11.7	0.40	28
2.)	"	0.1	11.68	0.41	"
3.)	"	0.05	9.27	0.55	-
4.)	"	0.05	9.25	0.55	-
5.)	borate	0.1	9.08	0.62	-
6.)	"	0.1	9.05	0.53	-

pyruvate determined by a modification of the method of Friedemann and Haugen (59).

This difficulty was avoided in the Warburg manometer runs and subsequently in the special reaction vessels by using neutralized thiamine in the side arm in place of thiamine hydrochloride. In the Thunberg tube runs it was found easier to put the pyruvate in the cap, and the remainder of the reaction mixture, including the thiamine, in the tube. This modified procedure was used in obtaining the data on pH dependence in Figure 11. Controls containing thiamine and buffer alone and pyruvate and buffer alone were always run. The total volume after mixing was usually 2.5 ml. For measuring the kinetics of acetoin and acetolactate formation, larger volumes were incubated in the special reaction vessels.

When acetaldehyde was required in a reaction mixture, the evacuated Thunberg tubes were filled with nitrogen to a pressure slightly in excess of one atmosphere. After several minutes in the water bath, excess pressure was released by opening the tube momentarily. The tube was then again opened momentarily and the acetaldehyde injected by means of a syringe.

Warburg Flasks - carbon dioxide evolution was determined in the Warburg apparatus using flasks with two side-arms. The first side-arm contained the thiamine solution, which was tipped at zero time; the second, 0.1 ml of 5 N sulfuric acid to stop the reaction and release carbon dioxide. Excessive carbon dioxide absorption from the air during sample preparation was avoided by working rapidly and by neutralizing the thiamine solution placed in the side-arm so that a smaller excess of base would be required in the main chamber before mixing. This also prevented destruction of pyruvate in triethanolamine buffers as described previously. No carbon dioxide was produced when pyruvate and buffer were incubated alone. A very slight apparent carbon dioxide production occurred as thiamine blanks were incubated, amounting to less than 0.3 mM (0.3 millimoles of carbon dioxide produced per liter of reaction mixture) in three hours. Since this amount is barely significant no correction was made. Reaction mixtures into which the acid was tipped at zero time evolved about 1.2 mM of carbon dioxide. All data were

corrected through the use of such controls with each experiment. Samples were run in triplicate and the results averaged. Carbon dioxide evolution from carbonate was complete 10 minutes after the acid was tipped but when acetolactate was present a full 50 minutes was needed.

E. Sources and Synthesis of Thiamine Analogs

O-acetyl thiamine hydrochloride and oxythiamine dihydrochloride were purchased from California Foundation for Biochemical Research.

Pyrithiamine hydrobromide and 2-methyl-4-amino-5-aminomethyl dihydrochloride were obtained from Nutritional Biochemicals Corp. All compounds were used without further purification.

4-Methyl-5-(2-hydroxy ethyl) thiazole as obtained from Merck and DuPont was yellow and gave a strong carbonyl peak when examined by infrared analysis. Redistillation (108°/0.8 mm) of the pooled products gave a clear, almost colorless, viscous liquid which was now free of carbonyl contamination. The purified product was stored at -20° in a desiccator to hinder oxidation. A portion which was kept at room temperature quickly re-oxidized as evidenced by yellow coloration.

2-Methyl-4-amino-5-bromomethyl-pyrimidyl dihydrobromide was a technical product of Merck and Co. Although one sample proved adequate to synthesize an oxazolium analog of thiamine,

an older preparation used in an attempted condensation with 2-4-dimethyl thiazole was unsatisfactory. Titration with standard base showed it to have two dissociable groups, with pK_as at 2.5 and 6.5, respectively, with the lower pK_a requiring twice as much base as the higher one. Based on three equivalents of base needed the equivalent weight was 145. Attempts to recrystallize the salt have been unsuccessful, partly because of the reactivity of the bromomethyl group with polar solvents and partly because of the extreme hygroscopic nature of the purified salt.

Dihydrothiamine, in which the thiazolium ring carbon-nitrogen double bond is reduced, was prepared by the action of sodium trimethoxyborohydride on the free base form of thiamine according to the procedure of Bonvicino and Hennessy (60). The crude crystals from this preparation were converted directly to the dihydrobromide salt without further purification by use of the following procedure: 3.55 gm (0.0224 M) of the crude dihydrothiamine was placed in a 100 ml beaker and dissolved in 5.7 ml (0.05 M) of 48% hydrobromic acid. Immediately thereafter 60 ml of absolute ethanol was added and the solution rapidly filtered. The dihydrobromide salt crystallized out in the filter flask. The crystals were filtered, washed twice with absolute ethanol and recrystallized by dissolving in a minimum of water (ca. 3 ml), adding 40 ml of ethanol and cooling at 2° overnite. The yield was 1.15 gm of white crystals which melted at

199-201°. Additional crystals could be obtained by adding small quantities of ether and cooling.

Dihydrothiamine, besides having the expected structure, can also exist in two additional isomeric forms, all with distinctive melting points. These two isomers are supposedly the cis and trans cyclic ethers formed by the addition of the β-hydroxy group to the double bond of the newly formed thiazoline ring. It was originally assumed that the structural determinations by Bonvicino and Hennessy were correct, but Hirano, et al. (61) have since then presented evidence in opposition to these findings. Therefore, the exact structure of the dihydrobromide salt is not known with certainty.

Tetrahydrothiamine was prepared in a similar manner except that sodium borohydride was used as the reducing agent (60). The yield was 90% for a product melting at 135-138° (uncorrected); theory = 145°. 2.0 gm (0.008 M) of this material was converted to the dihydrobromide salt by the addition of 3.2 ml (0.028 M) of 48% hydrobromic acid. The salt crystallizes out immediately and was subsequently recrystallized by dissolving in 4 ml of water, adding 60 ml of absolute ethanol and cooling for two days at 2°. Yield = 2.2 gm (52%); Melting pt. = 226-232°, Theory = 226°; Neut. eq. = 211, Calcd. 211; % Br = 38.6, Calcd. = 38.1.

Salts of 4-methyl-5-(2-hydroxyethyl) thiazole - The 3-methyl, 3-o,m,p-nitrobenzyl, 3-benzyl, 3-α-methylbenzyl,

and 3-cyanomethyl salts of 4-methyl-5-(2-hydroxyethyl) thiazole were prepared essentially by the method of Clarke (62). The 3-methyl and 3-benzyl salts were prepared by D. E. Metzler (63). The 3-o,m,p-nitrobenzyl salts were prepared according to Livermore and Sealock (64).

The 3-α-methylbenzyl salt was prepared by dissolving 0.04 moles each of α-bromoethyl benzene (Eastman, redistilled 89°/15 mm) and 4-methyl-5-(2-hydroxyethyl) thiazole in 4 ml of thiophene free anhydrous benzene. The solution was heated overnight at 55°. The α-methylbenzyl salt separates out as a light yellow syrup. Attempts to crystallize this compound from a number of solvent systems were all unsuccessful. During crystallization attempts in hot absolute ethanol the salt was partially solvolyzed to the pyrimidylmethyl ethyl ether and 4-methyl-5-(2-hydroxyethyl) thiazolium hydrobromide. Nevertheless, the absorption spectra and the titration behavior very closely parallel that of the 3-benzyl salt. Determination of the % bromide shows that the oil is about 93% pure. These properties plus the method of preparation leave little doubt as to the character of the product.

The 3-cyanomethyl salt was prepared in a similar manner. 0.04 mole quantities of chloracetonitrile (Eastman white label, redistilled 24°/27 mm) and 4-methyl-5-(2-hydroxyethyl) thiazole were dissolved in 5 ml of thiophene-free anhydrous benzene in a test tube. The solution was well mixed,

stoppered and heated at 80° for 3 days. The benzene supernatant was discarded and the red resinous layer which remained was triturated twice with anhydrous ethyl ether to remove as much as possible of the starting materials and benzene solvent. The gummy mass remaining was dissolved in 25 ml of absolute ethanol and treated twice with ca. 0.5 gm of charcoal (Darco) to remove the polymeric contaminants. A clear yellow solution remained. Anhydrous ethyl ether was added to the point of incipient turbidity and the solution cooled overnight at 2°. The resulting dense, bright yellow crystals were filtered and washed with cold 50/50 ethanol and ethyl ether. The washed crystals were recrystallized again from ethanol and ethyl ether, washed and dried in a vacuum desiccator to give a yield of 3.23 gm. Repeated recrystallization and charcoal treatment did not remove the yellow color, though absorption spectra in both acid and base showed only negligible absorption above 300 mμ. Chromatography on Dowex 50 (X4) exchange resin (200 to 400 mesh) with 0.2 N hydrochloric acid as the developing solvent gave a single peak which was still yellow when concentrated. Infrared spectra revealed a weak carbonyl peak which probably belonged to a small amount of yellow contamination. The melting pt. was 132-133° (uncorrected). Analysis: Calcd. for $C_8H_{11}ClN_2OS$: C, 44.54; H, 5.14; Cl, 16.22; N, 12.99. Found: C, 43.93; H, 5.07; Cl, 15.9; N, 12.81.

In addition, titration with standard base (Figure 4) required the theoretical two moles of base, with the first mole being taken up very slowly. However, unlike thiamine there are two distinct breaks in the curve. This indicates that the pseudo-base is a discernable intermediate and that the pK_as for both steps may be obtained. They are 7.2 and 9.3. This is the first case where thiazolium salts have behaved in such a manner, and demonstrates markedly the strong inductive effect of the cyanide grouping. This latter effect on the basicity of the thiazolium nitrogen lowers the equilibrium constant for pseudo-base formation some 1,000 to 10,000 fold under that of similar compounds substituted with aromatic or aliphatic groupings.

Interestingly enough, however, infrared spectra failed to reveal any cyanide peak in the customary region of 4.5μ. A check of the literature shows that this is not uncommon for compounds in which the cyano group is attached to a carbon atom substituted by an electro-negative element. For example, the meso form of the dicyanohydrin of biacetyl gives a very weak -C≡N band and if the hydroxyl groups are esterified even this weak absorption disappears (65). As a further check 3-cyanomethyl-4-methyl thiazolium chloride was synthesized (see below). This salt, too, gave no -C≡N peak with infrared. Titration of this compound led to an unexpected result. Addition of a small amount of base gave a flocculent white precipitate which continued to drop out as

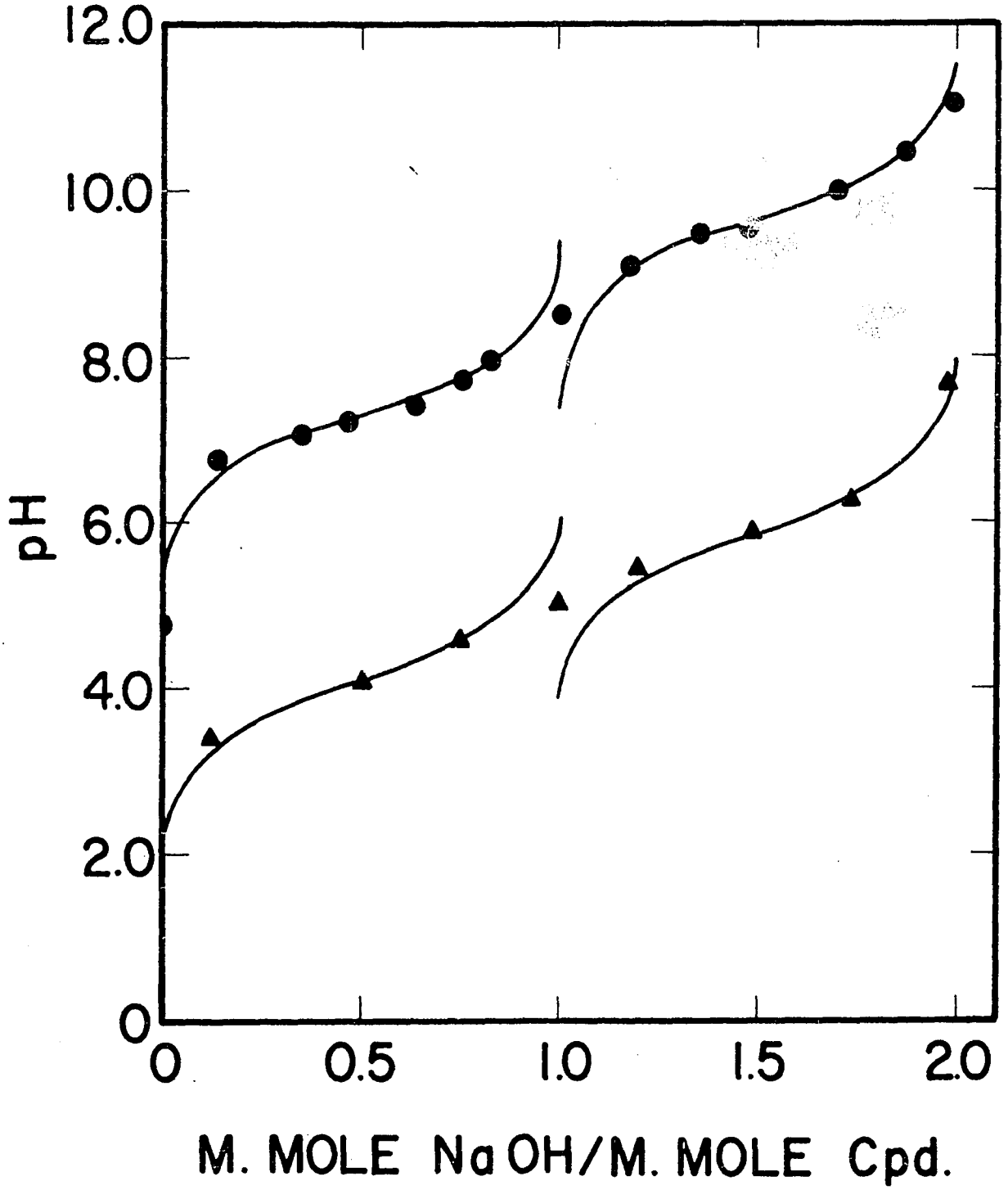


Figure 4. Titration curves of thiamine analogs

3-cyanomethyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride

4-methyl oxazolium analog of thiamine

more base was added. This precipitate is undoubtedly the pseudo base form of the thiazolium ring. Unlike the other cyanomethyl analog it does not have the additional hydroxyl group to help keep it in solution. Surprisingly enough the pseudo base is only sparingly soluble in excess base and then only with considerable "yellowing". A small amount of the white pseudo base was prepared by adding one equivalent of base to 50 mg of 3-cyanomethyl-4-methyl thiazolium chloride, filtering, washing with water and drying. Infrared spectra now showed a sharp band at 4.55μ indicating clearly the presence of the -C≡N grouping. Evidently the thiazolium nitrogen must be in the quaternary form before the aforementioned "quenching" of the -C≡N stretching takes place.

3-Cyanomethyl-4-methyl thiazolium chloride was synthesized by essentially the same procedure as that used for the other 3-cyanomethyl analog. The 4-methyl thiazole used was prepared by the method of Kurkjy and Brown (66). One run using 0.04 M quantities gave 0.75 gm of white crystals after two recrystallizations. The compound darkened at 225° and melted with decomposition at 230°. Calcd. for $C_6H_7ClN_2S$: C, 41.50; H, 3.48; Cl, 20.4; N, 16.17. Found: C, 41.40; H, 4.11; Cl, 20.3; N, 15.35.

O-hetero thiamine [3-(2-methyl-4-amino-5-pyrimidyl-methyl)-4-methyl-5-(2-hydroxyethyl) oxazolium chloride] was generously given to us by Dr. John F. Codington of the Sloan-

Kettering Institute for Cancer Research. Titration showed it to be about 75% pure.

3-(2-methyl-4-amino-5-pyrimidylmethyl)-4-methyl oxazolium bromide - This 4-methyl oxazolium analog of thiamine was prepared in the following manner: 4-methyl oxazole was prepared by the method of Cornforth and Cornforth (67). 1.66 gm (0.02 M) of 4-methyl oxazole was dissolved in 3 ml of freshly distilled n-butanol in a small test tube. This solution was heated to 95-100° and 2.50 gm (0.067 M) of 2-methyl-4-amino-5-bromoethyl pyrimidine dihydrobromide (Merck, technical grade) was added in small portions with mixing. After all the pyrimidine salt was added the test tube was tightly stoppered and heated for 30 minutes more at 125°, shaking at 4-5 minute intervals. The solution was cooled and the crystals which formed were filtered, washed with butanol and then ether. The washed crystals were dissolved in a minimum amount of water, 95% ethanol was added to the point of incipient turbidity, and the solution was cooled to 2° for two days. The resultant crystals were filtered, washed three times with cold ethanol and recrystallized again. This procedure yielded 0.23 gm of small, light tan crystals which melted with decomposition at 235° (uncorrected). Titration of this compound (Figure 4) takes up two equivalents of base and has pKs of 4.1 and 5.8. The first pK_a is undoubtedly for the 4-aminopyrimidine grouping and the higher pK_a for the formation of the pseudo base. It is not known whether the

ring opens similar to the thiazole ring or not. Analysis: Calcd. for $C_{10}H_{14}Br_2N_4O$: C, 32.88; H, 3.86; N, 15.3; Br, 43.66. Found: C, 32.84; H, 4.04; N, 15.3; Br, 43.7.

3-(3-aminopropyl)-4-methyl thiazolium chloride and 3-(4-amino-butyl)-4-methyl thiazolium chloride were from the collection of the late R. R. Sealock and were prepared by H. Sarver (68) using the method of Clarke (62).

3-benzyl-4-methyl thiazolium chloride was also from Sealock's collection and was prepared by P. L. White. Its titration behavior was strikingly similar to that of the 3-cyanomethyl-4-methyl thiazolium salt. The first addition of base gave rise to a white precipitate, the pseudo base, which unlike the pseudo base form of the 3-cyanomethyl salt, redissolves on standing to give the open ring thiol salt. The titration curve which results is the theoretical one for a two proton dissociation with no detectable monoprotic intermediate. The pK_{av} is 8.8, considerably lower than the 9.96 value of a similar compound, the benzyl analog of thiamine. There is no obvious reason for the large difference in these pK_{av} values.

III. RESULTS AND DISCUSSION

When pyruvate is incubated with thiamine in a slightly basic medium a small amount of the pyruvate is rapidly decarboxylated to give a thiamine-acetaldehyde intermediate. Depending on the other reactants present, a variety of products may result. The reaction of this intermediate with a proton, another pyruvate or acetaldehyde gives acetaldehyde, α-acetolactate or acetoin, respectively, as products. This has previously been depicted in equation 8. Since under the test conditions used, the concentration of pyruvate was many times higher than the concentration of hydrogen ions, no acetaldehyde was ever found in the reaction mixtures. α-acetolactate and acetoin were the only products ever detected.

Thiamine also catalyzes the direct acyloin condensation of two molecules of acetaldehyde to acetoin. However, acetaldehyde reacts much more slowly than pyruvate. When approximately 0.2 M acetaldehyde was incubated with 0.02 M thiamine at pH 9.2 in 0.08 M pyrophosphate buffer for two hours at 40°, only 0.1 millimolar acetoin was formed. This is only about 6% of that formed from 0.2 M pyruvate under comparable conditions. Breslow (48) reports that under Mizuhara's test conditions (39) acetaldehyde was 20% as effective as pyruvate in forming acetoin. Since these latter test conditions involve incubation for 40 hours, our lower yield undoubtedly is a reflection of the time lag required

for accumulation of sufficient intermediate to react with the second acetaldehyde molecule. The evidence for such an intermediate is given in the following section.

A. Kinetics and Evidence for an Unstable Intermediate

Early studies by E. Manzo (69) in this laboratory had shown that carbon dioxide production always exceeded acetoin production. In addition, the evolution of carbon dioxide was rapid and measurable after 15 minutes, while acetoin production lagged behind and was almost negligible up to one hour. This phenomenon was reinvestigated more carefully, and the results are given in Figure 5. Some of the kinetic runs were made in borate buffer, since it had been discovered (44) that borate, in contrast to other buffers, seemed to have a stimulatory effect on acetoin production. For example, compare the yields of acetoin in curve C (borate) with that in curve B (triethanolamine). It was later found that borate was simply functioning as a catalyst to decarboxylate α-acetolactate to acetoin. Other runs were made in triethanolamine buffers and the acetoin was assayed both with and without acid treatment. The acid treatment (see experimental section) served to decarboxylate any α-acetolactate remaining which had not been decarboxylated spontaneously. All acetoin values were multiplied by two, since two molecules of pyruvate must be decarboxylated to yield one of acetoin. It should

Figure 5. Acetoin (X2) and carbon dioxide produced by incubation of pyruvate with thiamine at pH 8.9, 40°

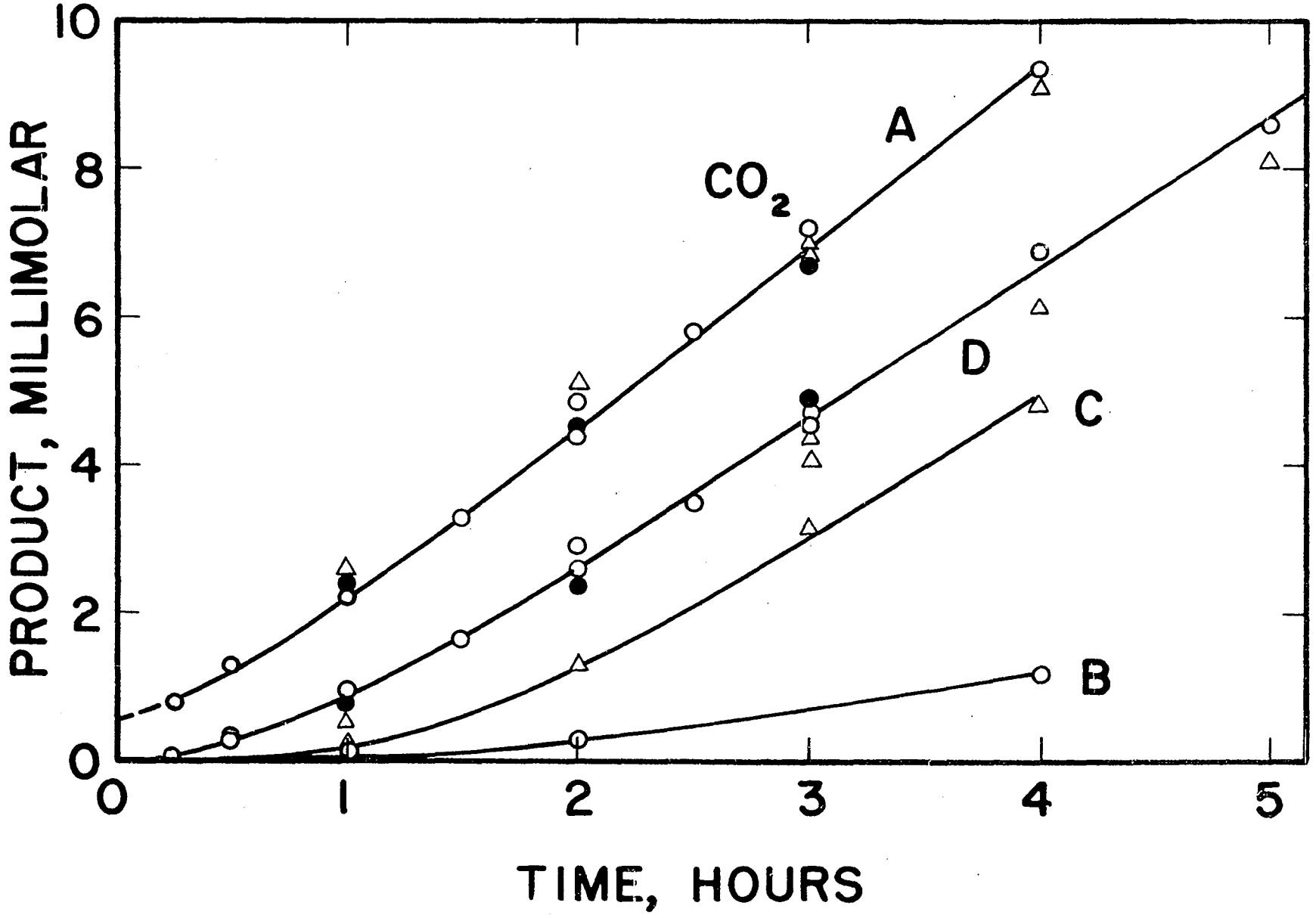
○ , ● , triethanolamine-buffered

△ , borate-buffered

○ , △ , 0.2 M pyruvate + 0.02 M thiamine

● , 0.4 M pyruvate + 0.01 M thiamine.

A, carbon dioxide evolved after acid treatment, B and C, acetoin without acid treatment. D, acetoin after acid treatment to decarboxylate α-acetolactate.


PRODUCT, MILLIMOLAR
TIME, HOURS
0
2
4
6
8
10
0
1
2
3
4
5
A
CO_2
D
C
B

be noticed that the final rate of carbon dioxide production is higher than that of the acetoin. This amounts to about a 17% difference in rate and has been attributed to decarboxylative reactions of thiamine with pyruvate which do not yield acetoin. As stated in the experimental methods, pyruvate does not decarboxylate by itself under these conditions.

The characteristic lag in acetoin production together with the rapid carbon dioxide evolution is indicative of the accumulation of one or more reactive intermediates. The kinetics of the reaction can be described approximately by assuming two consecutive first order reactions. The equations for this simply case (70) can be fitted to the data remarkably well, except at short reaction times. Initially the reaction is represented by the equation:

$$\text{pyruvate} + \text{thiamine} \longrightarrow \text{intermediate} + CO_2$$

After longer times, acetolactate appears and is partially decarboxylated to acetoin. Thus the rate of carbon dioxide evolution should increase with time. In the steady state the carbon dioxide evolution rate should be equal to about twice the initial rate corresponding to the overall reaction

$$2\ \text{pyruvate} \longrightarrow \text{acetoin} + 2\ CO_2$$

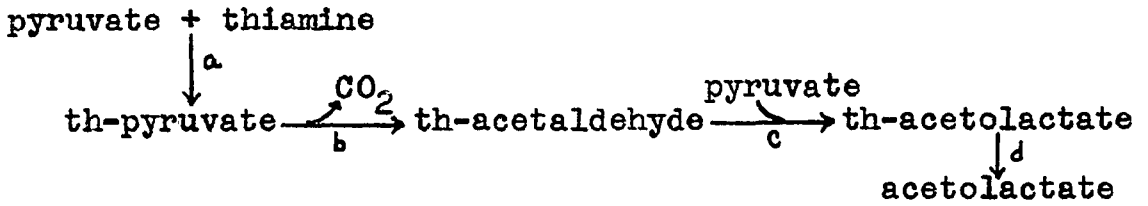
Actually, the observed CO_2 evolution rate is rapid at the start but quickly falls off to a lower steady-state level. It was felt that this initial fall off in rate might result

from some reversible reaction of thiamine with pyruvate to give catalytically inactive forms. For example, the amino group of thiamine or the sulfhydryl group of the thiol form of thiamine might react with pyruvate. However, titration of thiamine in the presence of a 30-fold excess of sodium pyruvate has given no indication of any such interaction. In addition, the spectrum at pH 9.2 of 5×10^{-5} M thiamine in a 1.5×10^{-3} M pyruvate solution is equal exactly to the sum of the spectra of separate solutions of the same concentrations.

Another possible explanation is the following. If there was a small impurity in the sodium pyruvate which was rapidly decarboxylated on mixing with thiamine at pH 9, then all of the carbon dioxide values except the zero time blank would be too high. If the CO_2 curve were transposed down about 0.5 millimolar units, a curve with the predicted lag period results. This 0.5 millimolar impurity represents about 0.25% of the pyruvate present. Since the sodium pyruvate is known to have a slight impurity (see chemicals and reagents) perhaps the result observed is not too surprising.

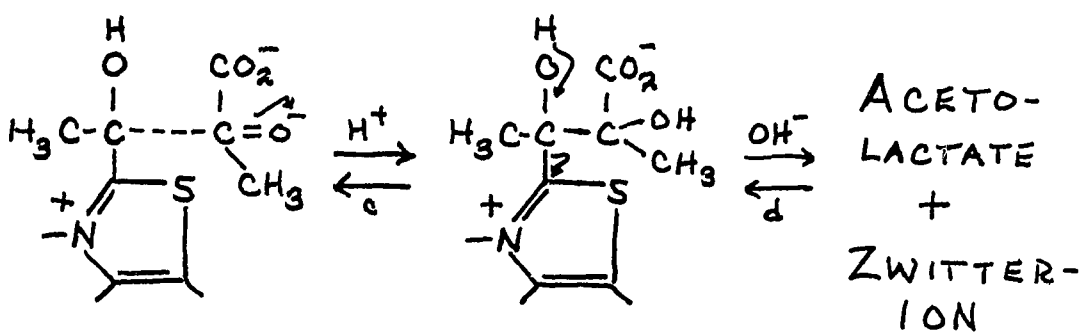
Nature of the Reactive Intermediate - Of interest now is, what is the nature of the intermediate which accumulates? At least four steps must be present in the overall reaction as shown in equation 10.

(10)



Pyruvate and thiamine react in step a to form some type of thiamine-pyruvate compound which can then decarboxylate in step b. Either step a or step b may be rate-limiting with respect to the initial CO_2 evolution. The second rate-limiting step could be either c or d, and the kinetically recognizable intermediate either a thiamine-acetaldehyde or a thiamine-acetolactate compound.

A look at the intimate details of the portion of the catalytic cycle dealing with the proposed intermediates might be revealing. As depicted below in step c, the reaction of thiamine-acetaldehyde with pyruvate requires the addition of a proton and therefore should be favored at lower pHs. However, step d, the breakdown of thiamine-acetolactate to products, requires the removal of a proton and should decompose at a faster rate under basic conditions.



Thus, lowering the pH should favor the formation of thiamine-acetolactate while at the same time retarding its breakdown. Raising the pH would increase the rate with which the acetolactate intermediate breaks down but would also slow step c, the rate of its formation. A study of the kinetics of these reactions versus pH should help decide if the above reasoning is valid.

Rate of Acetoin Production Versus pH - It was found possible to study the steady state pH-rate dependence of acetoin production from pH 7.5 to 9.0. Below pH 7.5 the rate is so slow that it precludes measurement. At pH 9.0 the steady state rate falls off after five hours, presumably because of decomposition reactions affecting both the pyruvate and thiamine. Pyruvate is known to undergo a variety of base catalyzed reactions (71) and thiamine is subject to hydrolytic cleavage at the thiazolium nitrogen in basic solutions. However, at lower pHs, once steady state conditions are reached, the rate is constant for at least 10 hours and at pH 8.0 and below for 30 hours. The steady state rates for 0.2 M pyruvate and 0.02 M thiamine in 0.1 M triethanolamine buffer at four different pHs are given in Table 5. The pH values were found to be essentially constant over the time ranges studied. The intermediate concentration was determined graphically by taking the differences between the line representing the steady state rate and a parallel line drawn through the origin representing the theoretical rate if there

Table 5. Effect of pH on rate of acetoin production

pH	Rate (mM/hr.)	Intermediate concentration (mM)	K_1 (hr^{-1}) ($\times 10^3$)	K_2 (hr^{-1})
7.47	0.074	0.22	0.37	0.34
8.0	0.25	0.40	1.25	0.62
8.47	0.56	0.53	2.8	1.06
9.0	1.10	0.60	5.5	1.83

were no intermediate. A typical curve used for determination of the intermediate concentration is shown in Figure 6.

K_1 was shown to be first order with respect to pyruvate and thiamine and the values given in Table 5 were calculated by dividing the rate of acetoin production by the pyruvate concentration. A plot of the log of K_1 versus pH (Figure 7) shows a linear relationship of slope one between pHs 7.5 and 8.0, but at higher pHs, K_1 falls off from linearity. This is the type behavior to be expected if the rate limiting step is the formation of a zwitter-ion from the thiazolium ion form of thiamine. Below a pH of 8.0, 99% of the thiamine is in the thiazolium ion form, but as the pH rises more and more of it is converted into the inactive thiol form. At the pK_{av} approximately half of the thiamine present is in the thiol form. If corrections are made for this "draining off of

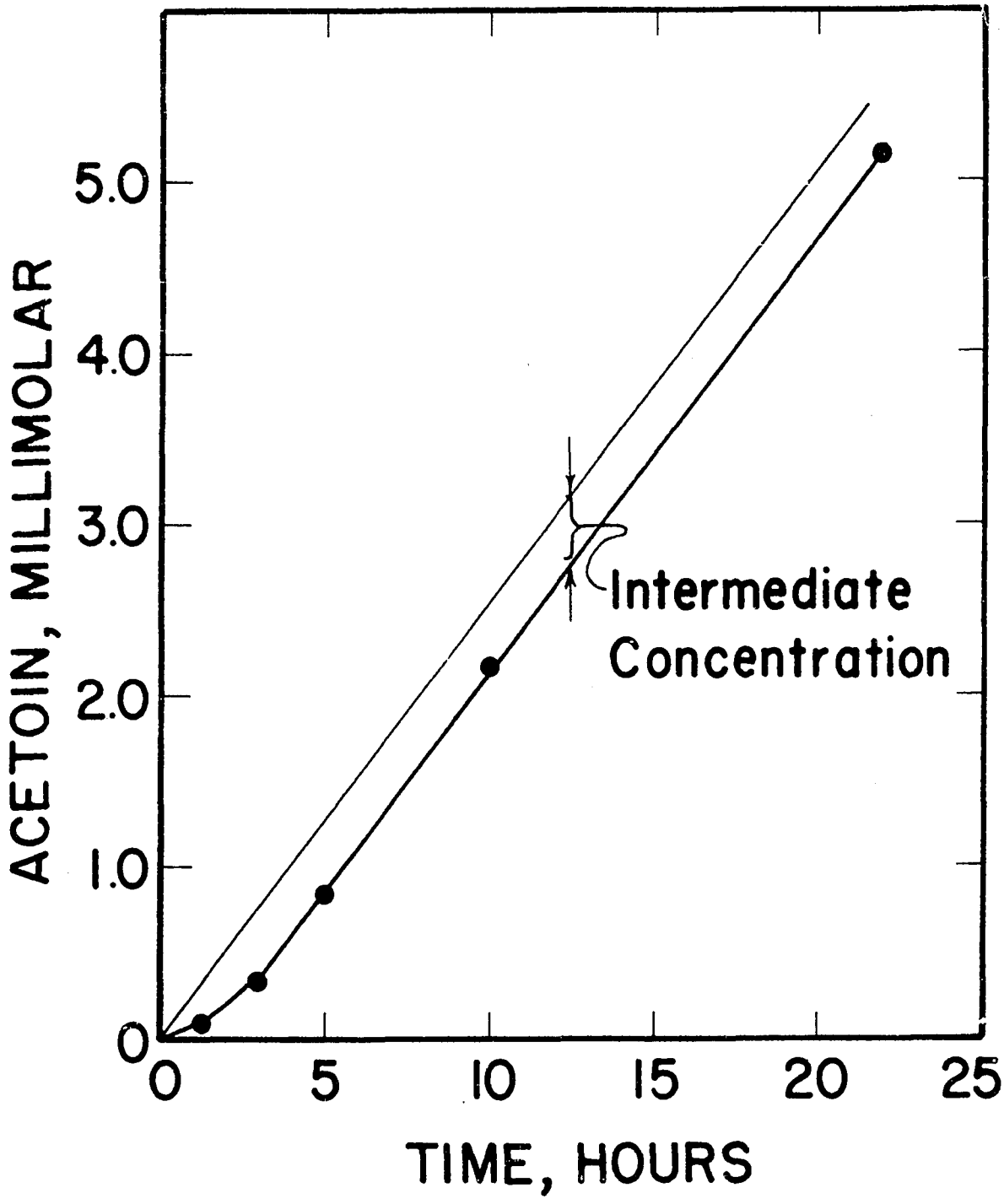


Figure 6. Acetoin produced at pH 8.0, 40°

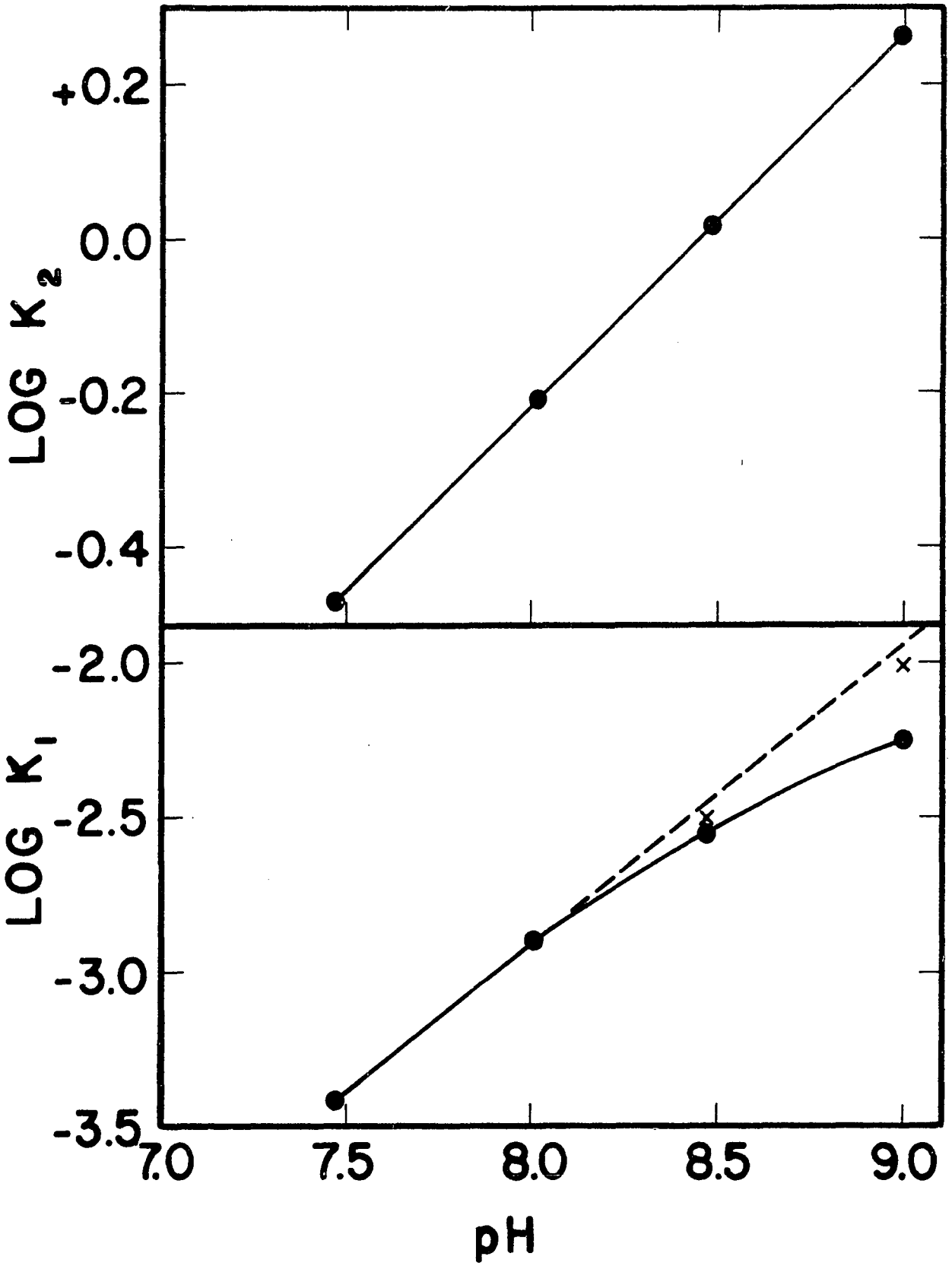
●, 0.2 M pyruvate and 0.02 M thiamine in 0.1 M triethanolamine buffer.

Straight line through origin represents the theoretical rate if there was no intermediate.

Figure 7. Effect of pH on kinetic constants K_1 and K_2 (see text)

Reaction mixtures were 0.2 M pyruvate and 0.02 M thiamine in 0.1 M triethanolamine buffer.

$\times$, corrected for thiamine converted to inactive thiol form.


+0.2
0.0
-0.2
-0.4
LOG K_2
-2.0
-2.5
-3.0
-3.5
LOG K_1
7.0
7.5
8.0
8.5
9.0
pH

catalyst", the corrected points lie close but not exactly on the extended line of slope one. This probably means that the above explanation is an oversimplification and that at these higher pHs other factors enter in.

In addition, K_2, which is the rate of acetoin production divided by the intermediate concentration, is also directly dependent on hydrogen ion concentration over the pH range tested. However, the slope of the log K_2 vs. pH (Figure 7) is not one but 0.5. This type of pH-rate dependence has no meaning mechanistically since it indicates catalysis by one half a hydroxide ion. Kinetically this type of dependence may be rationalized a number of ways. One such rationalization[1] which takes account of the proton required in step c and the hydroxide ion required in step d gives a final expression of Rate = $\frac{K' [OH]}{1 + K'' [OH]}$. If K' is factored out and K" [OH] is set equal to one at the midpoint of the pH range of linearity for log K_2, i.e., about pH 8, K" has a value of 10^6. The expression of Rate (pH 8)/K' = 0.5×10^{-6}. The values at pH 7 and pH 9 are 0.091×10^{-6} and 0.91×10^{-6}, respectively. Thus a pH change of two units gives a ten fold change in K" and the resulting slope of the line representing the pH dependence of log K" is 0.5 between pH 7 and 9. However, at pHs higher or lower than this the dependence deviates from linearity to give an overall S shaped curve. If

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[1]The author wishes to acknowledge the suggestion of Dr. Robert Hansen for this possible solution.

it were experimentally possible to measure K_2 for a wider pH range, it would be interesting to see if the above expression correctly defines its pH dependence. As it stands now, it may only be said that a kinetic relationship has been found which fits both the portrayed mechanistic picture and the observed pH dependence of K_2 over the pH range studied.

An additional experiment was performed to help distinguish if step c or step d of equation 10 was the rate limiting step. It was thought that if step c was rate-limiting, the addition of more pyruvate after steady state conditions had been reached, should rapidly give rise to an increase in products. If the break-down of the thiamine-acetolactate was rate limiting, the additional pyruvate would increase the rate, but only after a suitable lag period during which time more acetolactate intermediate could build up. 0.2 M pyruvate and 0.02 M thiamine in 0.1 M, pH 9.1 triethanolamine buffer (5 ml total volume) were incubated in a special reaction flask for 4 hrs. at 40°. At this time 1 ml of 2 M pyruvate was added and the increase in acetolactate and acetoin measured. (All the acetoin values after 4 hrs. were multiplied by a dilution factor of 6/5.) The results shown in Figure 8 would seem to support the proposition that the thiamine-acetolactate breakdown is the rate limiting step.

Reaction of Acetaldehyde with Accumulated Intermediate - The nature of the intermediate was also studied by its reaction with acetaldehyde. The experiment was conducted in the

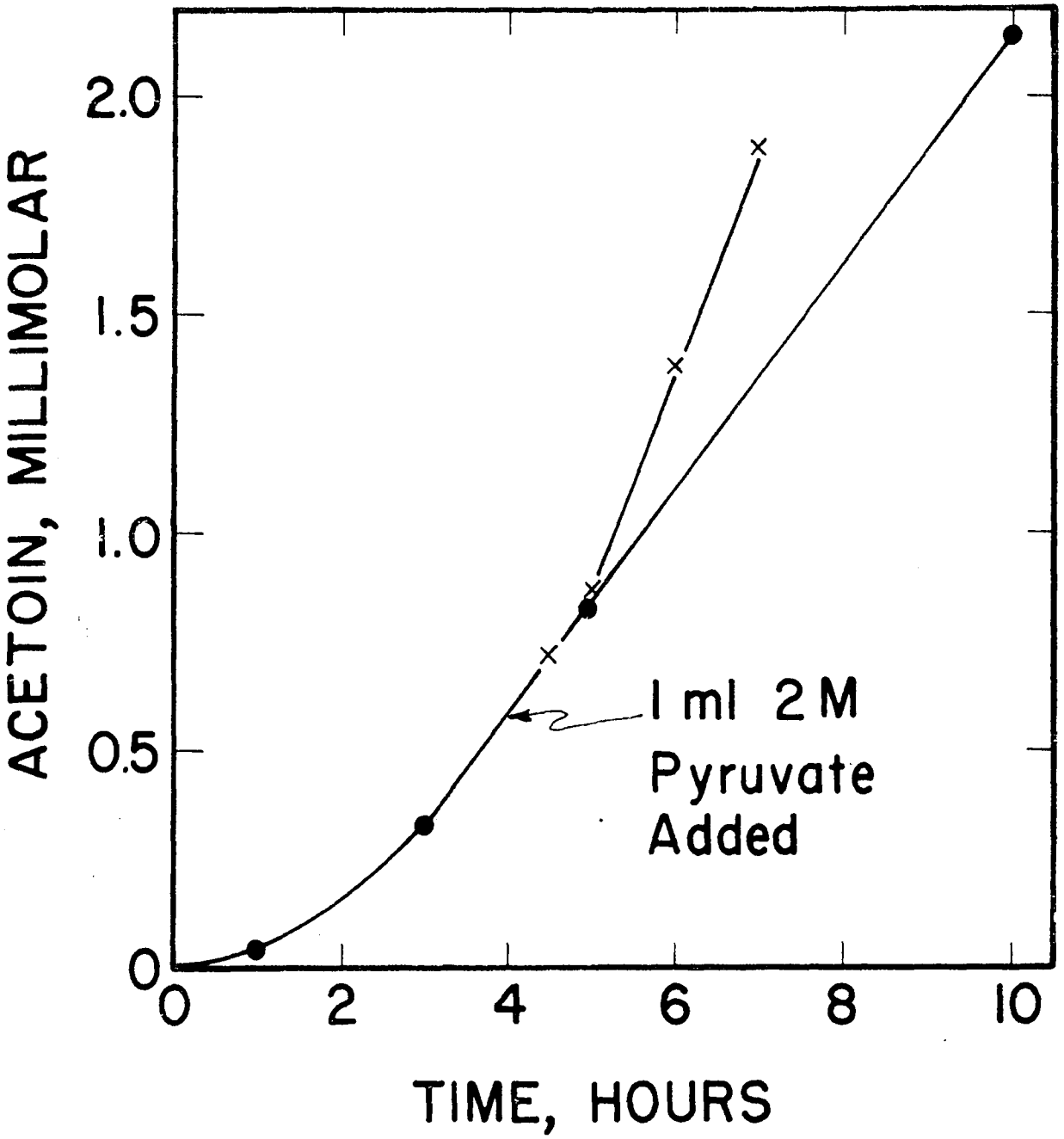


Figure 8. Effect of added pyruvate on rate of acetoin production

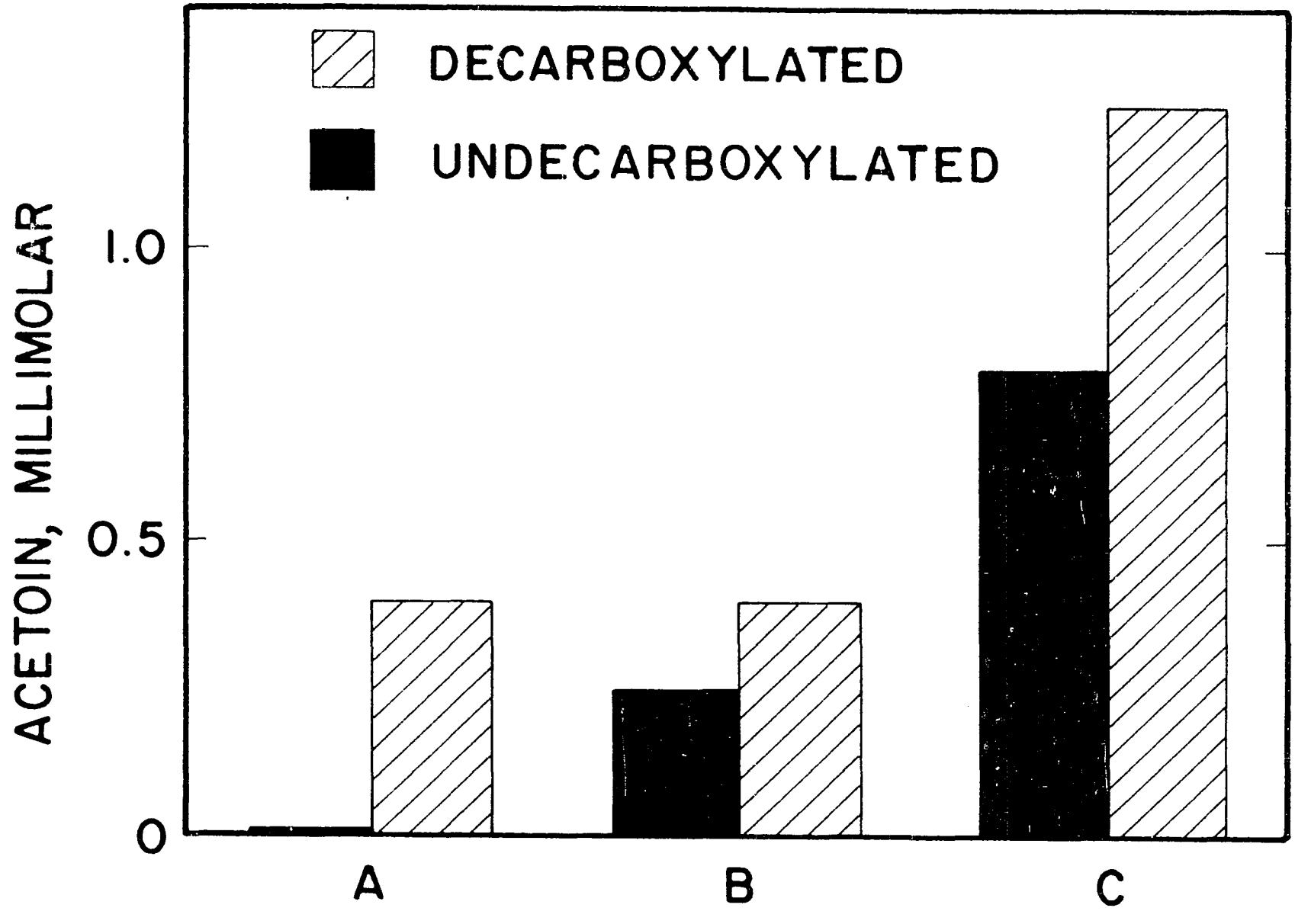
●, 0.2 M pyruvate and 0.02 M thiamine in 0.1 M triethanolamine buffer, pH 8.0. Total vol = 5 ml.

×, acetoin values (x 6/5) after addition of 1 ml of 2 M pyruvate to a duplicate sample of the above concentrations.

following manner: Pyruvate (0.2 M) and thiamine (0.04 M) were incubated in 0.08 M pyrophosphate buffer, pH 9.2, 40° for 30 minutes. Enough acetaldehyde to give a concentration of 0.2 M was then added to one sample (C) while to another (A) was added a comparable amount of water. Sample A was then placed in an ice bath to hinder further reactions. Sample C was incubated another 30 minutes and acetoin was determined without decarboxylation. Still a third sample (B) contained pyruvate, thiamine and acetaldehyde, all present initially and incubated for a total of 30 minutes. As shown in Figure 9, the amounts of acetoin (solid bar), formed in sample C far exceeded that in samples A and B combined. These results are interpreted simply if we assume that a thiamine-acetaldehyde intermediate accumulates in the first 30 minutes in sample C (a thiamine-acetolactate compound in equilibrium with a thiamine-acetaldehyde compound would be an equally possible intermediate). When acetaldehyde was added some of the intermediate reacted with it to give acetoin directly. In sample A the small amount of acetoin formed came indirectly through decarboxylation of acetolactate. In sample B a relatively small amount of acetoin formed because the intermediate had to accumulate before condensation with acetaldehyde could take place. If no intermediate existed the sum of the acetoin yields of samples A + B should equal that of sample C.

Figure 9. Reaction of acetaldehyde with accumulated intermediate

All samples contained 0.2 M pyruvate, 0.04 M thiamine and pyrophosphate buffer, pH 9.2 and were incubated for 30 or 60 minutes at 40°. Acetoin was measured directly as well as after acid treatment to decarboxylate acetolactate. A, 30 minutes, with no acetaldehyde; B, 30 minutes, with 0.2 M acetaldehyde; C, 60 minutes, with acetaldehyde (to give 0.2 M) added at 30 minutes.


DECARBOXYLATED
UNDECARBOXYLATED
ACETOIN, MILLIMOLAR
1.0
0.5
0
A
B
C

From this experiment it is also possible to compare the relative efficiencies of pyruvate and acetaldehyde as "acceptors" for the intermediate. This can be judged roughly by the amounts of α-acetolactate (mM) and of acetoin (mM) formed during the last 30 minutes of incubation of sample C. The α-acetolactate value was calculated by subtracting the sum of solid bar C and lined bar A from lined bar C in Figure 9. The acetoin value was taken as just equal to solid bar C since little acetoin *per se* is produced in one hour without acid treatment (see Figure 5) and acetaldehyde alone as a substrate gives very little acetoin. These values show that acetaldehyde is much superior to pyruvate as an "acceptor". A strikingly analogous behavior has been demonstrated by Juni and Heym (72) for pyruvic acid oxidase preparations from pigeon breast muscle. In this case, addition of acetaldehyde enhances the production of acetoin some twelve-fold and lowers the α-acetolactate formation. The α-acetolactate that is formed is optically inactive. Thus, this enzyme, as in the model system, exhibits no sterospecificity for the acceptor molecules. Also like the model system, acetaldehyde alone is a relatively poor substrate for the formation of acetoin.

Stability of the Intermediate - It was felt that if the intermediate was a thiamine-acetaldehyde compound it might be possible to break it down to acetaldehyde by treating with acid. However, repeated attempts to obtain acetaldehyde by either acid or base treatment have failed. At most less

than 2% of the intermediate has been obtained as acetaldehyde. Perhaps this is not too surprising in view of the pH dependence of the equilibrium between the thiamine-acetaldehyde and the thiamine-acetolactate compounds. Because of this dependence, as the pH of a reaction mixture is lowered the condensation reaction between pyruvate and thiamine-acetaldehyde is favored. If the pH is low enough the thiamine-acetaldehyde intermediate may react with a proton in place of pyruvate. However, since the hydroxide concentration is so low, the decomposition of either proposed intermediate should be very slow. It is possible, for example, to perform the following experiment. A reaction mixture similar to the one described for the reaction of the intermediate with acetaldehyde is incubated for 30 min. at pH 9.0, acid is added to lower the pH to 2.0 or below, and the mixture is allowed to stand for 3 hours at room temperature. Now if the pH is readjusted to 9.0 and acetaldehyde is added, the same stimulation of acetoin production is observed as that recorded in Figure 9. If the intermediate is allowed to stand under acidified conditions for periods of 24 hours or longer, addition of acetaldehyde gives a much diminished stimulation of acetoin production. Nevertheless, it would seem that the best chance for isolating this intermediate would be under acid conditions, in which some rapid method of separation could be used.

Attempts at Isolation of Intermediate - It seemed that ion exchange resins[1] offered the best possibilities for isolating the intermediate in an amount large enough to study independently. The problem lies in finding a resin which will absorb thiamine or thiamine-like compounds from a fairly acid solution but which will in turn release these compounds on increasing the acid concentration slightly. Sulfonic acid resins absorb thiamine so strongly that it requires 6N HCl to elute it. On the other hand, carboxylic acid resins (e.g., IRC 50) in the completely protonated form will not absorb thiamine at all. In a compromise isolation attempt a mixture of one third IRC 50 sodium form and two thirds acid form were used in a .9 x 150 cm column. A 10 ml, pH 9.0 sample of 0.2 M pyruvate and 0.08 M thiamine which had been incubated 45 min. was added. Elution was with 0.1 N HCl at a rate of 6-10 ml/hr. The effluent was monitored for thiamine and the intermediate by reading the absorption at 240 mμ. The pH of the solution before thiamine came off varied from 7 down to 5. Immediately following the thiamine peak at approximately 600 ml came a smaller trailing peak after which the pH dropped to one. This smaller peak was observed on two other similar runs, but on adding acetaldehyde, adjusting

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[1]A review of the use of ion-exchange resins in vitamin chemistry has been written by Hennessy (73) in Calmon and Kressman's book on the use of ion exchange resins in organic and biochemistry.

the pH to 9.0 and incubating 30 min. at 40°, little or no acetoin resulted from any of these tubes. Because the slow equilibrium rate of the carboxylic acid resins force a slow elution rate, it seems likely that all the intermediate had decomposed before it could be separated, and the trailing peak is undoubtedly some artifact. That the intermediate (probably thiamine-acetolactate at this pH) can decompose at pHs 5 to 7 was shown by allowing it to build up at pH 9.0 and then lowering the pH to the range 5.5 to 7.0 with HCl. A small amount of acetoin (0.4 to 0.7 μM) is slowly formed over a period of 24 hrs. and then levels off. This is of comparable concentration to the intermediate as measured kinetically. This additional production of acetoin is undoubtedly a reflection of the fact that the rate of decomposition does not fall off as rapidly with pH as does the initial rate of reaction of thiamine with pyruvate. Strangely enough, this effect is inhibited by phosphate buffers but not by cacodylate buffers.

Future isolation attempts should be cognizant of the above observations. Perhaps the use of very low cross-linked sulfonic acid resins in conjunction with C^{14} labeled pyruvate offer the best possible combination for isolation and detection of the intermediate. The low cross-linked resin should allow the use of higher acidities to stabilize the intermediate, while the coincidence of thiamine absorption plus

radioactivity should clearly mark the tubes containing the intermediate.

B. pH Optimum of Acetoin Production

One of the first things discovered about thiamine catalyzed acyloin condensations was the distinctive pH dependence of the reaction. Mizuhara *et al.* found from very limited studies (39) that the optimal yield came when two equivalents of base were added per thiamine hydrochloride molecule. The addition of one or three equivalents of base[1] gave much lower yields. Because of this pH-activity behavior Mizuhara proposed his rather implausible mechanism involving the pseudo base form of thiamine (VI). However, other forms of thiamine would also show this type of pH dependence, e.g., the formation of an "ylid" carbanion of Breslow's first mechanism. The finding that the thiazolium C-2 hydrogen was very labile also fits this type of pH dependence. In addition, if the rate limiting step were the formation of the zwitter-ion, it would be expected that the pH optimum would correspond to the pK_{av} of the ring opening reaction. That this is true may be reasoned out from the following set of equilibria (equation 10).

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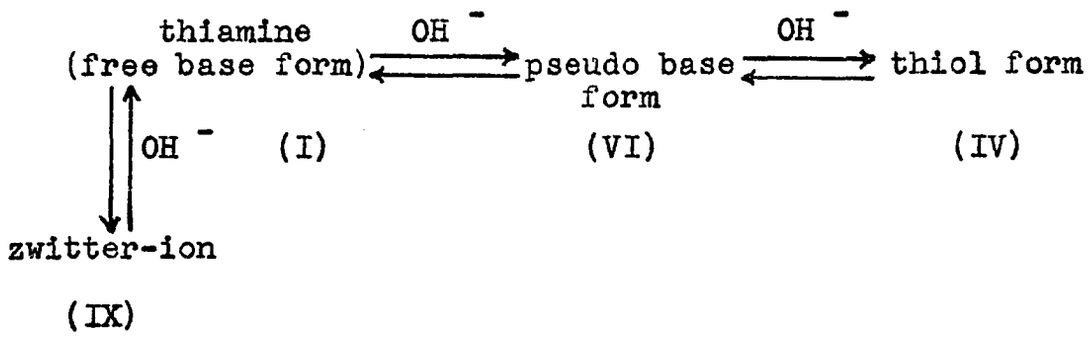
[1]The pH values reported for these samples are curiously low, being 5.8, 8.4 and 9.2 respectively for one, two and three equivalents of base added. More reasonable would be pH values of approximately a pH unit higher.

(10)



Equilibrium constants can be written for the formation of (VI) and (IX), both of which involve the action of one mole of base on the thiazolium ion (I). That is

$$k' = \frac{[VI]}{[OH^-][I]} \quad \text{and} \quad k'' = \frac{[IX]}{[OH^-][I]} \ .$$

These two equations can be combined to give the relationship $k'[IX] = k''[VI]$. From this latter equation it can be seen that the concentrations of the zwitter-ion form (IX) and the pseudo base form (VI) are directly dependent. Since the maximum concentration of the pseudo base is at the pK_{av} of the ring opening reaction, it follows that the concentration of the zwitter-ion will also be maximal here. However, neither form is in high enough concentration to effect the slope of the titration curve of thiamine (74, 75).

Because it seemed likely that the rate limiting step was the zwitter-ion formation, it was of interest to accurately measure both the temperature dependence of the pK_{av}

of the ring opening reaction and the pH optimum of acetoin formation. The pK_{av} of both thiamine and the benzyl analog show an almost linear temperature dependence[1] between 15° and 40° as shown in Figure 10. The pH optima for both thiamine and the benzyl analog are given in Figure 11. It should be noted that the pHs were measured at 25° while the actual reactions took place at 40°. It was found that the pH of the reaction mixtures dropped about 0.2 pH units on warming the solutions from 25° to 40°. Thus the optima for thiamine and the benzyl analog at 40° are 8.9 and 9.6, respectively. These values agree quite well with the measured pK_{av}s at 40° of 8.96 and 9.63, and may be said to furnish further indirect evidence for the zwitter-ion mechanism.

C. Action of Thiamine Analogs on Pyruvate

The ability of a number of thiamine analogs to catalytically form acetoin from pyruvate has been tested. Most of these are derivatives of the thiazole ring, since it had been discovered earlier (43) that the pyrimidine ring was nonessential for activity.

Testing Procedure - The compounds were tested by incubation with sodium pyruvate in evacuated Thunberg tubes at 50°

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[1]Watanabe et al. (75) has published a somewhat different dependence in which he reports values of 9.33 for thiamine at both 15° and 25°.

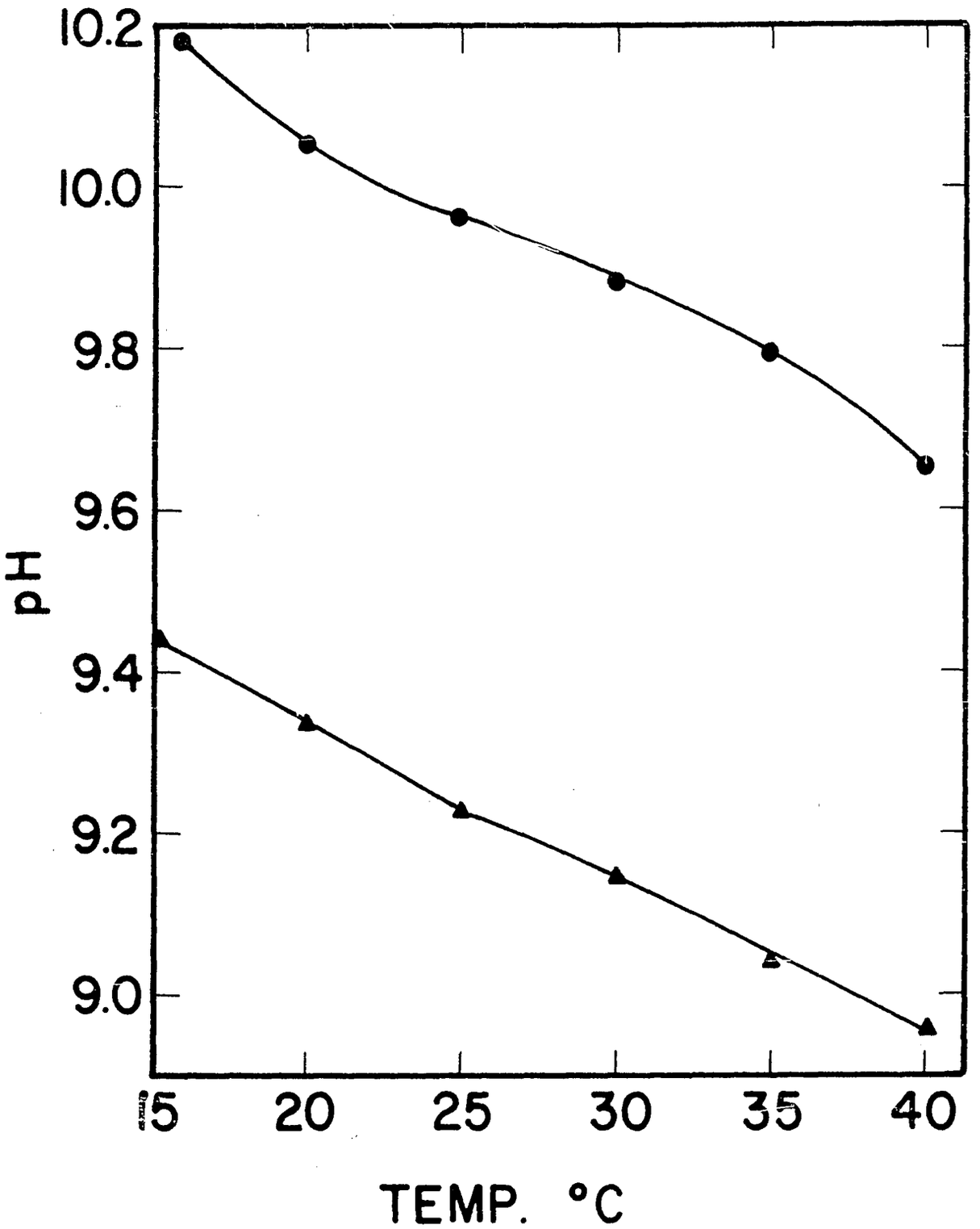


Figure 10. Temperature dependence of the equilibrium constant for thiazolium ring opening reaction

▲, thiamine; ●, 3-benzyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride.

Figure 11. pH dependence of acetoin formation catalyzed by thiamine and 3-benzyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride

Pyruvate (0.2 M) and catalyst (0.02 M) were incubated at 40° for 2 hours and the acetoin measured after acid treatment.

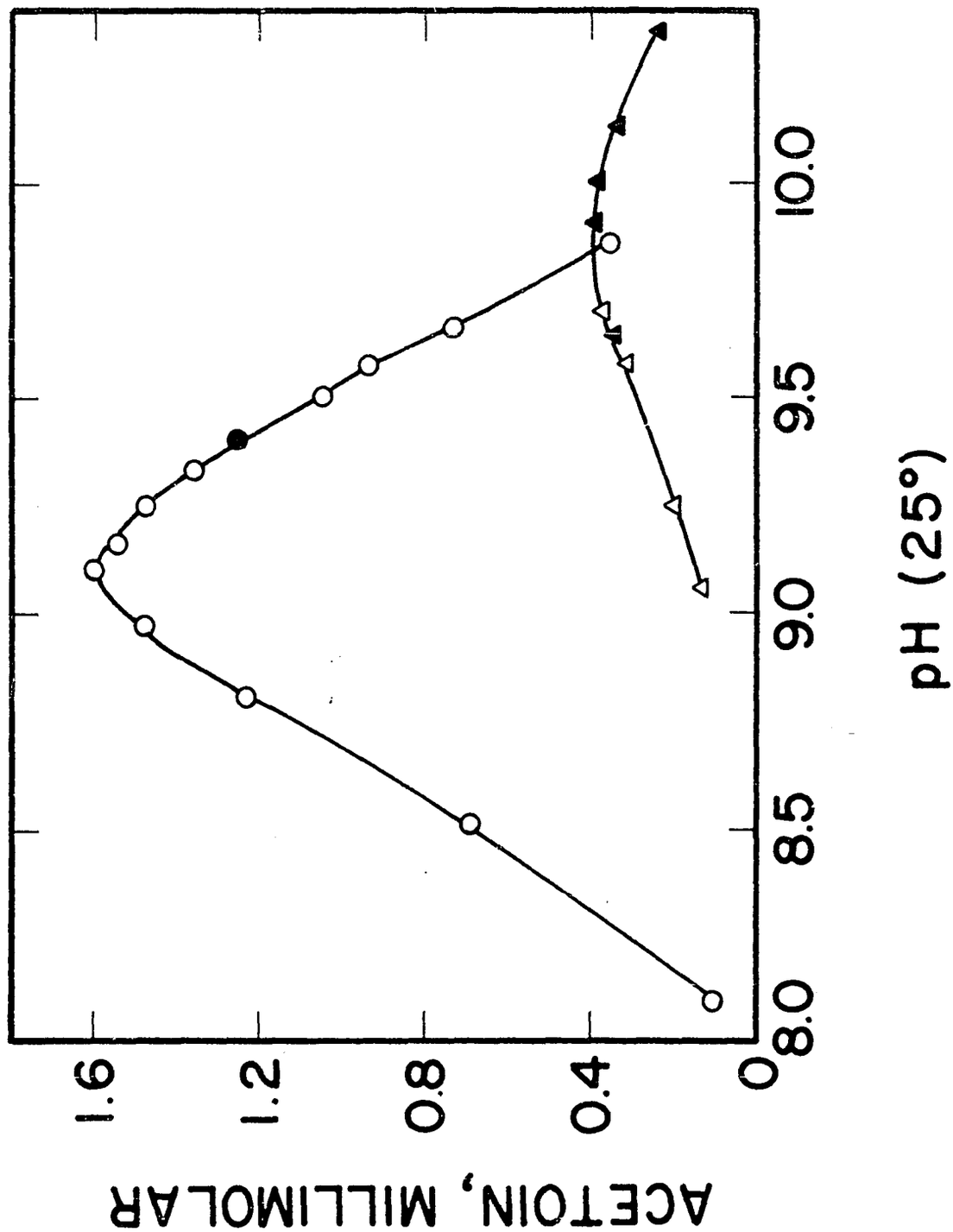
○ , ● , thiamine

△ , ▲ , 3-benzyl analog

○ , triethanolamine buffers

△ , pyrophosphate buffer

● , ▲ , no buffer added.


ACETOIN, MILLIMOLAR
1.6
1.2
0.8
0.4
0
8.0
8.5
9.0
9.5
10.0
pH (25°)

in borate buffer or at 40° in pyrophosphate buffer. Acetoin was then measured with acid treatment (i.e., acetolactate present was decarboxylated) for runs in pyrophosphate buffers but usually without acid treatment for runs in borate buffers.

Two factors combine to make estimation of the relative activities of the poorer catalysts especially difficult. One of these is the aforementioned lag period in acetoin production which at short times results in low yields. The other is the problem of high blanks. Sodium pyruvate and thiamine itself both cause a small amount of color. The color from thiamine may arise by hydrolytic breakdown to the ketol $CH_3-\overset{O}{\overset{\|}{C}}-\overset{OH}{\overset{|}{\underset{H}{C}}}-CH_2CH_2OH$. This would most probably give the color test since a similar compound, acetyl ethyl carbinol, does (56). The related thiol is a known degradation product of thiamine (76) and might also yield some color. The methyl thiazolium salt [3,4-dimethyl-5-(2-hydroxyethyl) thiazolium chloride] appears to break down in the same way. Since it is a much less active catalyst than thiamine, the blanks become a serious problem. At pH 9 or below these blanks were negligible, but above 9 they increased rapidly.

If the sulfur of thiamine is replaced by oxygen, the resulting oxazolium salt (O-heterothiamine) decomposes much more rapidly than thiamine to give color-yielding material. The formation of the previously mentioned ketol should occur

readily in this compound. An accurate estimate of its catalytic activity was therefore impossible. However, no such interference was observed with the 4-methyl oxazolium analog of thiamine. In this case the hydrolytic product would be methyl glyoxal, which is known to give very little color (56) in this test.

The kinetically observed lag period also complicates the interpretation of relative activities. For example, in one hour in borate buffer, the benzyl analog of thiamine produces only 15% as much acetoin as thiamine but in three hours it produces 30% as much. Thus the relative catalytic activities of two compounds cannot be compared by simply measuring the amount of acetoin produced in a given length of time. It is more proper to compare the lengths of time required for the production of a certain concentration of acetoin under specified conditions. Thus with the benzyl analog the time required to produce any amount of acetoin in pH 8.8 borate buffer at 50° is always 2.6 times that required with thiamine as catalyst. If the amounts of acetoin produced by action of the benzyl analog are plotted vs. reaction time divided by 2.6 the points fall on the curve of acetoin vs. time for thiamine catalysis (Figure 12). Thus we conclude that the benzyl analog is 1/2.6 times as active as thiamine at this pH. Breslow (48) and Mizuhara *et al.* (39) avoided this problem by using long incubation periods (40 and 34 hours, respectively) where all of the reaction mixtures were

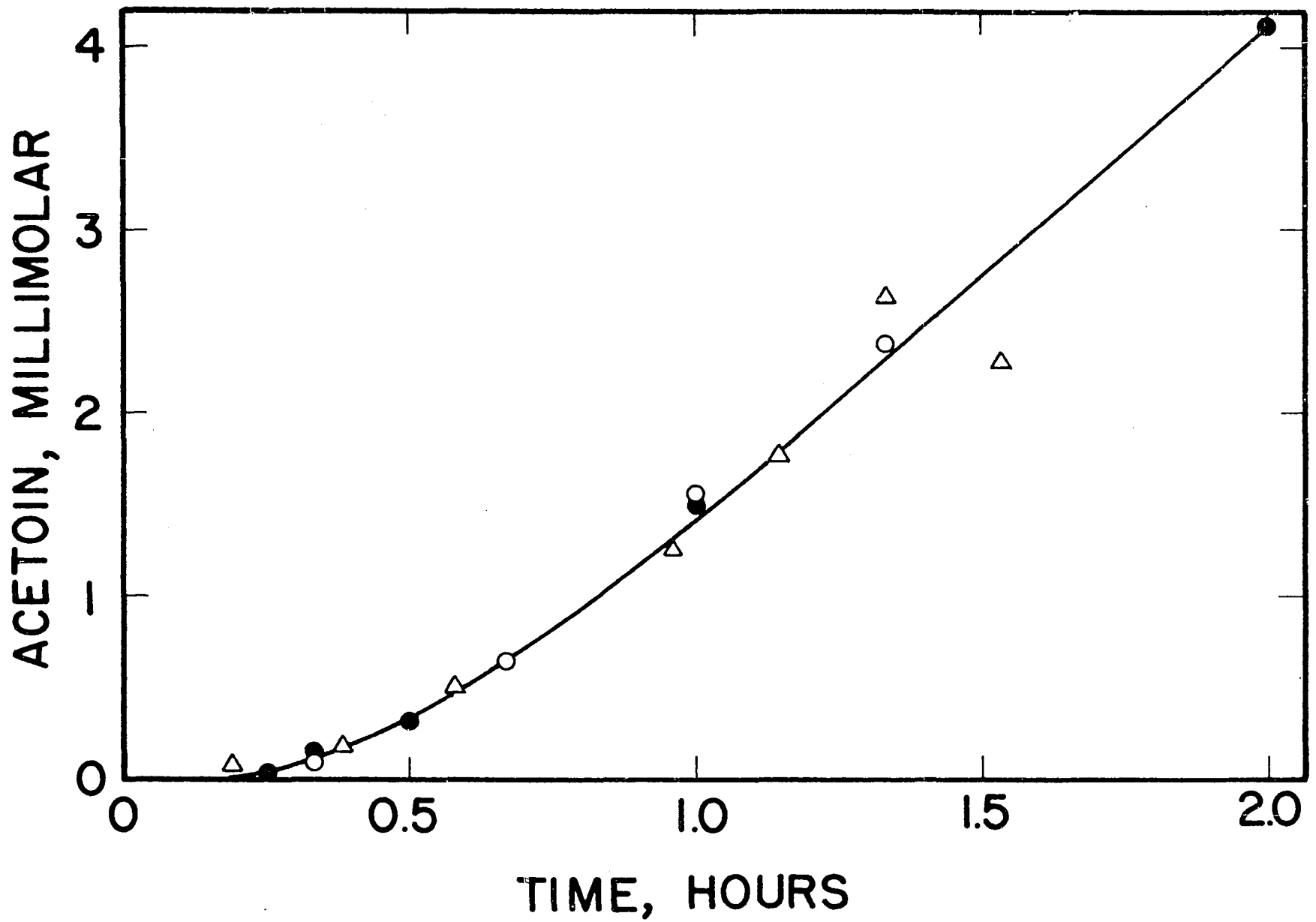
Figure 12. Acetoin production versus time for two catalysts

Pyruvate (0.2 M) plus catalyst (0.02 M) incubated at pH 9.8-9, borate buffer.

●, thiamine as catalyst, 50°

○, thiamine 40°, time divided by 3.0

△, 3-benzyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride, 50°, times divided by 2.6.


ACETOIN, MILLIMOLAR
0
1
2
3
4
0
0.5
1.0
1.5
2.0
TIME, HOURS

supposedly at steady state conditions. However, results obtained in this fashion would be much more dependent on the stability of the analogs under the test conditions than results obtained at shorter time periods. Therefore all the analogs were tested for relatively short times and their activities determined as described above.

In addition, the rate of production of acetoin by thiamine at 50° is just three times as fast as at 40° (Figure 12). Apparently temperature and catalyst structure influence the rate in similar ways.

The activities of a series of other catalysts have also been measured. These are summarized relative to that of thiamine in Table 6. Because of the importance of the pK_as of the analogs on their catalytic activity they have been tabulated in Table 7.

The results show conclusively that in this non-enzymic model system that either the methylene bridge or the thiazolium group of thiamine must be the site of interaction with pyruvate in catalysis of acetoin formation. The 3-methyl thiazolium analog (XV) is slightly active and the 3-benzyl analog (VIII) is 38% as active as thiamine. On the other hand, pyrithiamine (X) is completely inactive despite the fact that the amino pyrimidine group and the quaternary nitrogen are present as in thiamine. It is interesting to note in this connection that recently (77) it has been found that the exchange of hydrogens on both C-2 and C-6 of N-methyl

Table 6. Relative activity of thiamine analogs

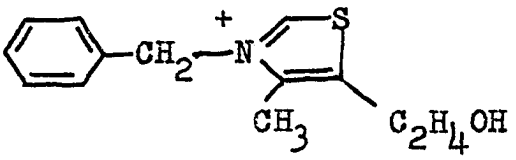
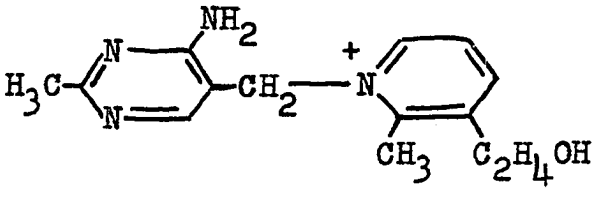
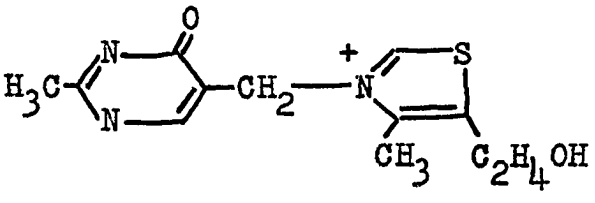
Analogs		Relative activity pH 8.7-9.0
(I)	Thiamine and o-acetyl thiamine	100%
(VIII)		38%
	3-benzyl analog of thiamine	
(X)		0
	pyrithiamine	
(XI)		trace (20% at pH 10.2)
	oxythiamine	
(XII)	O-heterothiamine (oxygen replacing sulfur in thiamine)	0

Table 6 (Continued)

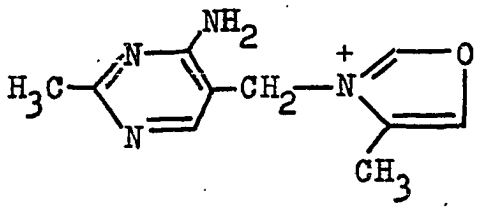
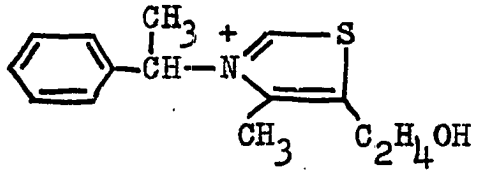
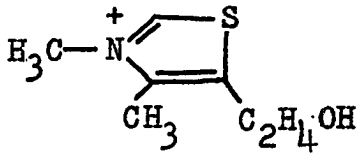
Analogs	Relative activity pH 8.7-9.0
(XIII) 	0
4-methyl oxazolium analog of thiamine	
(XIV) 	0 (0 at pH 10.2)
3-α-methyl-benzyl analog of thiamine	
(XV) 	2
3-methyl analog of thiamine	
(XVI) 3-cyanomethyl-4-methyl-5-(2-hydroxy-ethyl) thiazolium chloride	5
(XVII) 3-o,m,p-nitrobenzyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride	trace
(XVIII) 3-γ-aminopropyl-4-methyl thiazolium chloride	0

Table 6 (Continued)

Analogs		Relative activity pH 8.7-9.0
(XIX)	3-δ-aminobutyl-4-methyl thiazolium chloride	0
(XX)	dihydro and tetrahydro thiamine	0
(XXI)	2-methyl-4-amino-5-aminomethyl pyrimidine	0

Table 7. Tabulated pK_a values of thiamine analogs

Compound	pK_{a_1}	pK_{a_2}	pK_{a_3}
Thiamine	4.95		9.25[a]
O-hetero thiamine (XII)	4.1	5.8	
4-methyl oxazolium analog (XIII)	4.1	5.8	
3-benzyl-4-methyl thiazolium chloride		8.8[b]	
Benzyl analog (VIII)		9.96[b]	
α-methyl benzyl analog (XIV)		10.4[b]	
3-cyanomethyl analog (XVI)	7.25	9.3	
2-methyl-4-amino-5-aminomethyl pyrimidine (XXI)[c]	5.4	8.4	
Oxythiamine (XI)[d]	8.2		10.6[a]

[a]Average of pK_{a_2} and pK_{a_3}.

[b]Average of pK_{a_1} and pK_{a_2}.

[c]Titration performed by Lee Frank.

[d]Titration performed by Larry Levine.

nicotinamide is catalyzed by hydroxide ions. Thus the pyridinium salts can also form zwitter-ions as do thiazolium salts, but they do so only at a much higher pH. Nevertheless it is conceivable that under the proper conditions pyrithiamine might act to decarboxylate α-keto acids and to catalyze acyloin condensations.

When the sulfur of the thiazolium ring is replaced with nitrogen, Breslow has found that the resulting imidazolium salts are active as catalysts as long as C-2 remains unsubstituted (48). However, this does not hold true for oxazolium salts (oxygen substituted for the sulfur of the thiazole ring). Neither O-hetero thiamine (XII) or the 4-methyloxazolium analog of thiamine (XIII) are active as catalysts. This inactivity may be the result of the low pK_as for formation of the pseudo base form of the oxazolium salts. These pK_as are 5.8 for both compounds. This fact alone should not make the compounds inactive since, as previously discussed, the maximum concentration of zwitter-ion parallels that of the pseudo base. Two reasons can then be given for the observed inactivity. One is that the concentration of zwitter-ion formed is simply too small even at optimum conditions to catalyze any acetoin formation. The other is that the pseudo base form of oxazolium salts may very readily open to form the inactive open ring alcohols much as thiazolium salts give open ring thiols. However, in the case of oxazolium salts, the alcoholic group does not titrate with base and only one

equivalent of base per ring is taken up. There appears to be no way to distinguish between these two possible forms.

Further evidence that the Breslow mechanism (Figure 2) is correct has been afforded by the finding of Downes and Sykes (78) that 3-benzyl, 3-phenyl, and 3-allyl-2,4-dimethyl thiazolium salts are inactive as catalysts in acetoin formation. Ugai et al. (41) had previously reported 3-benzyl-2, 4-dimethyl thiazolium bromide to be inactive in catalysis of the furoin condensation, but their method of detection (crystal formation) would not have picked up small activities. The 2,4-dimethyl thiazole analog of thiamine was reported from this laboratory to be inactive (79). The status of this compound is now in doubt, however, and further attempts at synthesis must await the arrival of a suitable preparation of the pyrimidine half of thiamine.

According to the zwitter-ion mechanism the α-methyl benzyl analog of thiamine (XIV) might be anticipated to be an active catalyst. There may be two reasons for its inactivity. First, the addition of the methyl group on the methylene bridge may facilitate the solvolysis of the compound at the quaternary nitrogen so that the molecule splits before it has a chance to condense with pyruvate. Secondly, the additional methyl group may sterically hinder the attack of the zwitter-ion on pyruvate. Breslow (48) has recently reported one piece of evidence which supports this latter proposition. He finds that 3-isopropyl-4-methyl thiazolium

iodide, in contrast to 3-propyl-4-methyl thiazolium bromide, is inactive in catalyzing acyloin condensations. Structural models, though, would seem to indicate that this hinderance should not be too great. Thinking that perhaps the inactivity of the α-methyl benzyl analog was caused partly by steric hinderance and partly by the low testing pH, it was tested again at pH 10.2 near its approximate pK_{av} of 10.4. It was, however, still inactive.

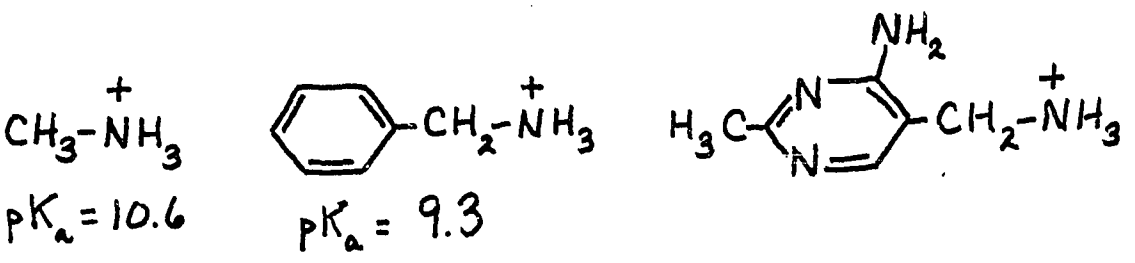
In addition, the series of o,m,p-nitrobenzyl analogs (XVII) and the γ and δ-aminoalkyl analogs (XVIII and XIX) theoretically should work as catalysts. However, it is apparent from the color of the reaction mixtures that these compounds are not stable under test conditions and little can be said about their catalytic activities.

As would be expected from Breslow's mechanism, reduction of the carbon-nitrogen double bond of the thiazolium ring causes complete loss of activity. This is illustrated by dihydro and tetrahydrothiamine (XX).

Oxythiamine (XI), in which the amino group of thiamine has been replaced by a hydroxyl, is almost inactive at pH 8.4 to 8.9 as reported by Breslow (43). However, at pH 10.2 it is 20% as active as thiamine is at pH 9.0. The pK for the ring-opening reaction is about 10.6. Titration of oxythiamine indicates that the oxypyrimidine group dissociates with a pK of about 8.2. Thus under the test conditions used the pyrimidine ring bears a negative charge and we would anticipate a

marked decrease in reactivity at C-2 of the thiazolium group and the observed increase in the pK of the ring opening reaction. Downes and Sykes (78) have attributed the low activity of oxythiamine to an actual hydrogen bond between the oxygen on the pyrimidine ring and C-2 of the thiazolium ring. However, it is unnecessary to postulate this unique hydrogen bond. There is also the possibility that the oxygen adds intramolecularly to the thiazolium ring as Breslow (48) has suggested. There is some precedent for this as Maier and Metzler (63) have shown that the amino group of thiamine adds to C-2 of the thiazolium ring at a higher pH.

The large difference in activity between the 3-methyl and 3-benzyl salts (VIII and XV) is surprising. Breslow (48) suggests that the difference in inductive effects of the methyl and benzyl groups is responsible. He states that any electron withdrawing group attached to the nitrogen should help stabilize both the zwitter-ion and the anion resulting from decarboxylation of the α-keto acid. As to the magnitude of the inductive effect he cites the large difference in pK_a values for methylamine (10.6) and benzylamine (9.3). Along this same line, the pK_a of the aminomethyl group of 2-methyl-4-amino-5-aminomethyl pyrimidine (XXI) has been found to be 8.4.



(XXI)

Thus the amino pyrimidine ring exhibits an even stronger inductive effect than the benzene ring in lowering the basicity of the aminomethyl group. Since thiamine is the most active of these catalysts, a qualitative relationship might be drawn between the basicities of the thiazolium nitrogens and the catalytic activities of their parent compounds.

Of interest here, also, is the 3-cyanomethyl analog (XVI). Because of the strong inductive effect of the cyano grouping[1] it might be expected from the above corollary that this would be an even better catalyst than thiamine. The validity of this reasoning, of course, is dependent on whether or not variations in the structure effect the formation constant of the zwitter-ion in the same way that they do the formation constant of the pseudo base. As was mentioned previously in the experimental section, the pK_a for pseudo base formation is lowered some 1,000 fold by substitution of a -C≡N for a hydrogen. It might be expected that the amount of zwitter-ion

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[1] For example, compare the pK_as of acetic acid (4.77), phenylacetic acid (4.31) and cyanoacetic acid (2.44). The pK_a for cyanomethylamine is not known.

should increase as the ease of pseudo base formation increases. However, the fact that the cyanomethyl analog is only 1/20 as active as thiamine would seem to indicate that it does not. It is conceivable, of course, that the 4-amino group on the pyrimidine ring aids somehow in acetoin formation. But it is not to be expected that its effect would be of such a large magnitude. The precise role of the 4-aminopyrimidine group in thiamine catalyzed reactions is yet to be discovered.

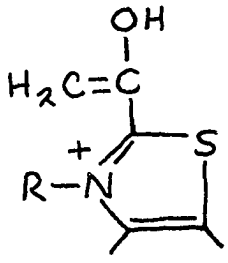
IV. CONCLUSION

The value of any model system reaction in biochemistry is directly dependent on whether or not it helps explain the mode of action of some enzyme. Since enzymes are so complex the use of model systems to simulate their action will undoubtedly continue to grow in the future. This seems particularly true in the case of the enzymes which require additional prosthetic groups in the form of co-enzymes. For example, Metzler et al. (30) were eminently successful in explaining all the known reactions of vitamin B_6 enzymes with amino acids by use of model systems. This work provided the stimulus to any number of investigators to further purify and study the mechanism of B_6 enzymes. It seems very likely that Breslow's mechanism will have the same effect on the study of thiamine containing enzymes. Since the results presented here essentially corroborate and in some cases extend the findings of Breslow, it is hoped they may also aid in some way to stimulate this study.

It might be added that the best thiamine enzymes to study appear to be those catalyzing the formation of acetoin or α-acetolactate. Unlike so many thiamine enzymes, they are easy to assay. In addition they are widespread in nature and thus a variety of source materials is available. When the intimate details of the reaction are studied the work given here will then become of especial interest. In any

case, the study of thiamine-containing enzymes does not promise to be as easy as that of B_6 enzymes. Unlike B_6, thiamine, at least non-enzymically, does not appear to give any spectral changes during its catalytic cycle.

There has recently been reported (80) a new thiamine enzyme, "phosphoketolase", which catalyzes the cleavage of D-xylulose-5-phosphate to acetyl phosphate and D-glyceraldehyde-5-phosphate. To do this it most likely goes through the intermediate (XXII), which should from all predictions



(XXII)

have a spectrum distinct from thiamine. One way to study this in model systems would be to investigate the decarboxylation of hydroxy pyruvic acid, $HO-CH_2-\overset{O}{\overset{\|}{C}}-COOH$, by thiamine. If the hydroxyl group is lost through β-elimination as with the enzyme, then a spectrally discernable intermediate should result. Its properties could then be studied more quantitatively than has been possible before with the thiamine intermediate described here.

V. SUMMARY

A non-enzymic model system in which thiamine in slightly basic solutions acts on pyruvate to yield acetoin, α-acetolactate and small amounts of unidentified products has been studied in detail. This study has centered on the stoichiometry and kinetics of the reaction and on the synthesis and testing of the catalytic action of a variety of thiamine analogs on pyruvate. The following facts have been ascertained:

(1) Acetaldehyde is not a product in this reaction. It will, however, to a lesser degree substitute for pyruvate as a reactant to give acetoin.

(2) Carbon dioxide evolution begins at once but α-acetolactate and acetoin production exhibit a characteristic lag. The kinetics are approximately those of two consecutive first order reactions. The apparent first order rate constants for these reactions have been determined as a function of pH. The logs of both rate constants are linearly dependent on pH with log K_1 giving a slope of one and log K_2 giving a slope of 0.5.

(3) The kinetically recognized intermediate is either a thiamine-acetolactate or a thiamine-acetaldehyde compound. Evidence is presented which indicates that the two are in equilibrium but in which the rate limiting step is the breakdown of the thiamine-acetolactate compound. A mechanism is

presented which purports to show the effect of pH on this equilibrium. A kinetic expression which fits both this mechanism and the pH dependence of K_2 is given. The intermediate is relatively stable to acid conditions and the possibility of isolating it under acid conditions is discussed.

(4) Some indirect evidence for the validity of Breslow's zwitter-ion mechanism of thiamine action is given. The pH optima for acetoin formation for both thiamine and 3-benzyl-4-methyl-5-(2-hydroxyethyl) thiazolium chloride correspond to the pHs of maximum zwitter-ion concentration, i.e., at the respective pK_{av}s for the thiazolium ring opening reaction. These latter pK_{av}s have been measured as a function of temperature.

(5) A number of thiamine analogs have been synthesized and tested for catalytic activity in converting pyruvate to acetoin. The thiazolium ring is necessary for catalytic activity, but the pyrimidine ring is not. Oxazolium salts, pyrithiamine, dihydrothiamine and tetrahydrothiamine are inactive. Oxythiamine, contrary to previous reports, is active but only at substantially higher pHs. All other salts tested which had activity were in essential agreement with the zwitter-ion mechanism of Breslow's.

VI. BIBLIOGRAPHY

1. Harris, L. J., Vitamins and Vitamin Deficiencies, J. and A. Churchill Ltd., London (1938).

2. Funk, C., J. State Med. 20, 341 (1912), as quoted in Robinson, F. A., The Vitamin B Complex, p. 9, John Wiley and Sons, New York (1951).

3. Funk, C., The Vitamines (2nd German ed. authorized translation by Dubin, H. E.), Williams and Wilkins Company, Baltimore (1922).

4. Williams, R. R. and Spies, T. D., Vitamin B_1 and its Use in Medicine, Macmillan Company, New York (1938).

5. Suzuki, U., Shamimura, T. and Okadi, S., Biochem. Z. 43, 89 (1912).

6. Edie, E. S., Evans, W. H., Moore, B., Simpson, G. C. E. and Webster, A., Biochem. J. 6, 234 (1912).

7. Cline, J. K., Williams, R. R. and Finkelstein, J., J. Amer. Chem. Soc. 59, 1052 (1937).

8. Todd, A. R. and Bergel, F., J. Chem. Soc. 1937, 364.

9. Andersag, H. and Westphal, K., Ber. 70, 2035 (1937).

10. Williams, R. R., Williams-Waterman Fund, The Research Corporation, New York (1956).

11. Funk, C., Z. Physiol. Chem. 89, 378 (1914).

12. Kinnersly, H. W. and Peters, R. A., Biochem. J. 24, 711 (1930).

13. Peters, R. A. and Thompson, R. H. S., Biochem. J. 28, 916 (1934).

14. Peters, R. A., Lancet 1, 1161 (1936).

15. Sherman, W. C. and Elvehjem, C. A., Biochem. J. 30, 785 (1936).

16. Neuberg, C., An. Rev. Biochem. 15, 435 (1946).

17. Auhagen, E., Z. physiol. Chem. 204, 149 (1932).

18. Lohmann, K. and Schuster, P., Naturwiss. 25, 26 (1937); Biochem. Z. 294, 188 (1937).

19. Lipmann, F., Nature 140, 25 (1937).

20. Ochoa, S., Physiol. Rev. 31, 56 (1951).

21. Lipmann, F., Nature 138, 1097 (1936).

22. Lipmann, F. and Perlman, G., J. Am. Chem. Soc. 60, 2574 (1938).

23. Karrer, P., Graf, W. and Schukri, J., Helv. Chim. Acta 28, 1523 (1945).

24. Karrer, P. and Krishna, H., Helv. Chim. Acta 33, 555 (1950).

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

26. Zima, O., Ritsert, K. and Moll, T., Z. physiol. Chem. 267, 210 (1941).

27. Peters, R. A., Nature 158, 707 (1946).

28. Gunsalus, I. C., in McElroy, W. D. and Glass, B., eds., The Mechanism of Enzyme Action, pp. 545-580, The John Hopkins Press, Baltimore (1954).

29. Gunsalus, I. C., in Symposium on Vitamin Metabolism, p.6, The National Vitamin Foundation, Inc., New York (1956).

30. Metzler, D. E., Ikawa, M. and Snell, E. E., J. Am. Chem. Soc. 76, 648 (1954).

31. Langenbeck, W., Ergebn. d. Enzymforsch. 2, 314 (1933).

32. Neuberg, C. and Simon, E., Biochem. Z. 156, 374 (1925).

33. Stern, K. G. and Melnick, J. L., J. Biol. Chem. 131, 597 (1939).

34. Weil-Malherbe, H., Nature 145, 106 (1940).

35. Weisner, K. and Valenta, Z., Experientia 12, 190 (1956).

36. Karrer, P., Bull. soc. chim. 1947, 149.

37. Johnson, B. C., An. Rev. Biochem 24, 419 (1955).

38. Takeuchi, Y., Okuda, K., Hayakawa, S. and Mizuhara, S., J. of Biochem. (Japan) 42, 93 (1955).

39. Mizuhara, S., Tamura, R. and Arata, H., Proc. Jap. Acad. 27, 302 (1951).

40. Mizuhara, S. and Oono, K., Proc. Jap. Acad. 27, 705 (1951).

41. Ugai, T., Tanaka, S. and Dokawa, S., J. Pharm. Soc., Japan 63, 296 (1943).

42. Koffler, H. and Krampitz, L., Bact. Proc. 113 (1955).

43. Breslow, R., Chem. and Ind., British Industries Fair Review, p. R28 (April 1956).

44. Manzo, E. Y. and Metzler, D. E., Fed. Proc. 16, 391 (1957).

45. Ingraham, L. and Westheimer, F., Chem. and Ind., 846 (1956).

46. Breslow, R., J. Am. Chem. Soc. 79, 1762 (1957).

47. Breslow, R., Chem. and Ind., 893 (1957).

48. Breslow, R., J. Am. Chem. Soc. 80, 3719 (1958).

49. Price, V. E. and Levintow, L., in Ball, E. G., ed., Biochemical Preparations Vol. 2, p. 23, John Wiley and Sons, Inc., New York (1952).

50. Umbarger, H. E. and Magasanik, B., J. Am. Chem. Soc. 74, 4253 (1952).

51. Fieser, L., Experiments in Organic Chemistry, 3rd ed., D. C. Health and Co., Boston (1955).

52. Stotz, E., J. Biol. Chem. 148, 585 (1943).

53. Krampitz, L. O., in Colowick, S. P. and Kaplan, N. O., eds., Methods in Enzymology Vol. 3, p. 277, Academic Press Inc., New York (1957).

54. Westerfeld, W. W., J. Biol. Chem. 161, 495 (1945).

55. Eggleston, P., Elsdent, S. R. and Gough, N., Biochem. J. 37, 526 (1943).

56. Green, D. E., Westerfeld, W. W., Vennesland, B. and Knox, W. E., J. Biol. Chem. 145, 69 (1942).

57. Ennor, A. H. and Stocken, L. A., Biochem. J. 42, 557 (1948).

58. Neidig, B. A. and Hess, W. C., Anal. Chem. 24, 1627 (1952).

59. Metzler, D. E. and Snell, E. E., J. Am. Chem. Soc. 74, 979 (1952).

60. Bonvicino, G. and Hennessy, D. J., J. Am. Chem. Soc. 79, 6325 (1957).

61. Hirano, H., Iwatsu, T. and Yurugi, S., Yakugaku Zasshi 77, 241 (1957). [C. A. 51, 16485 (1957)].

62. Clarke, A. T., J. Am. Chem. Soc. 66, 652 (1944).

63. Maier, G. D. and Metzler, D. E., J. Am. Chem. Soc. 79, 4386 (1957).

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

65. Kitson, R. E. and Griffith, N. E., Anal. Chem. 24, 334 (1952).

66. Kurkjy, R. P. and Brown, E. V., J. Am. Chem. Soc. 74, 5778 (1952).

67. Cornforth, J. W. and Cornforth, R. H., J. Chem. Soc. 1953, 93.

68. Sarver, H., Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1948.

69. Manzo, E. Y., Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1957.

70. Moelwyn-Hughes, E. A., Physical Chemistry, Cambridge University Press, London (1951).

71. Waldman, E., Prey, V. and Jelinek, F., Monatsh. 85, 872 (1954).

72. Juni, E. and Heym, G. A., J. Biol. Chem. 218, 365 (1956).

73. Hennessy, D. J., in Calmon, C. and Kressmann, T. R. E., eds., Ion Exchangers in Organic and Biochemistry, p. 520, Interscience Publishers, New York (1957).

74. Williams, R. R. and Ruehle, A. E., J. Am. Chem. Soc. 57, 1856 (1935).

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

76. Watanabe, A. and Asahi, Y., J. Pharm. Soc. Japan 77, 153 (1957).

77. Duff, H. E., Saunders, M. and Wang, J. H., J. Am. Chem. Soc. 80, 1767 (1958).

78. Downes, J. E. and Sykes, P., Chem. and Ind. 1957, 1095.

79. Yount, R. G. and Metzler, D. E., Fed. Proc. 17, 341 (1958).

80. Heath, E. C., Hurwitz, J., Horecker, B. L. and Ginsburg, A., J. Biol. Chem. 231, 1009 (1958).

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