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I Metal-pyridoxal-amino acid chelates II Studies on the purification and kinetics of threonine dehydrase

Leodis Davis
Iowa State University

I. METAL-PYRIDOXAL-AMINO ACID CHELATES

II. STUDIES ON THE PURIFICATION AND KINETICS OF THREONINE DEHYDRASE

by

Leodis Davis

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I. INTRODUCTION

Pyridoxal phosphate (PPal, pyridoxal-5-phosphoric ester) is the functional co-enzyme form of vitamin B_6 (Figure 1). The apparent activity of the other forms of vitamin B_6 (Figure 1) is explained by the presence of enzymatic systems which have the ability to convert them to pyridoxal phosphate.

PPal functions in several and varied physiological reactions as a co-enzyme. The first enzymes shown to require PPal as a co-enzyme were glutamate-pyruvate and glutamate-oxalacetate transaminases (1-2-3) and amino acid decarboxylases (4-5). Subsequently a number of other enzymes were isolated which had PPal as a prosthetic group. The enzyme-catalyzed reactions which have been reviewed recently by Braunstein (6), are all characterized by action of one sort or another upon amino acids.

An understanding of the manner in which PPal functions as a co-enzyme was greatly aided by the proposal of a general mechanism by Braunstein and Shemyakin (7) and by Metzler et al. (8). Both groups suggested that PPal functions through the formation of Schiff bases (imines) with amino acids. The mechanism of formation of these Schiff base intermediates is presented in (Figure 2). The amino group of the amino acid reacts with the 4-formyl group of PPal to

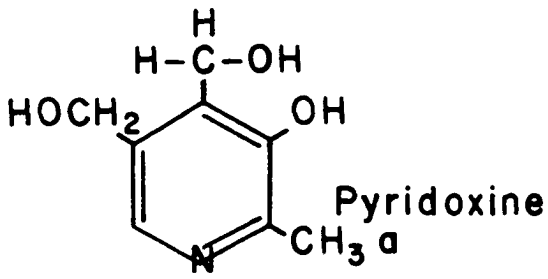
Figure 1. The forms of vitamin B_6

a. pyridoxine

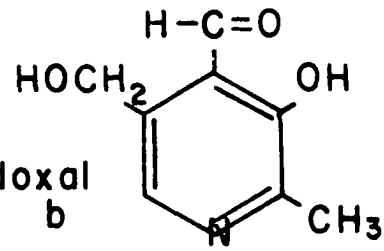
b. pyridoxal

c. pyridoxamine

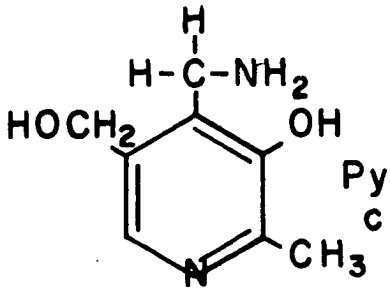
d. pyridoxal-5-phosphoric ester



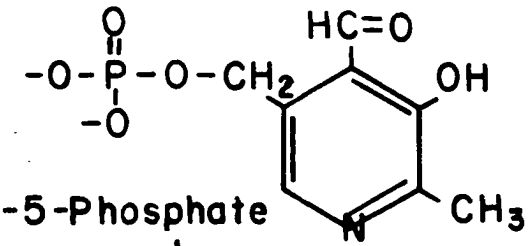
Pyridoxine
a



Pyridoxal
b

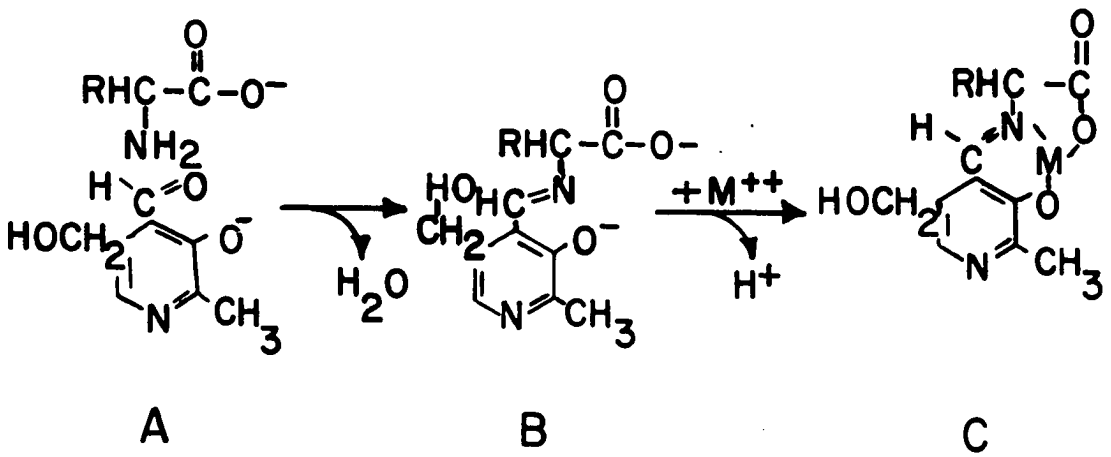


Pyridoxamine
c

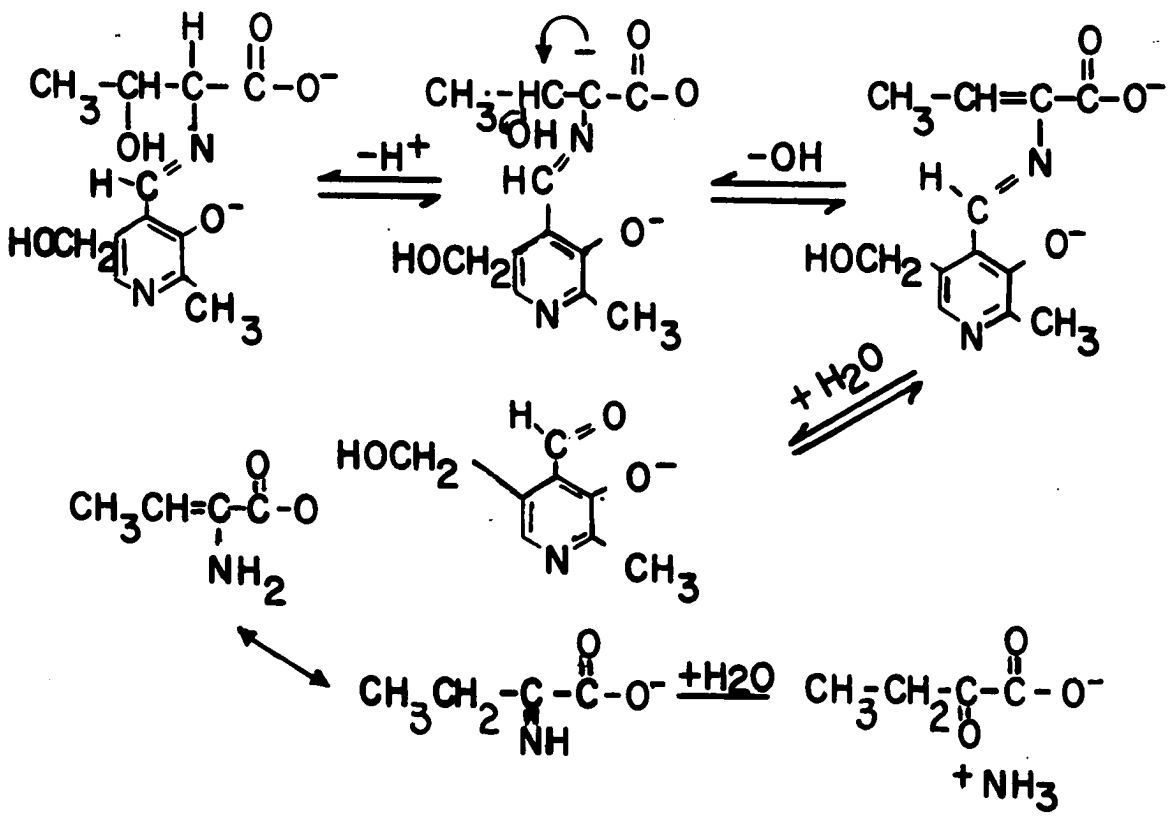


Pyridoxal-5-Phosphate
d

Figure 2. Imine and chelate formation; conversion of threonine to α-ketobutyric acid



A B C



form a carbinolamine which then loses water to form the imine. One result of this imine formation is activation of the α-hydrogen of the amino acid, which facilitates many of the amino acid transformations observed. The dissociation of the α-hydrogen of amino acids under the influence of PPal enzymes has been demonstrated by exchange reactions with deuterium. (9). An example of the role of activation of the α-hydrogen of Schiff bases in catalysis by PPal enzymes is shown in Figure 2. A proposed mechanism for the conversion of threonine to α-ketobutyrate is shown. Activation and subsequent removal of the α-hydrogen is probably the first in a sequence of steps which finally results in conversion of threonine to α-ketobutyrate (Figure 2).

Another fact which contributed to the elucidation of the way in which PPal functions as a co-enzyme was the discovery that pyridoxal and PPal (Figure 1), in model non-enzymatic systems, will catalyze most of the reactions that are normally catalyzed by enzymes which contain this vitamin.

In studies of non-enzymatic pyridoxal catalyzed amino acid transformations a catalytic effect of metal ions was noted (10-11). The theory was advanced in 1954 that these reactions occur via an intermediate pyridoxylidene amino acid metal chelate (Figure 2) (10). Such complexes are probably more reactive than the free imine, and it is possible that

such chelates are intermediates in the action of some PPal-dependent enzymes.

Little quantitative information is available on these chelates in solution. The aim of the work presented in the first section of this thesis is to gain knowledge about the basic chemistry of vitamin B_6 and about the stability and composition of these chelates which might aid in explaining how PPal functions in enzymes. Some preliminary details on these chelates appear in the author's Masters thesis (12). Other investigations done in conjunction with this work, but not conducted specifically by the author have been submitted for publication (13).

To gain information on how PPal functions as a co-enzyme a presumedly PPal dependent enzyme, threonine dehydrase, has been purified and studied. This enzyme, isolated from sheep liver, catalyzes the degradation of threonine to ammonia and α-ketobutyric acid. Several PPal enzymes have been isolated which degrade hydroxy amino acids (14-18). Since dehydration and deamination occur concomitantly these enzymes have been referred to both as deaminases and dehydrases.

Threonine dehydrase unlike the enzymes which act on the other hydroxy amino acids, serine and homoserine, has not been studied extensively, but it has been partially purified

by Sayre and Greenberg (18). The authors obtained a 600 fold purification of threonine dehydrase using sheep liver as an enzyme source.

The author's first effort with this enzyme was an attempt to purify it. A 4000 fold purification has been obtained using heat treatments (70°C), ammonium sulfate fractionations and diethylaminoethyl cellulose column chromatography.

Although a crystalline enzyme has not been obtained, a study of the effects of specific inhibitors, variation of the kinetic functions (maximum velocity and Michaelis constant) with pH, substrate specificity and cofactor requirement, has presented information on mechanism of action of threonine dehydrase. A large part of the second section of this thesis is devoted to a study of pH effects on threonine dehydrase. From such an investigation information of the following type may be obtained : stability of the enzyme at various pH values, the state of ionization, most favorable for reaction, of the enzyme and of the substrate, and pK's of groups at the active center.

II. PYRIDOXAL-AMINO ACID-METAL CHELATES

A. Review of Pertinent Literature

Metal chelates of pyridoxylidene amino acids form readily in solution. Eichhorn and Dawes (19) showed this by spectral analysis of solutions of pyridoxal plus -amino acids and metal salts and of pyridoxamine plus keto acids and metal salts.

When an amino acid is added to a solution of pyridoxal, very distinct spectral changes occur. Whereas pyridoxal alone shows very little absorption in the 400 mμ region, strong bands appear in this region when an amino acid or amine is added to an aqueous pyridoxal solution. At the same time the 317 mμ band characteristic of the internal hemiacetal form of pyridoxal decreases and new bands appears at 280 mμ and at 410-415 mμ attributed to the imine. These changes result from the formation of a double bond in conjugation with the aromatic nucleus. Pyridine derivatives which lack a double band in conjugation with the aromatic ring absorb at wavelengths below 330 mμ (20). The free aldehyde forms of pyridoxal and pyridoxal phosphate absorb at 390 mμ (20). The 414 mμ band of the imine shift to 367 mμ with increasing pH (21). The rest of the spectrum shift accordingly. This is probably due to dissociation of a proton from some group on

the imine. This proton is probably lost from the phenolic group where it is hydrogen banded to the imino nitrogen (Figure 2) (21).

Upon the addition of certain metal ions to a mixture of amino acids and pyridoxal, further changes in the ultraviolet spectrum not characteristic of the imine occur. Eichhorn and Dawes (19) showed these changes to be a direct result of metal complex formation between the pyridoxal imine and the metal ion. The metal ion probably displaces the hydrogen bonded proton in structure B (Figure 2) since a drop in pH is observed concomitantly with the spectral changes. Further proof of the existence of these metal complexes of pyridoxal imines has been obtained by isolation and characterization of several of them. Christensen (22) as well as the author prepared several crystalline chelates. The nickel and zinc compounds contained the metal and imine in the ratio of 1:2; whereas, the copper compounds contained a 1:1 ratio of imine to metal ion.

These chelates were isolated from alcoholic solutions. The nickel and zinc compounds were only slightly soluble in water and very rapidly decomposed to give a mixture of the 1:1 chelate and free pyridoxal. The copper compounds are much more stable in aqueous solution and in the presence of a suitable concentration of copper valinate almost no dissociation of the imine chelate occurs (22). Metzler *et al* (11)

isolated a solid aluminum chelate of the imine of pyridoxal with the amino acid "β-pyridoxyl serine" (formed by condensation of pyridoxal with glycine). The spectra and dissociation constants for this compound have been measured (11). Baddiley (23) made some preliminary observations on the color changes which occur when certain metal ions are added to a mixture of pyridoxal and amino acids. From the results he concluded that chelate formation had taken place. He was also able to isolate and characterize the copper compound of pyridoxylidene tyrosine. Crystalline chelates of pyridoxal imines have also been separated by chromatography and electropharesis. Fasella et al. (24) were able to isolate, by these methods, fluorescent intermediates from transamination reactions to which copper had been added. These compounds were identified as metal complexes of Schiff base (imine) type compounds between pyridoxal and, in this case, alanine and of pyridoxamine and pyruvate (24).

As a method of determining the ratio of metal to imine in these metal pyridoxal amino acid chelates the method of continuous variation has been employed (19-24). This method depends on the fact that the chelate will be in highest concentration in a solution which contains the metal and ligand in the ratio in which they appear in the compound.

Matsuo has described the ultraviolet absorption spectra of pyridoxal phosphate amino acid imines in aqueous solutions

(25). He reported spectrophotometric evidence for the chelation of these compounds with a number of metal ions.

The aim of the work presented here is to obtain quantitative information about the extent of formation, the composition and basic chemistry of these pyridoxal-amino acid metal salt solutions.

B. Experimental

1. Materials and apparatus

All spectral analyses were done on Beckman model DU and Cary model 14 spectrophotometers. For stability constant measurements the cell compartments were thermostated at 25°. pH measurements were made with a Beckman model G pH meter using "general purpose" glass electrodes.

The pyridoxal HCl was obtained from Nutritional Biochemicals Co. and Merck and Co. Concentration of stock solutions were routinely checked in 0.1N HCl spectrophotometrically. (Molar absorbancy index, a_m at 288 mu = 8.75×10^3 at 2×10^{-4} M).

The neutral form of pyridoxal was obtained by adjusting a concentrated solution of the pyridoxal HCl to pH 6.5 with KOH and allowing the pyridoxal to crystallize. The crystals were collected on filter paper and dried under vacuum over P_2O_5. These were recrystallized twice from water to a constant spectrum.

The amino acids used were from commercial sources. Valine, used in much larger amounts than the other, was obtained from Dow Chemical Co. and from the California Corporation for Biochemical Research. Standard carbonate-free KOH was prepared by an ion exchange method using amberlite IR-410 in the hydroxyl form (26).

Copper pyridoxylidene valine and copper pyridoxylidene glycine were prepared according to the procedure of Christensen and Collins (27).

Copper pyridoxylidene valine dihydrate: To one millimole of DL-valine, dissolved in 2 mg. of aqueous NaOH, was added 1 millimole of pyridoxal HCl to form the sodium salt of the imine. With the addition of one millimole of copper acetate the solution was filled immediately with a very fine light green crystalline product. After standing overnight, the crystals were collected, washed with cold 95% ethanol and dried in a desiccator over KOH. Calcd. for $C_{18}H_{16}N_2O_4Cu \cdot 2H_2O$: Cu, 17.5. Found: Cu, 17.0.

Copper pridoxylidene valine hydrochloride: An aqueous solution containing one millimole of pyridoxal HCl in a minimal amount of water was added to a suspension of 1 millimole of copper valinate dissolved in 50 ml. H_2O. The suspension was shaken for a few minutes until all the copper valinate dissolved or reacted. The greenish blue solution was then taken nearly to dryness on a rotary concentrator,

taken up in absolute alcohol and placed in a refrigerator (5°) overnight. The next day the product was filtered off and dried. Calcd. for $C_{13}H_{17}N_2O_4$ Cu·Cl: C, 42.8; H, 4.7; N, 7.69; Cu, 17.5; Cl, 9.73. Found: C, 44.2; H, 5.1; N, 7.6; Cu, 17.7; Cl, 6.0.

Copper valinate and glycinate were prepared by adding a copper acetate solution (Satd.) to a solution (0.5 M) of the amino acid, filtering and drying. Calcd. for Cu valinate . $2H_2O$: Cu, 25.8. Found: Cu, 26.5.

Standardization of copper, nickel and zinc perchlorates: One tenth molar solutions of the metal perchlorates were titrated with a 0.1 M solution of disodium ethylenediamine tetraacetic acid (Na_2EDTA) which had previously been standardized against a sample of pure zinc. Erichrome Black T (F241) was used as an indicator (28) for zinc and PAN(1-(2-pyridylazo)-2-naphthol) (29) for the copper and nickel. The nickel was determined by titrating the free EDTA, with a standard copper solution using PAN as an indicator, after a measured amount in excess had been added to the nickel solution (29).

2. Evaluation of equilibrium constants

Spectrophotometric and pH measurements have been used to evaluate the formation constants of the imine chelates and their dissociation constants as acids. (Note that these and

all other equilibrium constants used in this thesis are apparent constants expressed in terms of concentrations rather than activities and with pH assumed to equal - log $[H^+]$. (They have been measured at ionic strength 0.1 whenever possible).

In addition to the reaction between the imine and metal ion, all of the reactions indicated in (Figure 3) must be considered as of possible significance in the pH range 4.5-9 and may affect the evaluation of the desired formation constant. The secondary equilibria have been taken into account as will be illustrated in the following method for computing formation constants of imine chelates.

Solutions containing pyridoxal hydrochloride, amino acid, metal perchlorate and KOH in varying proportions were prepared in 25 ml. volumetric flasks, the metal salt being added last. Blanks without pyridoxal were prepared at the same time. The solutions were allowed to equilibrate at 25° for 1-3 hours before the pH and light absorption measurements were made. In the case of the copper system equilibration was much slower. In this case solutions were usually prepared from crystalline 1:1:1 chelate and valine or from copper divalinate and pyridoxal, equalibration then being more rapid.

The overall formation constant, $K = [\text{chelate(all forms)}] / [M^{++}][pV^{-}]$ was calculated as follows: For the selected

wavelengths, e.g. 270, 315, 380, or 420 mμ, the absorbancy index of the pyridoxal was calculated, and that of the chelate was obtained from experimental data e.g. (Figure 4) at the pH of the test solution. From these absorbancy indices and the observed absorbancy of the test solution, a first estimate of the fraction of chelated pyridoxal was computed. This estimate had to be corrected for the presence of free imine, where concentration was estimated from the approximately known concentration of free valine and pyridoxal and the known formation constant for the imine (21). The contribution of the imine to the observed absorbancy was then subtracted, and a new calculation of the fraction of chelateal pyridoxal was made. Consistent values were obtained from calculations at the different wave lengths. For solutions containing a great deal of free imine, e.g. at high pH, a different procedure was used. An initial estimate of the free pyridoxal present was made from 315 mμ data. Then, using data at 380 mμ and 420 mμ, and two simultaneous linear equations, the fraction of imine and chelate were computed.

The total metal concentration minus the amount in the form of the chelate MPV was now distributed among the forms M^{++}, MV^{+}, and MV_2 using known equilibrium constants (12). The large excess of valine in most solutions simplified the problem.

With the free M^{++}, pyridoxal, valine and chelate concentrations established it was a simple matter to evaluate the desired equilibrium constants. The equilibrium constant thus obtained is pH dependent since the chelate can pick up a proton on the ring nitrogen at different pH values. The pH dependence of K permits calculation of K_c ($K_c = \frac{[MPV]}{[M][PV^{=}]}$), the formation constant of the unprotonated chelate, and gives an independent measurement of K_{mpv}.

C. Results and Discussion

The copper pyridoxylidene chelates of valine and glycine can be prepared in solid form. When the solid was dissolved in water in the presence of a suitable concentration of copper valinate, almost no dissociation of the imine chelate occurs (22). Under these conditions the absorption spectrum of the copper pyridoxylidene valine chelate can be measured directly (Figure 4). The glycine chelate is very similar and not shown. These chelates, probably of structure c (Figure 2) accept protons on the ring nitrogen with pK's of 5.60 as shown by Christensen (22) and 6.05 for the valine and glycine compound respectively. These pK's have been determined by potentiometric and spectrophotometric methods in the case of the valine compound and by spectrophotometric in the case of the glycine compound. The titration curves are

Figure 3. Equilibria in mixtures of pyridoxal with valine and metal salts.

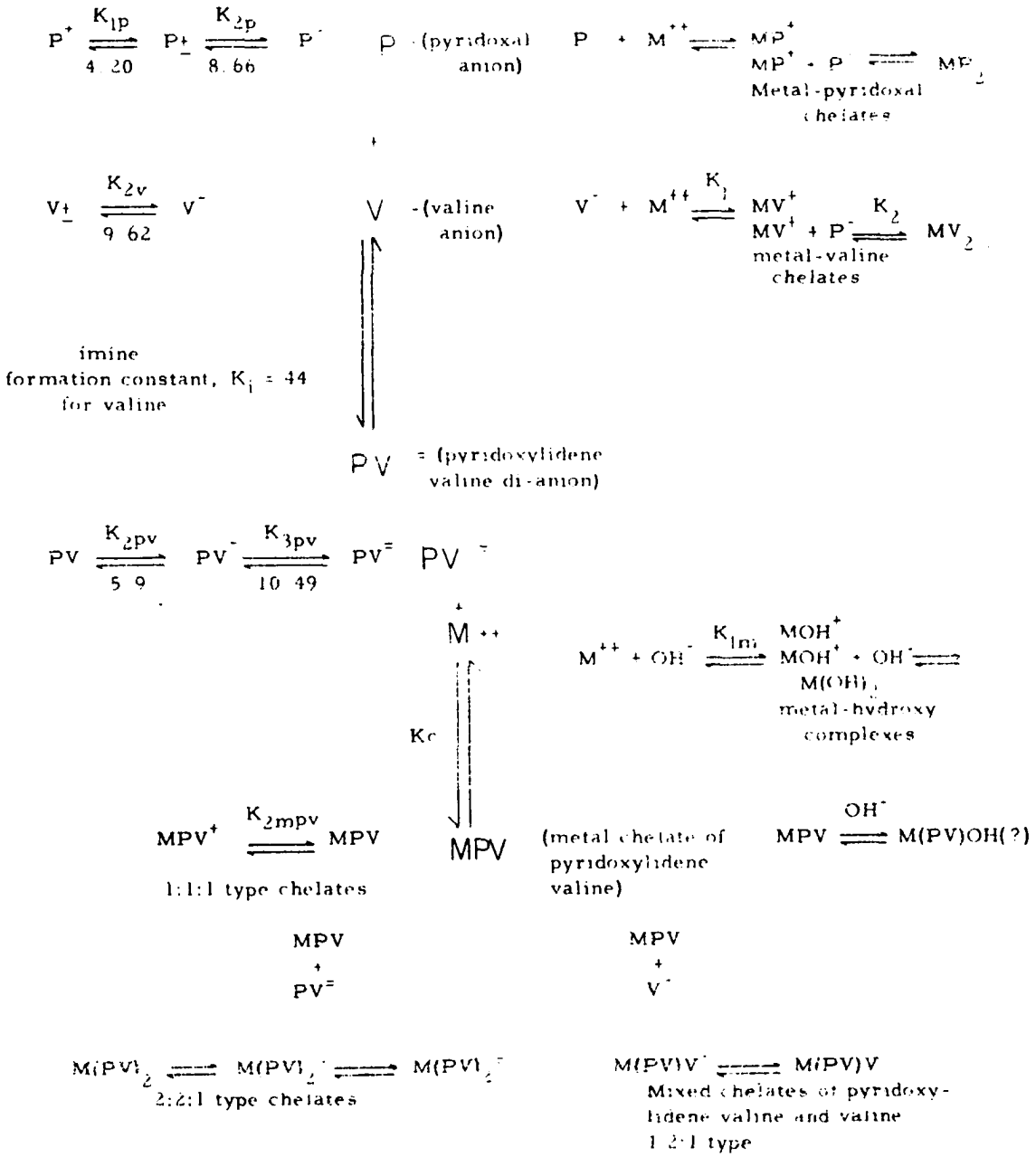
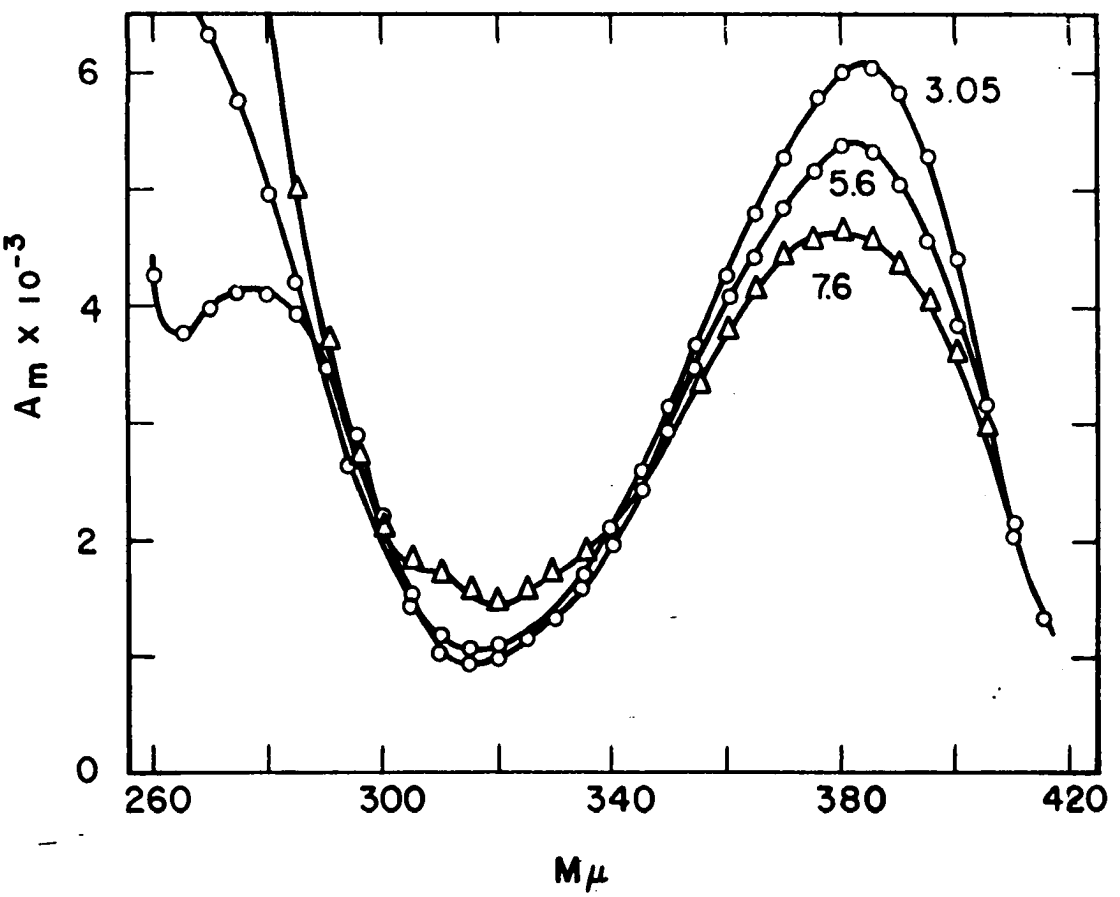

P+ K1p P± K2p P-
4.20
8.66
P (pyridoxal anion)
P + M++ ⇌ MP+
MP+ + P ⇌ MP2
Metal-pyridoxal chelates
V± K2v V-
9.62
V (valine anion)
V- + M++ K1 MV+
MV+ + P- K2 MV2
metal-valine chelates
imine formation constant, K1 = 44 for valine
PV = (pyridoxylidene valine di-anion)
PV K2pv PV- K3pv PV= PV3-
5.9
10.49
M++
Kc
MPV
MPV+ K2mpv MPV
1:1:1 type chelates
(metal chelate of pyridoxylidene valine)
M++ + OH- K1m MOH+
MOH+ + OH- ⇌ M(OH)2
metal-hydroxy complexes
MPV OH- M(PV)OH(?)
MPV + PV=
MPV + V-
M(PV)2 ⇌ M(PV)2- ⇌ M(PV)23-
2:2:1 type chelates
M(PV)V- ⇌ M(PV)V
Mixed chelates of pyridoxylidene valine and valine
1:2:1 type

Figure 4. Spectra of copper pyridoxylidene valine adjusted to the indicated pH values. Copper di-valine (4×10^{-4} M) was added


6
4
2
0
$A_m \times 10^{-3}$
260
300
340
380
420
Mμ
3.05
5.6
7.6

presented in (Figure 5). The solid curves are theoretically drawn for the indicated pK values. The hydrochloride of copper pyridoxylidene valine has also been isolated in crystalline form (Experimental).

Using solutions of pyridoxal, valine and copper valinate the formation constant K as a function of pH for the copper chelate has been evaluated. A plot of log K vs. pH (Figure 6) is linear with a slope of approximately one below the pH value equal to pK_{mpv}, and with a slope of zero above this pH. This indicated that single protons are taken up by the chelates, and that the form of the chelate above this pH is neutral.

K_c the constant for formation of the 1:1 imine-metal chelate is defined by the reaction of the imine dianion plus the metal (II) ion to form a neutral chelate.

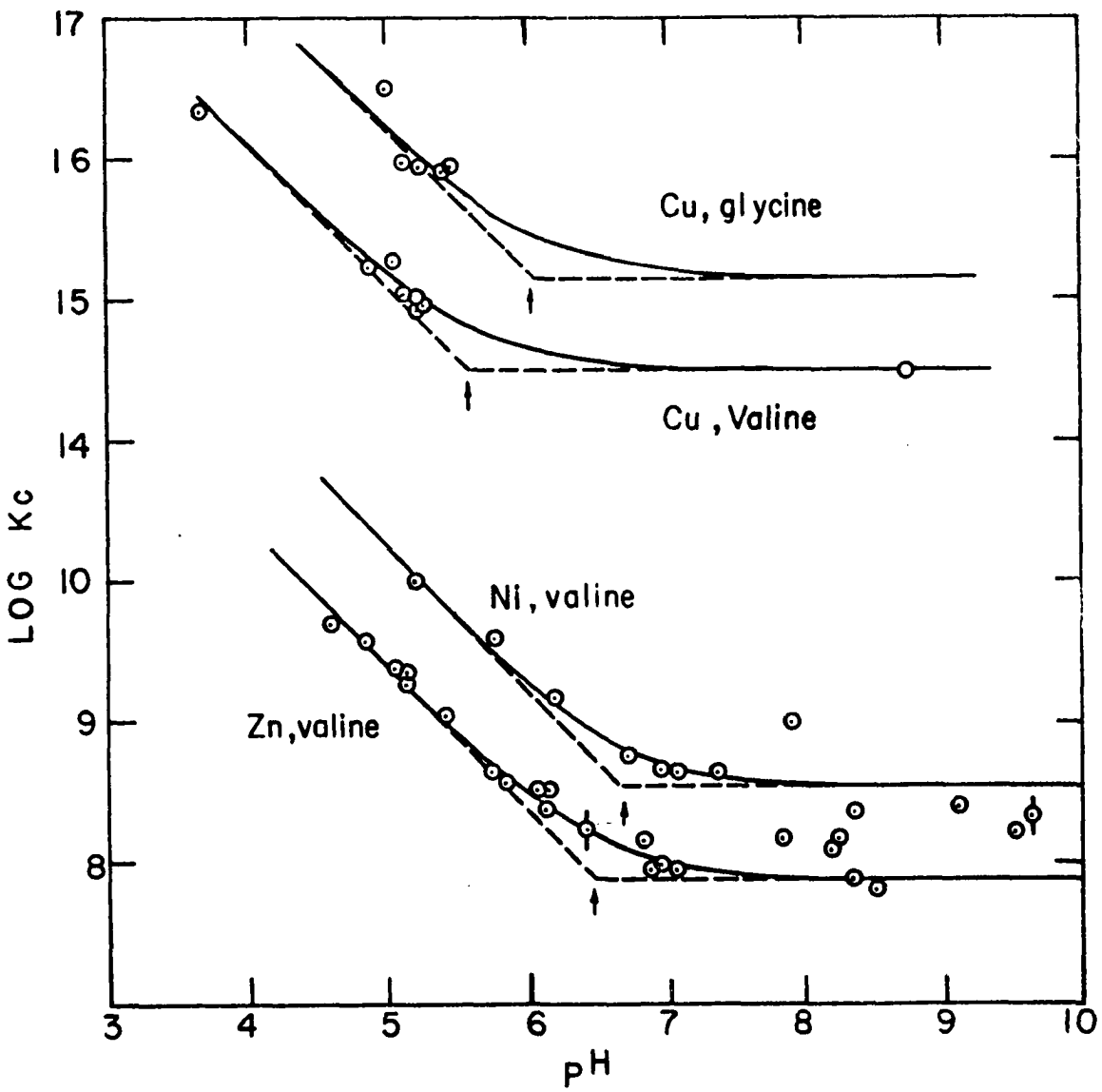
The value of log K_c = 14.5 (Figure 6) compares favorably with the value 14.4 obtained by Christensen (22). Data for the glycine chelate were obtained similarly (Figure 6).

Although the nickel chelate is less stable than the copper, conditions were found over a wide range of pH under which nearly complete conversion to the imine chelate occurs. The absorption spectrum resembles that of the copper chelate, but does not change appreciably with pH. The long wave length absorption in the nickel compound occurs at 388 mμ while that of the copper compounds occurs at 379 mμ. Because

Figure 5. Potentiometric and spectrophotometric titration curves for copper pyridoxylidene glycine -□-□- and valine. Circles represent both spectrophotometric and potentiometric data for copper pyridoxylidene valine

$A_M \times 10^{-3}$
4
5
6
10
9
8
7
6
5
4
3
2
pH
0.1
5.0
0
MEG. $[OH^-]$

Figure 6. Apparent formation constants for 1:1:1 metal chelates of pyridoxylidene valine and glycine versus pH. The solid lines are theoretical curves based on the assumption that the chelate accepts a single proton with the pK indicated by the vertical arrows. These pK values were independently obtained, except in the case of the nickel-valine system


17
16
15
14
10
9
8
LOG Kc
Cu, glycine
Cu , Valine
Ni , valine
Zn , valine
3
4
5
6
7
8
9
10
pH

the spectrum does not change appreciably with pH the pK for protonation of the ring nitrogen could not be evaluated directly from spectrophotometric data. However, the calculated values of K (Figure 6) depend on pH in such a way as to indicate that the pK is about 6.7. The increase scatter of points above pH 7 makes it difficult to obtain precise values of pK or of K_c.

A summary of the formation constants and pK's for the three pyridoxylidene amino acid chelates investigated is presented in Table I. In Table 2 is presented compositions of typical solutions used in determination of chelate formation constants.

The proposed structure for the 1:1 compound is shown in (Figure 7).

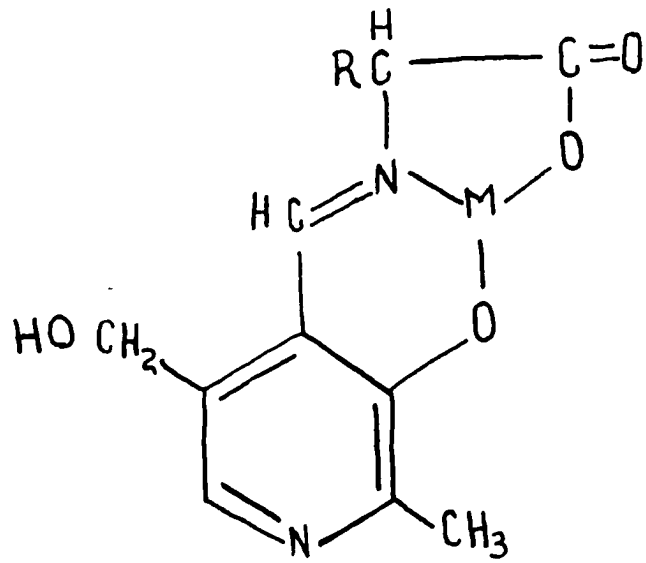


Figure 7. 1:1 Chelate

Table 1. Formation constant, K_c, and pK for protonation of ring nitrogen in ligand of chelate pK_{2mpv} of metal complexes of pyridoxylidene amino acids. Constants are for the reaction of the di-anion of the imine with the metal ion to form the 1:1:1 chelate

Metal ion	pK_{2mpv}	Ligand	Log K_c
Cu^{++}	5.6	pyridoxylidene valine	14.5
	6.05	pyridoxylidene glycine	15.0
Ni^{++}	6.7	pyridoxylidene valine	10.8
Zn^{++} (13)	6.5	pyridoxylidene valine	7.94

The tridentate nature of the ligand probably leads to a greater stability of the chelate through the formation of two rings. This is born out by the fact that imines of pyridoxal with amines, in which the third point of attachment to the metal is eliminated, do not form chelates in any measurable amount (13). The order of the stability of the chelates Cu > Ni > Zn is the same as that observed for other chelate series (30). This in turn is also the order of their capacity to catalyze non-enzymic amino acid transformations (31).

The composition of the chelates studied here is one imine per metal ion. This could easily be established in the case of the copper chelates since they could be prepared, isolated and then used to prepare stock solutions for inves-

Table 2. Compositions of typical solutions used in chelate formation constant determination. Total pyridoxal = 2×10^{-4} M for Cu^{++} and Ni^{++} containing samples

pH	Valine Total	Valine Free	Total $\times 10^4$	Molar concentrations: Metal[a] M^{++}	MV^+	MV_2	Fraction of total pyridoxal: Imine Chelate	Free Pyridoxal	Imine, PV^-
3.68	.05	.049	4[b]	$.41 \times 10^{-4}$	1.54×10^{-4}	$.44 \times 10^{-4}$	.81	.19	---
5.22	.20	.20	4	1.58×10^{-8}	$.07 \times 10^{-4}$	3.12×10^{-4}	.40	.54	.06
8.77	.30	.30	4	4.05×10^{-15}	1.41×10^{-8}	3.33×10^{-4}	.38	.0010	.67
				Metal[c]					
5.21	.20	.019	9.94	8.7×10^{-4}	$.99 \times 10^{-4}$	5×10^{-7}	.12	.87	.01
6.20	.20	.019	9.94	4.24×10^{-4}	4.54×10^{-4}	$.29 \times 10^{-4}$	.43	.56	.01

[a]Copper.

[b]Added as Cu valinate.

[c]Nickel.

tigation. The composition of the nickel chelate was more difficult to establish. The one to one ratio in this case was arrived at from the consistency in the formation constants obtained when this assumption was made and by working in solutions of low free pyridoxylidene valine and high metal concentration. In such solutions it is expected that only a small amount of the chelates will contain two or more imines ligands per metal ion.

In non-enzymic catalysis by pyridoxal of amino acid conversions the catalytic effect of metal ions may be due to a number of things. For one, the metal ion may force the amino acid and the pyridoxal to lie in the same plane which would increase the ease with which electrons are able to move in the whole system. In a number of these non-enzymic reactions catalyzed by pyridoxal the removal of the α hydrogen from the amino acid is an important step. The greater the pull on the electrons associated with the α carbon the greater the ease with which the hydrogen is lost. Therefore, a second possible role of metal ions in catalyzing non-enzymic amino acid conversions mediated by pyridoxal is to increase the drain on the electrons around the α carbon.

The catalytic effect of metal ions in reactions mediated by pyridoxal in model systems strongly suggest the possibility that these pyridoxylidene amino acid metal chelates function

as important intermediates in many of the vitamin B_6 - catalyzed amino acid transformations.

However, the role which these chelates play in reactions catalyzed by vitamin B_6-containing enzymes is still very much in contention. The two vitamin B_6-requiring enzymes, homoserine dehydrase (26) and glutamic aspartic transaminase (27), which have been obtained as nearly pure homogeneous preparations show no metal requirement. This can by no means be taken to discount the role which metal ions might play in other vitamin B_6 enzymes for it may be that fortuitously these enzymes are exception rather than general examples.

Moreover, it is very plausible that the role played in non-enzymic catalysis by vitamin B_6 by metal ions may in the enzyme be taken over by some group at the active center.

D. Summary

Pyridoxylidene amino acid chelates form readily in solution as is evident from spectral observations. The formation of these chelates with Cu(II) and Ni(II) has been studied. It was found that the principal chelates formed contained pyridoxal, metal ion and amino acid in the ratio 1:1:1. This was shown in the case of the copper chelate by isolation and analytical determination of composition and in the nickel case from the consistency in the formation constant, under conditions of low imine and high metal con-

centrations, when a ratio of 1:1:1 was assumed. Furthermore, the chelates accept one proton at acidic pH values on the ring nitrogen.

Formation constants for these chelates have been evaluated, along with the dissociation constants for the conjugate acids. The hydrochloride of the copper pyridoxylidene valine chelate has been isolated and its composition determined. A comparison is made between the stability of these chelates and their capacity to catalyze non-enzymic amino acid transformations.

III. THREONINE DEHYDRASE

A. Review of Pertinent Literature

The pioneer work on enzymes which mediate the formation of pyruvate and of α-ketobutyrate from serine and threonine respectively was done by Gale and Stephenson in 1938 (32) working with Escherichia coli. They were able to show the presence of an enzyme or enzymes which would convert DL-serine to pyruvate. Yanofsky isolated an enzyme from Neurospora which mediated the conversion of D-threonine and D-serine to the corresponding ketoacids (15). Reissig later isolated from Neurospora an enzyme which had activity only towards the L-isomers (14). Also a partially purified preparation from cells of E. coli has activity towards D-serine and DL-threonine (16).

In no case examined has an enzyme been isolated specific for threonine and free from serine activity or conversely.

Umbarger and Brown have studied the L- and D- dehydrases present in E. coli (33). A surprising finding was that there are two distinct L-threonine dehydrases in E. coli (33). One is activated by pyridoxal phosphate, AMP and glutathione and is adaptive. The second one requires only pyridoxal-5-phosphate and is constitutive. Umbarger observed that the constitutive enzyme was inhibited by isoleucine (34). This

isoleucine inhibition may have a biological control significance since isoleucine is synthesized from threonine (35).

Walker (36) has purified an L-threonine dehydrase from the rumen microorganism. Pyridoxal-5-phosphate, the cofactor, can be reversibly dissociated from the enzyme by lyophilization in the presence of glutathione and ammonium sulfate.

Much more difficulty has been experienced in trying to resolve the mammalian tissue threonine dehydrase. Sayre and Greenberg (18) in 1956 isolated L-serine and L-threonine-specific enzymes from sheep liver. However, each enzyme had activity towards the other compound. It is not quite clear from their paper how the authors distinquish between the two enzymes. They reported some reactivation of lyophilized samples with pyridoxal phosphate.

Nishimara (37) has extended the preliminary work done by Sayre and clains to be able to successfully resolve threonine dehydrase and to reactivate it with pyridoxal phosphate.

A review on threonine dehydrase and the enzymes which act on the other hydroxyamino acids, serine and homoserine, is forthcoming in volume 5 of The Enzymes, eds. Boyer, P.D., Lardy, H and Myrback, K., Academic Press.

B. Experimental

1. Stock solutions and reagents

Tris carbonate buffer pH 8.95 50 ml. of 1.0 M tris-hydroxymethylaminomethanl, obtained from Sigma Chemical Co., 4.2 gm. sodium bicarbonate and 2.3 meq. of hydrochloric acid were mixed and diluted to 100 ml.

Buffered substrate, 0.1 M 0.2978 gm. of L-threonine and 1.05 meq. of sodium hydroxide were mixed and diluted to 25 ml.

2,4-Dinitrophenylhydrazine reagent 250 mg. of 2,4-dinitrophenylhydrazine was dissolved im 500 ml. of 2.00 N hydrochloric acid and the solution filtered.

Sodium hydroxide, 2.5 N Bakers reagent grade sodium hydroxide was dissolved in boiled, deionized, distilled water.

25% Trichloroacetic acid 25 gm. of trichloroacetic acid was diluted to 100 ml.

Ammonium sulfate Was obtained in five pound lots from Mallinckrodt (analytical reagent grade). It was added as the solid form in all ammonium sulfate fractionations.

Amino acids Were commercial products with the exception of aspartic acid which was kindly supplied by Dr. H. J. Sallach.

Dipotassium diethylstilbestrol disulfate, dipotassium estradiol - 17 β disulfate and dipotassium β - diphenyl disulfate were kindly supplied by Dr. Merle Mason.

Diethylaminoethyl and carboxymethyl celluloses were obtained from Bio-Rad Laboratories.

Sheep liver was obtained warm from the slaughter room at Iowa Packing Co. and immediately cooled. The liver obtained in 10 to 20 pound lots was used right away or was frozen for future use.

Buffer solutions Buffers used were acetic acid-sodium acetate, sodium formate,-hydrochloric acid, potassium dihydrogen phosphate-disodium hydrogen phosphate, triethanolamine-hydrochloric acid, tris-hydroxymethylaminomethane-hydrochloric acid, cacodylic acid-sodium hydroxide. The molarity of these buffers is the total concentration of weak acid or base and its salt.

2. Analytical methods

The assay used is essentially that of Friedenman and Haugen (38) with a modification by Sayre and Greenberg (18). It is based upon the color produced by an akaline solution of the 2,4-dinitrophenylhydrazone of α keto acids. The product formed has an absorption maximum at 520 mμ. Sayre and Greenberg's modification consists of the addition of 2 ml. of 95% ethanol before the color developing base is added

in order to prevent the cloudiness which is sometimes observed on the addition of the base.

Standard curves for the determination of α-ketobutyric acid, pyruvic acid and α-ketoisovaleric acid were obtained using the 2,4-dinitrophenyl hydrazine method. Under our assay conditions, in a volume of 10 ml., an O.D. of one at 520 mu corresponded to 1.2 and 1.0 μ moles of α-ketobutyric acid and pyruvic acid, respectively. The standard curve for the α-ketoisovalerate was non-linear. Directions for assay are:

1. Place in a 16 x 150 mm test tube 0.3 ml. of pH 8.95, 0.5 M triscarbonate buffer. (See stock solutions and reagents).

2. Add 0.9 ml. consisting of the enzyme sample plus water or any other additives.

3. Let stand in a 37°C bath for at least 5 minutes.

4. Add 0.3 ml. of 0.1 M DL threonine and mix well.

5. Incubate at 37°C for 30 minutes.

6. Stop the enzyme action by adding 0.5 ml. of trichloroacetic acid (25% by weight).

7. Centrifuge down the denatured protein.

8. Pipette 1 ml. of the supernatent into a test tube containing 2 ml. of 0.05% 2,4-dinitrophenyl hydrazine in 2 N HCl. Let stand 15 minutes.

9. Add 2 ml. 95% ethanol, then 5 ml. of 2.5N NaOH. Mix well. Let stand for 10 minutes; then read in the spectrophotometer (520 mu) against a reagent blank.

Determination of protein In all the isolation and purification work described the protein was determined by the optical density at 280 mμ of a dilute solution of protein. As a general rule an optical density at 280 mμ of 1.0 for a 1.00-cm cell corresponds to about 1 mg. of protein per milliliter (39).

Definition of enzyme unit An enzyme unit has been arbitrarily designated as that amount of enzyme which will produce one micromole of ketoacid after incubation at 37°C for one hour at pH 8.95 in the presence of 0.02 M substrate.

Purity index The purity index used to follow the purification has been defined as the units per ml. divided by the optical density of one ml. of protein solution.

C. Results

1. Purification and isolation

a. Aqueous extract Fresh or thawed fresh frozen sheep liver was washed with cold distilled water to remove blood. The liver was ground in a meat grinder after removal of large vessels and connective tissue, to facilitate homogenization. The minced liver was then homogenized in a waring blender for 0.5 minutes with 2 volumes of 0.1 M pH 7.2

phosphate buffer. The activity obtained was used as the starting level, and all expressions of purification based on this value.

b. First heat treatment The crude homogenate was heated to 70°C in a boiling water bath and cooled, in ice baths, immediately when this temperature was reached. The head treated material was centrifuged in 250 ml. polyethylene bottles at 6000 R.P.M. for 15 minutes in a Serval model SS-4 in the cold room. The clear reddish-green supernatant was retained and the precipitate discarded.

c. First ammonium sulfate precipitation The clear supernatant from step b was made 40% saturated with ammonium sulfate at room temperature. In all ammonium sulfate fractionations solid material was added. The amount of solid ammonium sulfate to be added was taken from a table in Methods of Enzymology I, 76.

The 40% saturated solution was allowed to stand for 4 hours and then filtered with a Horman filter using a D-3 filter. Enough Celite hydroflow filter aid being added to form a ½ in. pad and to facilitate the filtration. The pads were discarded and the filtrate saved.

The solution from above was made 70% saturated with ammonium sulfate. The saturated solution was allowed to stand, usually overnight, sometimes longer. Usually at the end of this period of time the flocculant precipitate of

brownish-green protein had settled to the bottom of the beakers. This allowed the supernatant to be decanted by suction resulting in a smaller volume to be centrifuged. The suspension remained was then centrifuged for 15 minutes in tared 250 ml. polyethylene bottles at 6000 R.P.M. in the Serval model SS-4 in the cold room. The precipitate was retained and the supernatant discarded. More protein suspension was added and the centrifugation repeated until all the protein had been collected.

The weight of the protein pad plus 70% ammonium sulfate was obtained by weighing the polyethylene bottles plus protein and ammonium sulfate and knowing the weight of the bottles.

The protein was dissolved in sufficient 0.1 M pH 7.2 phosphate buffer to give an optical density reading at 280 mu of 30 to 40 O.D. units per ml. which corresponded to about 30 to 40 mg. of protein per ml. Knowing the concentration and total weight of protein, the amount of 70% saturated ammonium sulfate remaining in the solution was calculated. This amount was then substracted from subsequent amounts to be added for further fractionations.

d. Second ammonium sulfate precipitation The solution from step c was made 30% saturated with ammonium sulfate, the precipitate discarded and the supernatant

retained. The solution was then made 50% saturated which resulted in a precipitate with the highest enzyme activity. The enzyme was resuspended in phosphate buffer pH 7.2, 0.1 M.

e. Second heat treatment The solution from step d in 500 ml. erlenmeyer flasks, was heated in a constant temperature bath set at 70°C. Once the solution reached 70°C it was heated for five minutes and then cooled in an ice bath. The denatured protein was centrifuged out in the large head of the Spinco model L refrigerated centrifuge at 30,000 R.P.M. for 30 minutes. The supernatant was a clear amber solution while the protein discarded was dark green or black in color.

The enzyme at this point was reprecipitated from 60% saturated ammonium sulfate at room temperature three or four times. The enzyme at this stage was ready for further purification on diethylaminoethyl cellulose or for use in kinetic work.

A sample purification chart is presented in Table 3 for working up several batches of sheep livers.

f. Preparation of the adsorbent column Two cellulose adsorbents DEAE (diethylaminoethyl) cellulose and CM (carboxymethyl) cellulose were used (40). First the adsorbents was adjusted to the desired pH with phosphate buffer. The adsorbent was washed several times with buffer of the chosen pH and ionic strength on a filter. Non-sedimenting material was removed by decantation. The adsorbent was then poured as a

Table 3. Purification of threonine dehydrase

Fraction	Volume (ml.)	Milligrams[a] protein	Threonine units	Specific[b] activity
Homogenate	10,000	1,620,000	64,000	0.04
1st Heat Treatment	9,080	270,000	77,500	0.28
1st $(NH_4)_2SO_4$ Fraction	1,820	80,080	68,000	0.85
2nd $(NH_4)_2SO_4$ Fraction	400	9,000	69,200	7.55
2nd Heat Treatment	400	2,250	67,200	30.00
DEAE Fraction 31	10	20	3,960	198
DEAE Fraction 32	10	20	4,000	200
Homogenate	9,000	1,710,000	51,750	0.03
1st Heat Treatment	8,000	470,000	59,500	0.13
1st $(NH_4)_2SO_4$ Fraction	1,630	97,000	40,500	0.42
2nd $(NH_4)_2SO_4$ Fraction	300	10,000	41,000	4.1
2nd Heat Treatment	300	2,500	39,700	15.9
Homogenate	18,000	2,880,000	165,000	0.057
1st Heat Treatment	16,000	624,000	170,000	0.27
1st $(NH_4)_2SO_4$ Fraction	3,300	21,450	103,080	4.83
2nd $(NH_4)_2SO_4$ Fraction	800	12,000	98,072	8.17
2nd Heat Treatment	760	2,300	97,040	42.17

[a]Milligrams protein taken to equal total optical density at 280 mμ.

[b]Specific activity = $\frac{\text{micro moles ketoacid}}{\text{hour mg. protein}}$

slurry into a column of desired size. The column was allowed to settle under gravity. After the settling was nearly complete the adsorbent was further compacted by the application of air pressure at 10 p.s.i. until a constant column height was obtained. After such preparation the column would not run dry under gravity flow.

The packed column was then mounted above a fraction collector in a cold room (5^{0}) and washed with several column volumes of the same buffer to ensure pH and temperature equilibration. Approximately 10 g. of adsorbent was used to prepare packed columns of 20-25 x 2 cm. The exact height depended on the absorbent employed.

g. Preparation of the sample The enzyme sample was obtained directly from the second heat treatment step in the purification scheme. It was reprecipitated from 60% saturated $(NH4)_2SO_4$ 4 or 5 times. The last time the sample (approximately 100 mg.) was taken up in about six ml. of buffer of same pH and ionic strength of that with which the column had been equilibrated. The clear amber solution was applied to the column and allowed to enter the adsorbent under the conditions imposed by gravity.

h. Development of the column After the enzyme sample had been applied to the column it was further washed on with several 1 ml. portions of buffer. After this washing was completed the continuous flow was started. Potassium

phosphate buffers were used, as well as potassium phosphate buffers with added KCl. Flow roles from 8 to 10 ml. per hour were achieved with a total hydrostatic head of from 30 to 40 in. After the completion of the runs, the adsorbents were regenerated with 0.1 N NaOH (40).

i. Examination of the effluent The content of each test tube was examined in a Beckman DU Spectrophotometer at 280 mμ (39). Volume and pH measurements when necessary were made at room temperature. The activity of various fractions was determined by the 2-4-dinitrophenyl-hydrazone method.

Samples ranging in size from 25 to 100 mg. were placed on prepared DEAE and CM cellulose columns. At this time we have not been able to effect any purification of the enzyme using CM cellulose. When a 100 mg. sample was placed on the column and an elution gradient attempted using 0.005 M to 0.1 M phosphate buffer at pH 7.2 the protein came off the column without any separation. Probably the CM cellulose may prove effective at some other pH or different elution gradient. This problem demands further investigation.

More success was obtained using DEAE cellulose. A gradient elution was created using phosphate buffer. The gradient was run from 0.005 M buffer to 0.1 M and in a separate experiment from 0.5 M phosphate to 0.1 M phosphate. This column purification step usually resulted in from a 6

to 7 fold purification depending on the purity of the material placed on the column.

An elution pattern for the chromatography of the enzyme on DEAE is shown in (Figure 8). A large broad peak came off early in the elution which contains little or no activity. Later the main peak came off in tubes 30 to 35. This peak accounts for most of the enzyme activity. It is rather symmetrical which indicates a fair degree of homogenity in the enzyme.

2. Substrate specificity

It was found that the purest threonine preparation was active for L-threonine, L-serine and DL-allo threonine. The enzyme showed no activity toward a number of other amino acids substituted in the β and ϑ positions. The results of the specificity of threonine dehydrase are summarized in Table 4. This specificity confirms the report of Nishimara (37).

3. Stability of enzyme as a function of pH

To determine the stability of the enzyme at various hydrogen ion concentrations the following experiment was carried out. A two ml. sample of a stock solution of enzyme which had been diluted one to twenty five with distilled water was mixed with two ml. of 0.01 M buffer. Buffers used were ammonium formate, ammonium acetate,

Figure 8. Elution pattern for the chromatography of threonine dehydrase from diethylaminoethyl cellulose. -⊡- Threonine units.

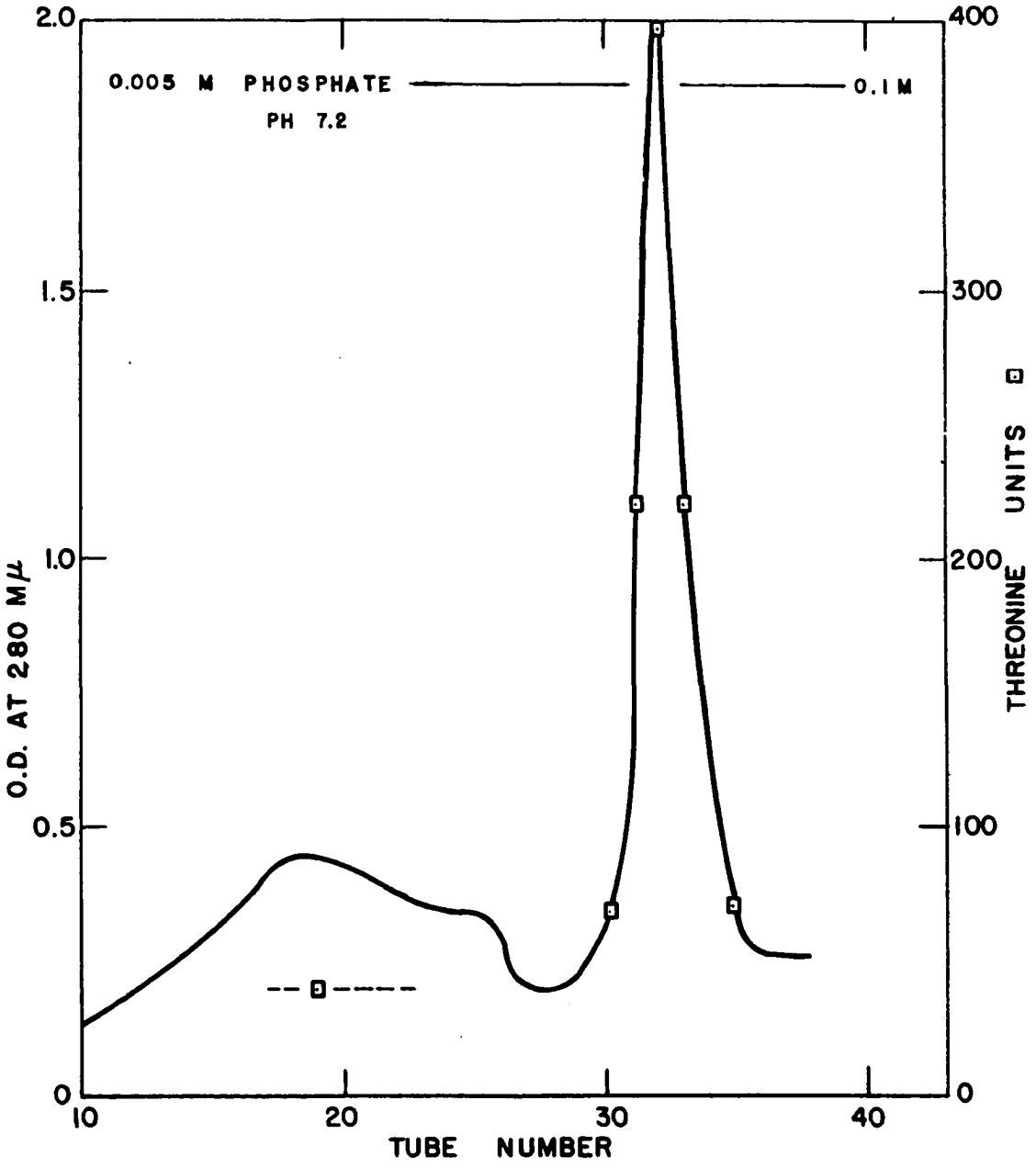

0.005 M PHOSPHATE
PH 7.2
0.1 M
O.D. AT 280 Mμ
THREONINE UNITS
TUBE NUMBER
2.0
1.5
1.0
0.5
0
400
300
200
100
0
10
20
30
40

Table 4. Substrate specificity

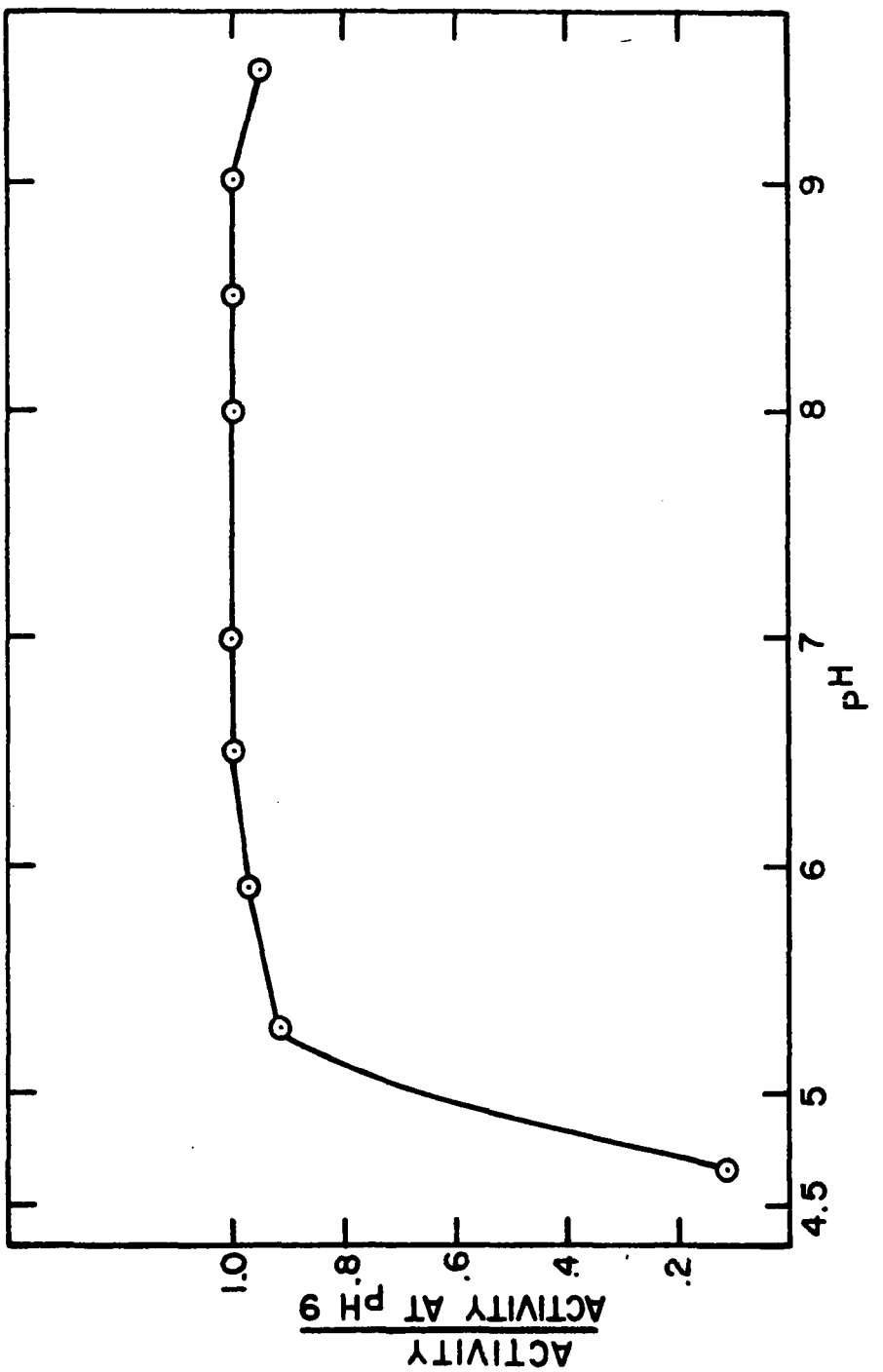
Substrate	μ moles keto acid produced by 1 unit[a]
L-threonine	1.00
L-serine	.14
D-threonine	00
D-serine	00
DL-(allo) threonine	.27
DL-(allo) hydroxy aspartic	00
DL-(threo) hydroxy aspartic	00
DL-seryl alanine	00
DL-serine methyl ester	00
O-phosphoserine	00
L-cysteine	00

[a]1 unit of enzyme produces 1 μ mole keto acid per hour at pH 8.95 (tris carbonate buffer) at 37° in the presence of .02 M substrate.

succinate, phosphate, tris, tris carbonate, and TEA. The samples thus prepared were allowed to stand at room temperature for four hours. A sample was taken from each and diluted one to five and 0.9 ml. of this sample used to assay for enzyme activity at pH 8.95 using tris carbonate buffer.

Figure 9 shows the results obtained. The enzyme is stable over the pH range 5.5 to 9.5. On either side of these

Figure 9. Stability of threonine dehydrase as a function of pH


ACTIVITY
ACTIVITY AT pH 9
1.0
.8
.6
.4
.2
4.5
5
6
7
8
9
pH

two pH values the enzyme is inactivated. These results define the range in which it is safe to investigate various properties, such as the maximum velocity and the Michaelis constant as a function of pH without inactivation. Some indications have been obtained that the range of 5.5 to 9.5 may be extended in the presence of substrate. This will bear further investigation. If this proves to be true it will allow an extension of measurements over a wider pH range.

4. Ketoacid production as a function of time and enzyme concentration

Under assay conditions, that is high substate, the production of α ketobutyric acid was linear up to two hours (Figure 10). After this time it starts to fall off due to a lowered concentration of substrate. In the presence of excess substrate the reaction was zero order with respect to substrate.

When the production of α ketobutyric acid was followed as a function of enzyme concentration straight lines were obtained at 10 and 30 minutes. (Figure 11). This implies that under conditions of high substrate the reaction was first order with respect to enzyme concentration.

When serine was used as substrate there was an initial burst of product with a very rapid termination of enzyme

Figure 10. α-Ketoacid production as a function of time

A. Substrate serine pH 9
15.5 enzyme units

B. Substrate threonine pH 9
1.06 enzyme units

C. Substrate serine pH 7
15.5 enzyme units

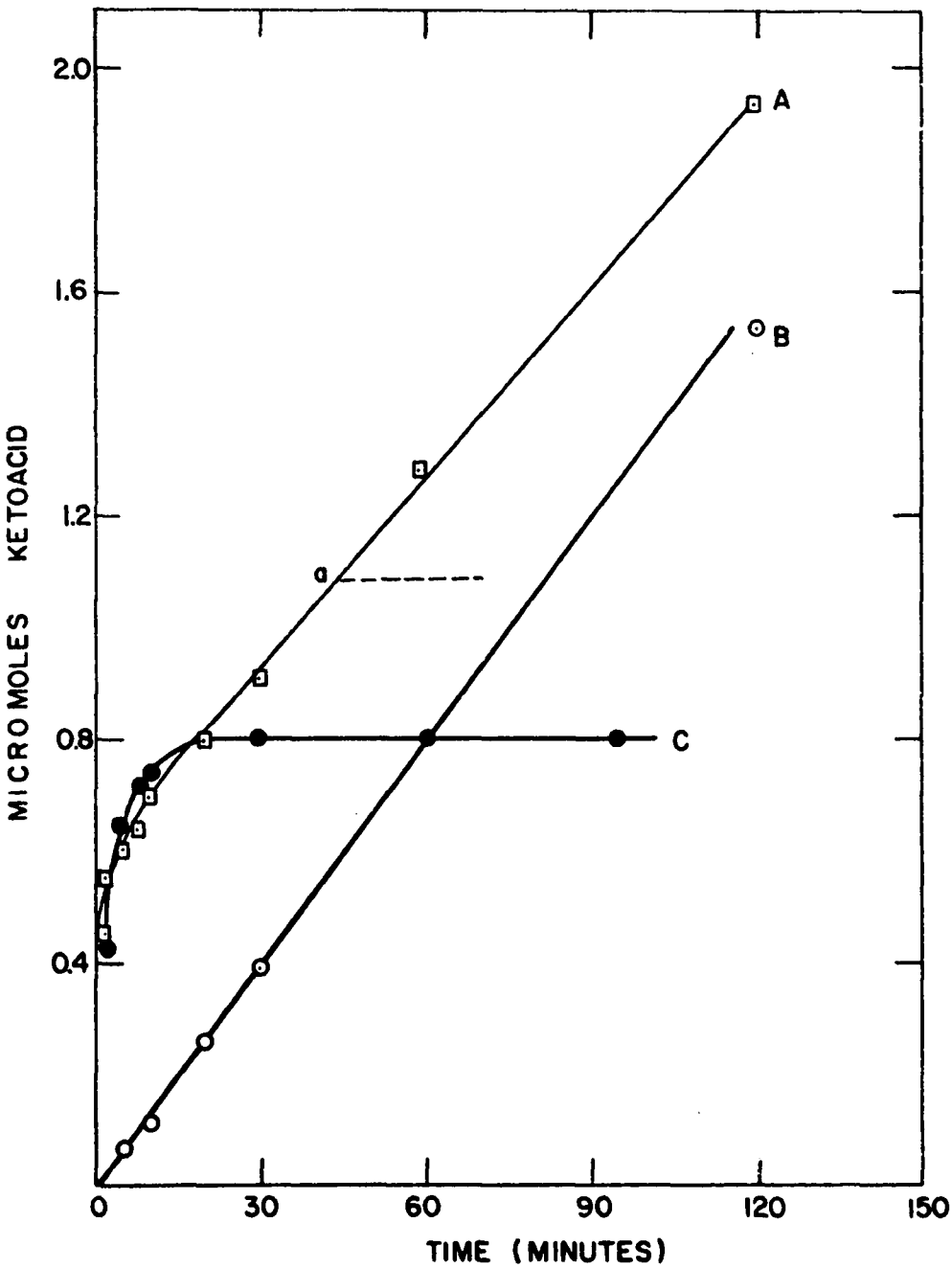
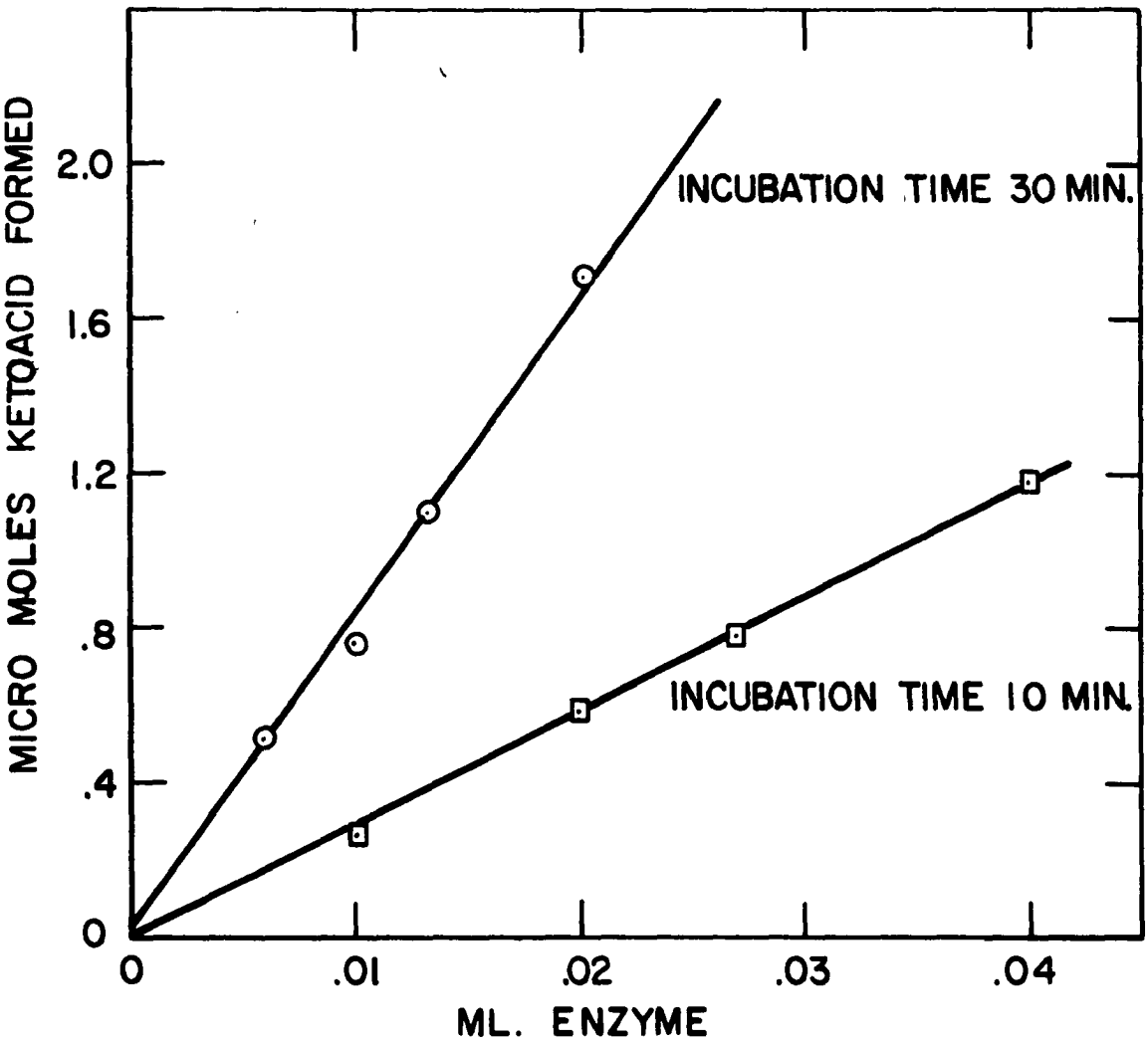

2.0
1.6
1.2
0.8
0.4
MICROMOLES KETOACID
A
B
C
a
0
30
60
90
120
150
TIME (MINUTES)

Figure 11. α-Keto acid production as a function of enzyme concentration


MICRO MOLES KETOACID FORMED
2.0
1.6
1.2
.8
.4
0
0
.01
.02
.03
.04
ML. ENZYME
INCUBATION TIME 30 MIN.
INCUBATION TIME 10 MIN.

activity when the reaction was run at pH 7 (Figure 10). When the reaction was run at pH 9 the initial burst was observed but at this pH the reaction did not cease but continued for up to two hours, but at a slower rate.

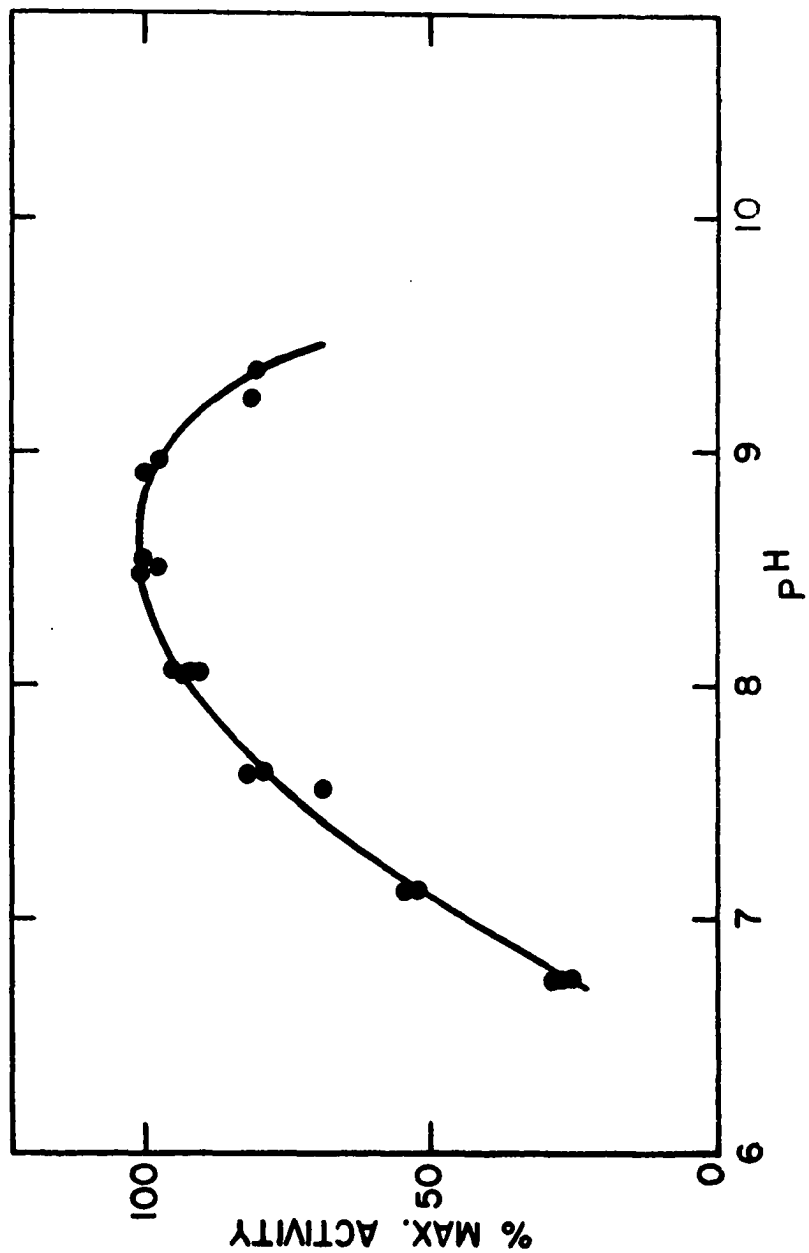
Now if threonine was added say at point B on curve 3 (Figure 10) the further production of product either from serine or threonine stopped. Therefore it appears that the enzyme has both serine and threonine activity when incubated with each alone. In the presence of both, however, the enzyme has no activity towards either.

5. pH Optimum

The pH optimum of threonine dehydrase was determined. Contrary to Sayre and Greensberg's report (18) it was found to have a fairly broad peak of maximal activity over the pH range 8.5 to 9 and not at 7. The optimum pH was determined using phosphate, tris, tris-carbonate and triethanolamine buffers (see methods and materials). A range from pH 6.75 to 9.4 was covered. Figure 12 shows per cent of maximum activity as a function of pH. The maximum activity was taken as the activity observed under assay conditions; that is pH 8.95 in tris-carbonate buffer in the presence of 0.02 M substrate.

The pH optimum of 8.5 to 9 corresponds very favorably with those of serine dehydrase from rat liver (41-42) and

Figure 12. pH Optimum of threonine dehydrase


% MAX. ACTIVITY
100
50
0
6
7
8
9
10
pH

the homoserine dehydrase from rat liver (43). The values are given below in Table 5.

Table 5. pH Optima for dehydrases

Serine	8.3
Homoserine	8.0
Threonine	8.5-9

These pH optima are a little high for physiological conditions; however, it is often observed that pH optima are a function of substrate concentration. The pH optimum of threonine dehydrase was determined under conditions of high substrate concentration. However, when the activities were measured under conditions of low substrate in the determination of the Michaelis constant as a function of pH the same maximum occurred. Therefore, the pH optimum of threonine dehydrase does not change as a function of substrate concentration.

6. Variation of Michaelis Constant and maximum velocity with pH

A series of experiments were set up which differed only in the concentration of substrate. Using the linear relationship developed by Lineweaver and Burk (44) (vide

infra) 1/v against 1/s was plotted which resulted in a family of straight lines when the pH was varied (Figure 13). By drawing the most extreme lines possible through the 1/v vs. 1/s data a variation of $\pm 1 \times 10^{-4}$ in K_m was observed. As most values of K_m were 1×10^{-3} or above, a maximum variation on the logarithmic scale of ± 0.1 logarithmic unit can occur. 1/V was obtained from the intercepts and $\frac{K_m}{V}$ from the slopes. K_m was obtained by dividing the slope by the intercept.

$$1/v = \frac{(K_m)(1/s)}{V} + 1/V$$

v = initial velocity of one unit of enzyme[a]

K_m = Michaelis constant

s = substrate in moles per liter

V_{max} = maximum velocity

From graphs like the ones shown in Figure 13 and others not shown it is observed that the V_{max} values do not change with pH. When the values of V_{max} are plotted against pH a straight line is obtained.

It may be well at this time to point out some of the assumptions made in dealing with kinetic functions, K_m and V_{max}. First, it is assumed that the enzyme reacts with the

---

[a]One unit of enzyme produces an optical density of one under assay conditions (pH 8.95, .02 L-threonine, 30 min.).

Figure 13a. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture

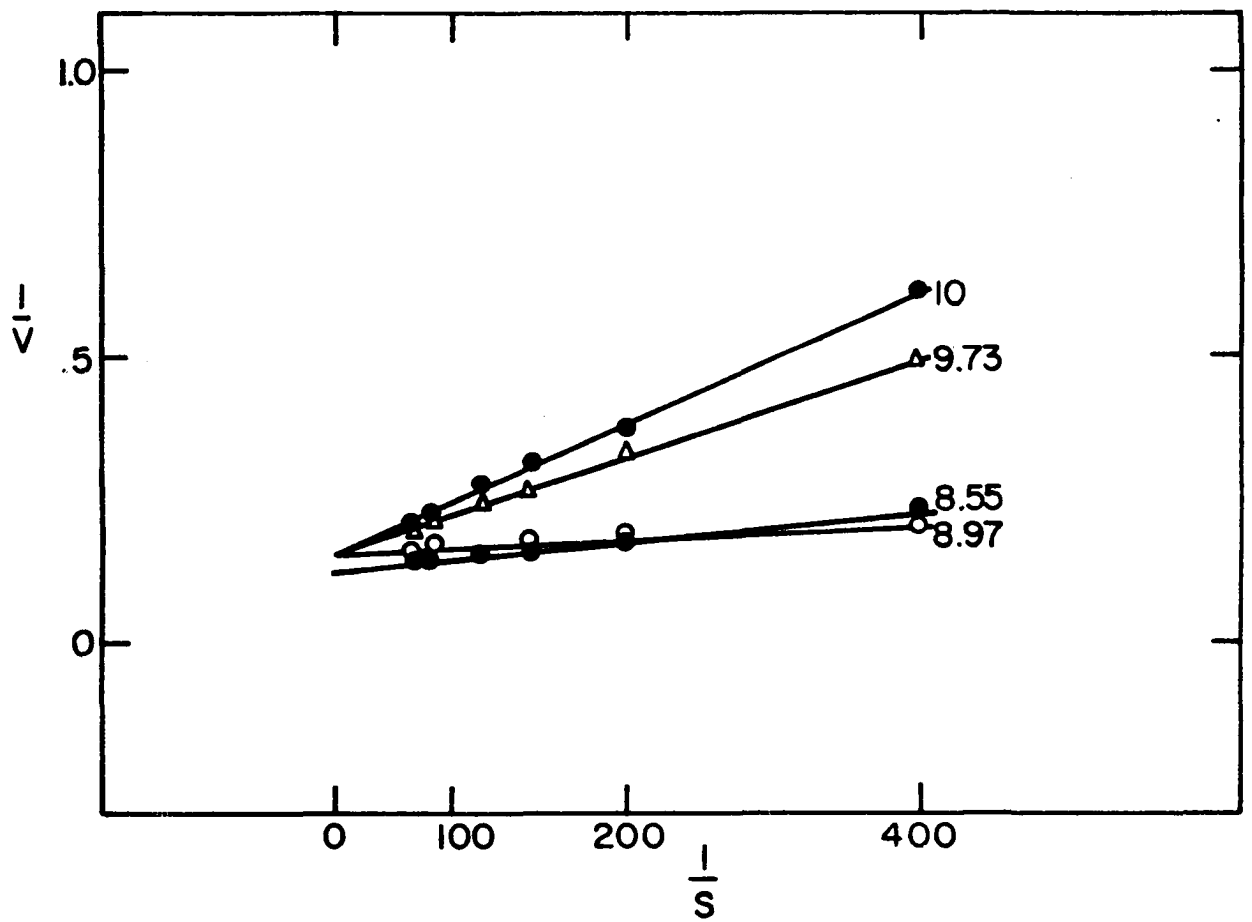

1.0
.5
0
1/V
10
9.73
8.55
8.97
0
100
200
400
1/S

Figure 13b. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture

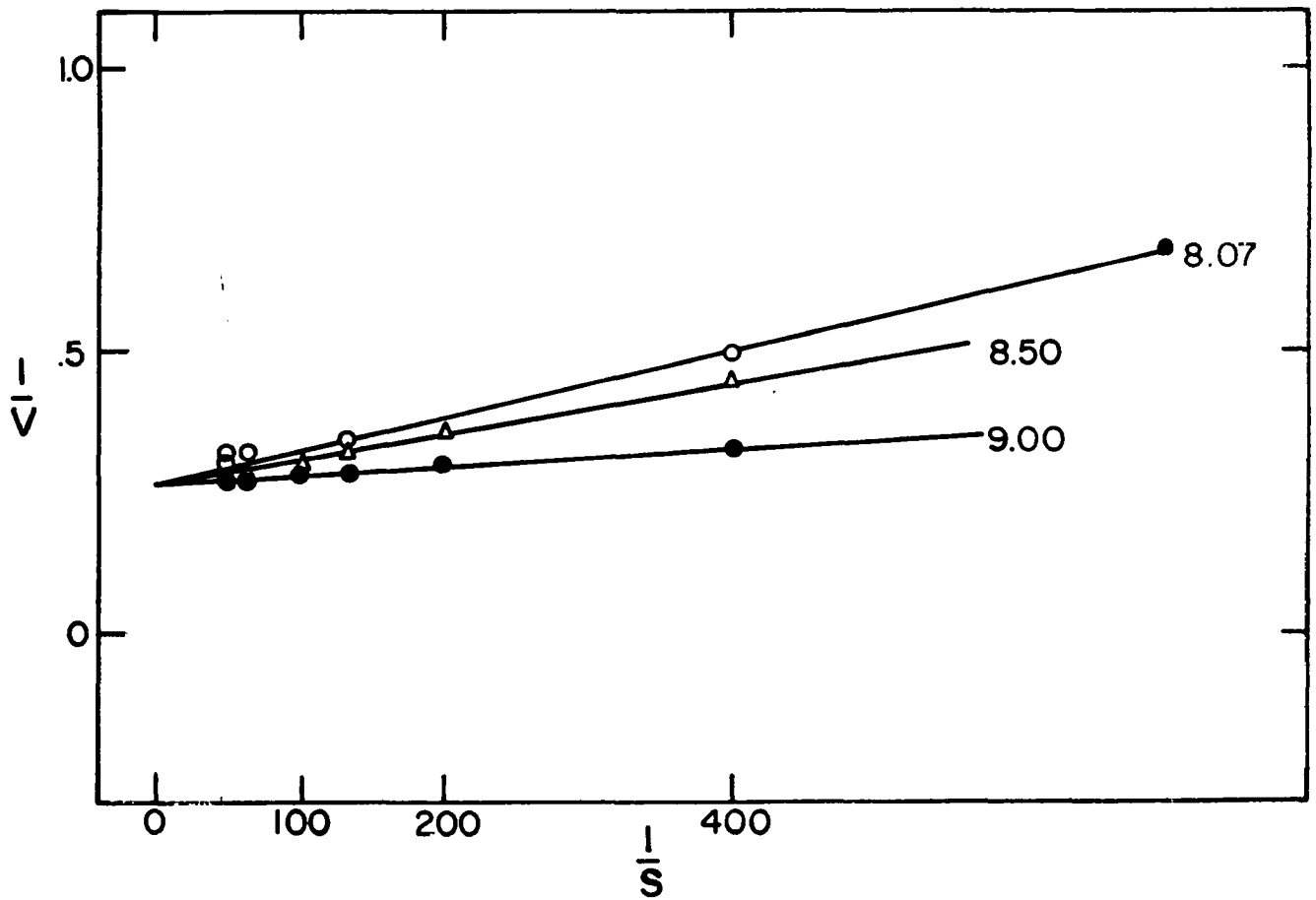

1.0
.5
0
1/V
0
100
200
400
1/S
8.07
8.50
9.00

Figure 13c. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture

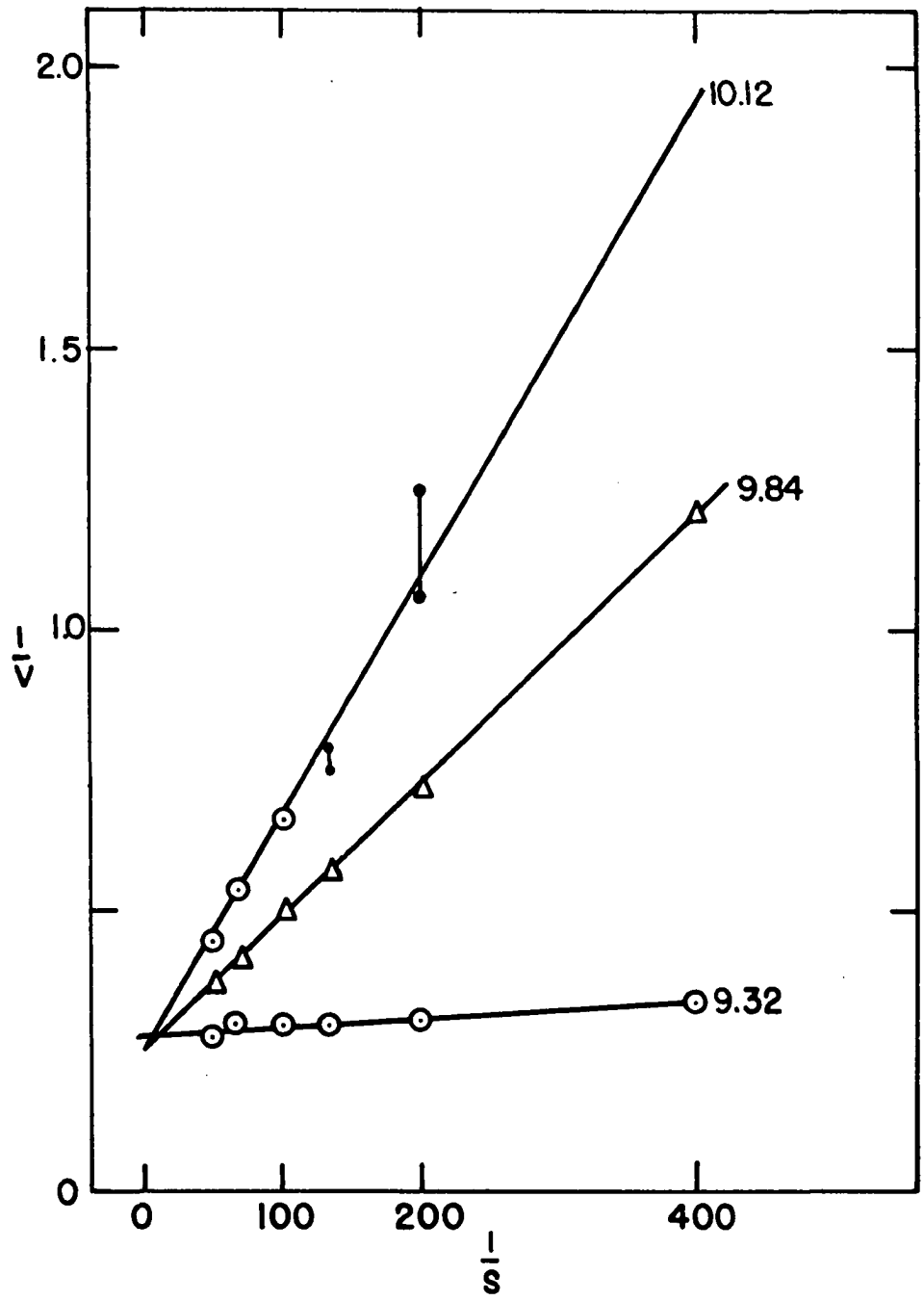

2.0
1.5
1.0
0
1/V
0
100
200
400
1/S
10.12
9.84
9.32

Figure 13d. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture

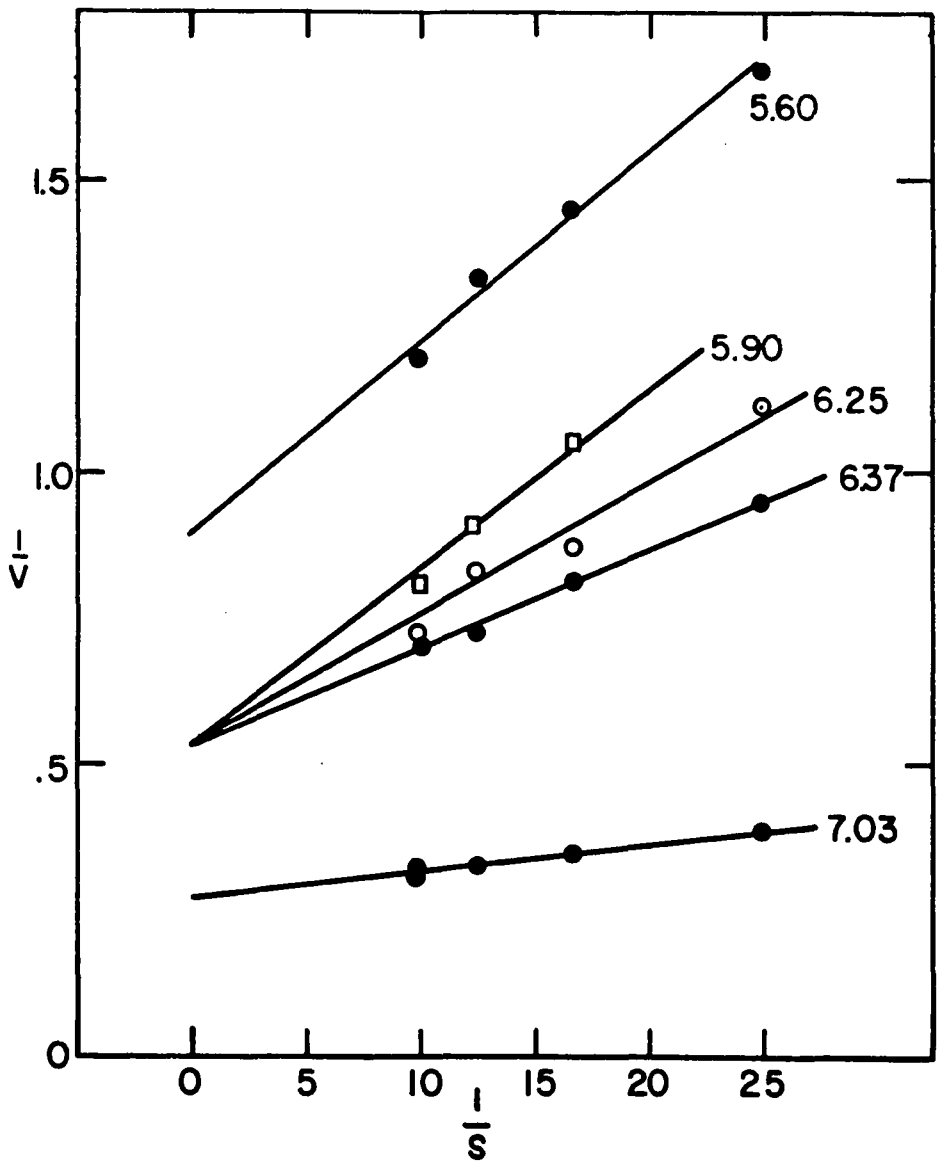

5.60
5.90
6.25
6.37
7.03
1.5
1.0
.5
0
1/V
0
5
10
15
20
25
1/S

Figure 13e. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture

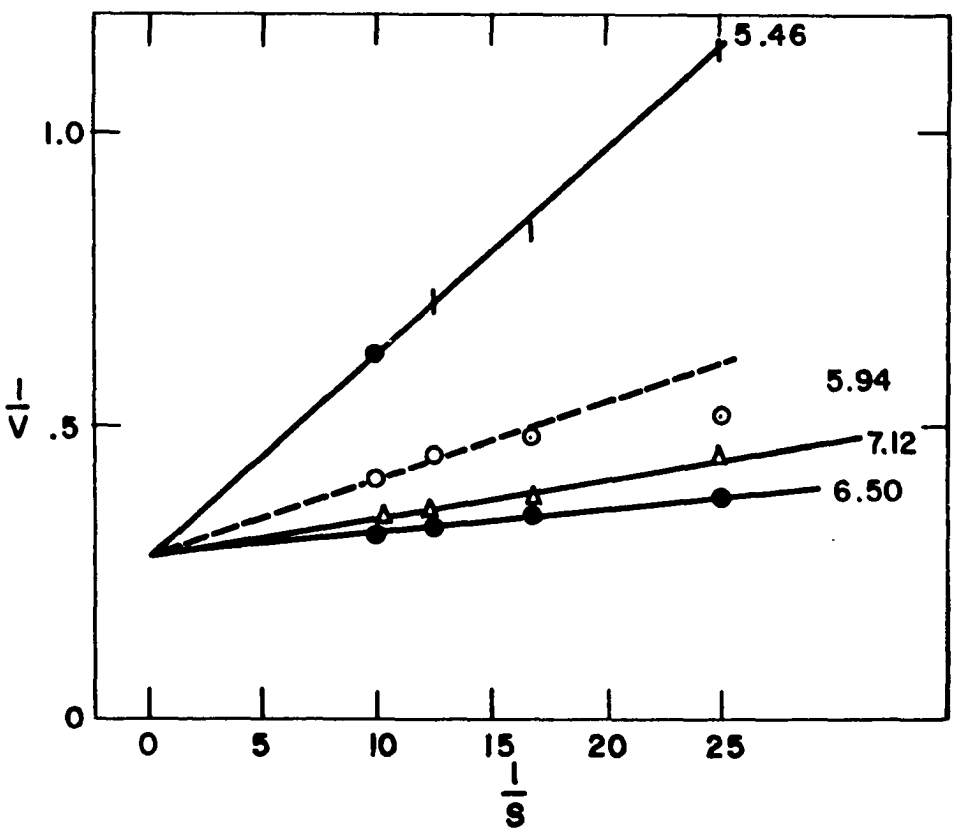
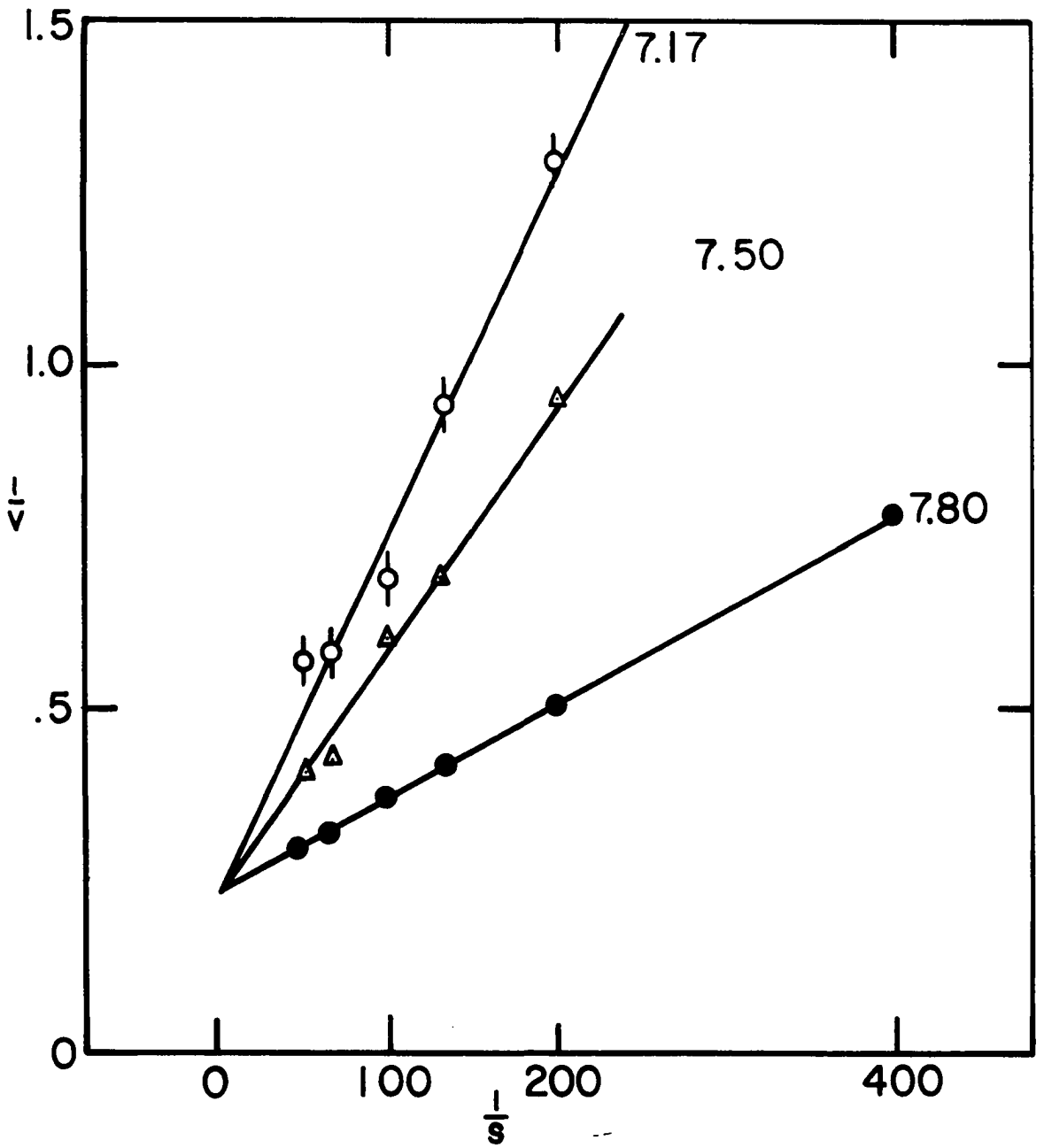

5.46
5.94
7.12
6.50
1.0
.5
0
1/V
0
5
10
15
20
25
1/S

Figure 13f. Reciprocal 1/s vs. 1/v for threonine dehydrase. Numbers on graph indicate pH at 25° of reaction mixture


1.5
1.0
.5
0
1/v
0
100
200
400
1/s
7.17
7.50
7.80

substrate to form a Michaelis complex which can either decompose into product and free enzyme in a non-reversible fashion (reaction 3 vida infra) or by a reverse of reaction 1, -2 into enzyme and substrate.

$$E + S \underset{1}{\overset{-2}{\rightleftharpoons}} ES \xrightarrow{3} E + P$$

E = enzyme

ES = activated complex

S = substrate

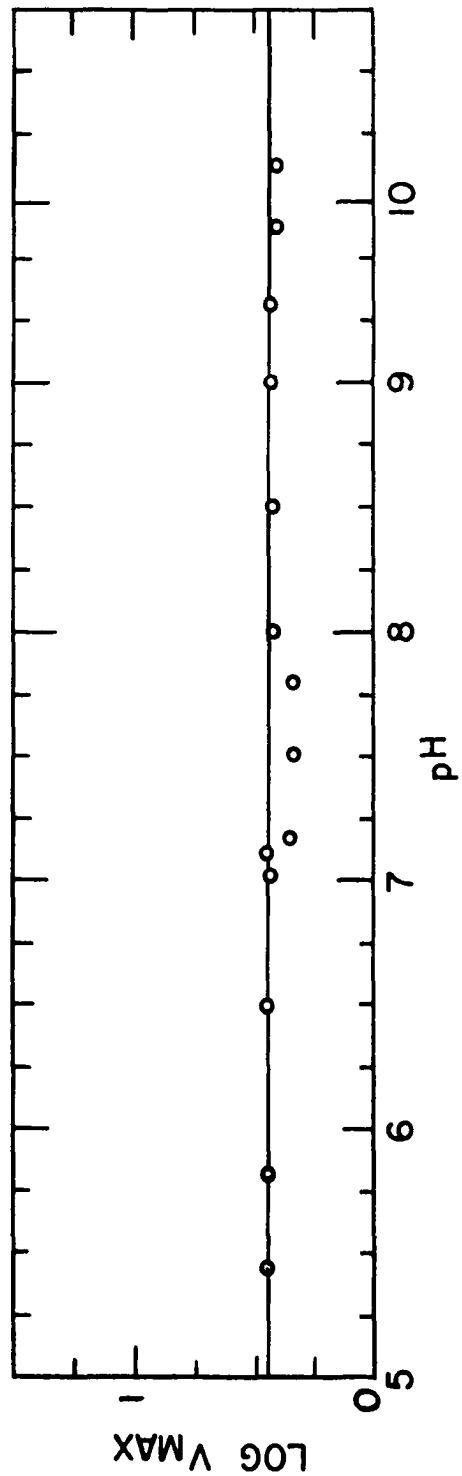
P = product

The conditions under which V_{max} was determined, that was high substrate, all the enzyme is in the ES form and the velocity should only be affected by factors which affect k_3 the rate constant for the breakdown of ES into E and P. Therefore, if there were any dissociable groups on this ES complex which had any affect on the rate of its breakdown they would manifest themselves in a change in V_{max} (see Dixon and Webb (45)).

Since as shown in Figure 14 a graph of log V_{max} against pH over the range 5.5 to 10 results in a straight line we have concluded that ES has no groups which dissociate within this pH range.

According to the theory of Webb which gives general rules for interpretation of changes in V_{max} and K_m as a function of pH; changes in K_m as a function of pH are due

Figure 14. Log_{10} of maximum velocity as a function of pH for threonine dehydrase

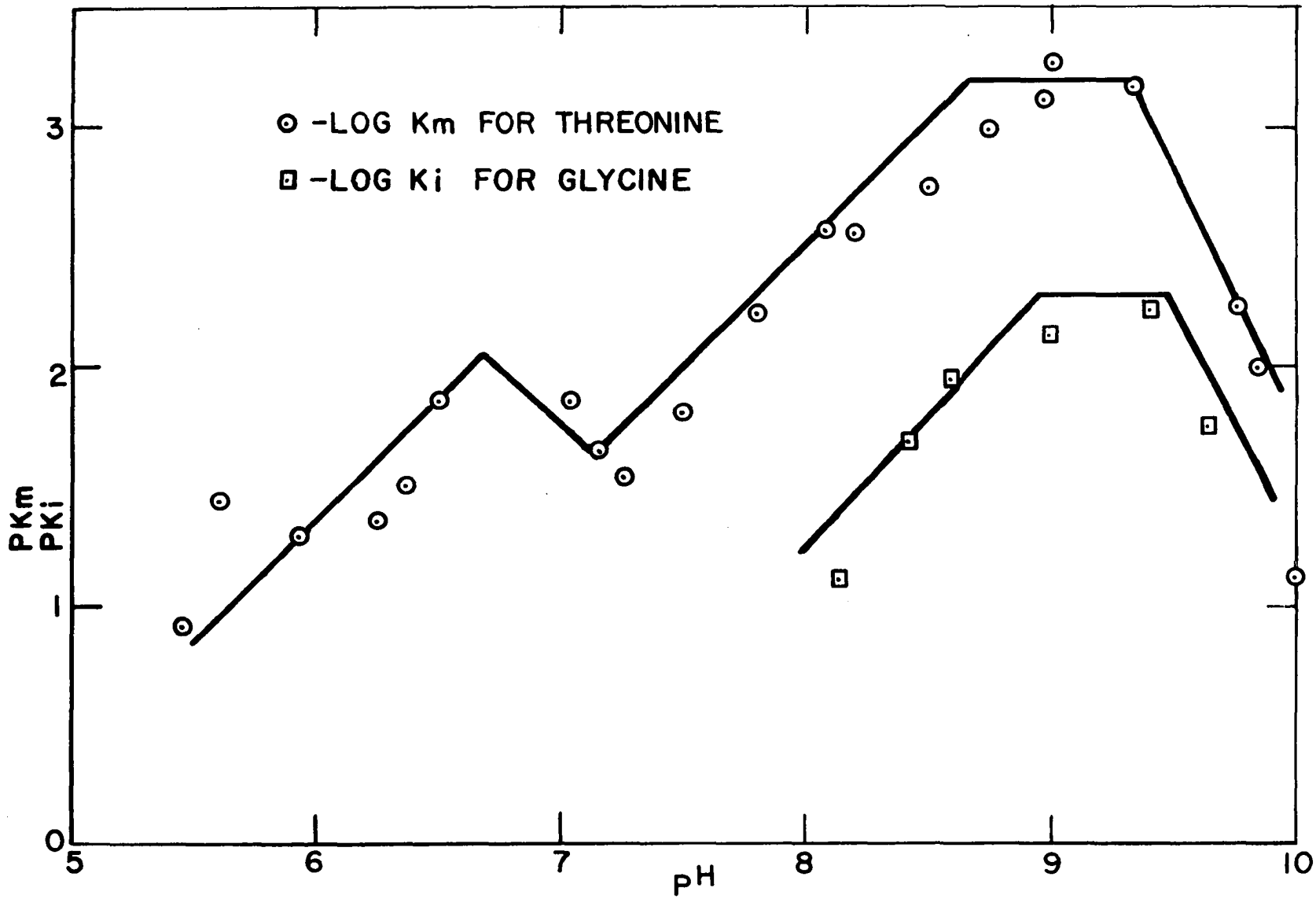

LOG VMAX
0
-1
5
6
7
8
9
10
pH

to dissociable groups present on the free enzyme, the free substrate and the enzyme substrate complex.

The rules of Webb for interpretation of changes in slope as depicted for threonine dehydrase in (Figure 15) are:

a. The graph of pK_m will consist of straight-line sections (if the pK values are sufficiently separated) joined by short curved parts.
b. The straight portions have integral slopes, i.e. zero or one-unit or two unit slopes, positive or negative.
c. Each bend indicates the pK of an ionizing group in one of the components and the straight portions when produced intersect at a pH corresponding to the pK.
d. Each pK produces a change of 1 unit in the slope.
e. Eack pK of a group situated in the ES complex produces an upward bend, i.e. an increase of (positive) slope with increase of pH, or, in other words, a bend with the concave side upwards; each pK of a group situated in either the free enzyme or the free substrate produces a downward bend.
f. The curvature at the bends is such that the graph misses the intersection point of the neighboring straight parts by a vertical distance of 0.3 units

Figure 15. Negative $\log_{10}$ of the Michaelis constant and inhibitor constant for glycine as a function of pH


⊙ -LOG Km FOR THREONINE
⊡ -LOG Ki FOR GLYCINE
PKm
PKi
0
1
2
3
5
6
7
8
9
10
pH

(=log 2); if two pK's occur together the distance is equal to log 3.

In (Figure 15) we have graphed the negative log of K_m as a function of pH. We note that the values of K_m are highly pH dependent in contrast to the maximum velocity.

The significance of the slopes and inflections in this graph are as follows. First the inflections indicate pK's of groups associated with the free enzyme and the free substrate. We have already established the fact that there are no dissociable groups associated with the enzyme substrate complex from the behavior of V_{max} as a function of pH. Therefore, these changes we observe in K_m as a function of pH must be due to dissociable groups on the free enzyme and substrate.

We are able to determine the pK of the substrate (threonine) independently. It was found to be 8.3 at 37°. Therefore the inflection at pH 8.5 is in all probability due to the pK of the substrate. The line is drawn with a slope of one which means one proton is released or there is one group present with this pK. This implies that the non-protonated form of the substrate (the threonine anion) is bound more readily by the enzyme than the dipolar ion. This is consistent with the proposed mechanism of catalysis by pyridoxal-5-phosphate whereby the amino group of the amino acid reacts with the 4- formyl group of pyridoxal-5-

phosphate in the initial step of the dehydration. It is known from model system studies that the amino acid anion reacts much more readily with pyridoxal to form Schiff bases than the dipolar ion (21). This is due to the fact that the lone pair of electrons on the nitrogen are more available for a electrophilic attack on the carbonyl group of pyridoxal.

The curve at pH 9.3 changes from a slope of 0 to a slope of 2. This indicates two groups on the enzyme which when ionized affect the binding of the substrate to the enzyme. That is to say when these groups are ionized threonine is not bound to the enzyme as readily as when these groups are protonated. Probably when these groups ionize negative charges are produced which oppose the combination of the negative threonine anion with the active center.

Little quantative data could be obtained from the region of pH 5.5 to 7. This was due to the fact that a definite but non-predictable buffer effect was noted. The substrate seemed to be bound more readily in the presence of phosphate and cacodylate than in the presence of imidazol. In order to be able to follow the reaction by the 2-4-dinitrophenylhydrazine method in this region the amount of enzyme had to be increased. Since the enzyme was not pure and there was contaminating protein present, a large amount of buffer had

to be added to main buffering capacity. The pH behavior in this region can probably be more critically evaluated when purer enzyme preparations are available.

In order to account for the pH optimum in the Michaelis constant it is necessary to postulate two ionizable groups on the enzyme and one on the substrate. In the following scheme the enzymic site is represented as a dibasic acid EH_2

$$\begin{array}{ccccc} E & & & & \\ \downarrow\uparrow\ K_{ae} & & & & \\ EH & & SH & & \\ \downarrow\uparrow\ K_{be} & + & \downarrow\uparrow\ K_{as} & & \\ EH_2 & & S^- & \underset{k_1}{\overset{k_2}{\rightleftharpoons}} & H_2ES \xrightarrow{k_3} H_2E + P \end{array}$$

The acid dissociation constants K_{ae}, K_{be} and K_{as} are of the usual type for a dibasic acid

$$K_{ae} = \frac{(H^+)(EH)}{(EH_2)}, \quad K_{be} = \frac{(H^+)(E)}{(EH)}, \quad K_{as} = \frac{(H^+)(S^-)}{(HS)}$$

The steady state treatment of this mechanism has been worked out by several investigators (46-48). This treatment yields the Michaelis equation for the initial velocity with the pH dependence of the maximum initial velocity V_m and the Michaelis constant K_m given below with Eo equal to enzyme in all forms.

$$V_m = \frac{k_3 (Eo)}{\left(1 + \frac{(H^+)}{K_{aes}} + \frac{K_{bes}}{(H^+)} \quad \ldots.\right)} \qquad (1)$$

$$K_m = \frac{k_2 + k_3}{k_1} \quad \frac{\left(1 + \frac{K_{ae}}{H^+} + \frac{K_{ae}\, K_{be}}{(H^+)^2}\right)\left(1 + H^+/K_{as}\right)}{\left(1 + H^+/K_{aes} \quad K_{bes}/H^+ \quad \ldots.\right)} \qquad (2)$$

It is observed that V_m has no pH dependence; therefore, the denominator for equation 1 is 1. Since K_m has the same denominator it must be 1 in this case also. This leaves us with the pH dependence in equation 2 dependent on ionization of enzyme and substrate. Since K_{as} can and has been determined independently this dependence $(1 + H/K_{as})$ can be removed by dividing K_m by this quantity. Therefore, when log $\frac{K_m}{(1+H/K_{as}}$ is plotted vs. pH and inflections in the curve must be due to groups on the enzyme. The results of such a treatment is presented in (Figure 16). The equation

$$\frac{K_m}{1 \quad H/K_{as}} = \frac{(k_2 + k_3)}{k_1} \left(1 + \frac{K_{ae}}{H^+} + \frac{K_{ae}K_{be}}{(H^+)^2}\right)$$

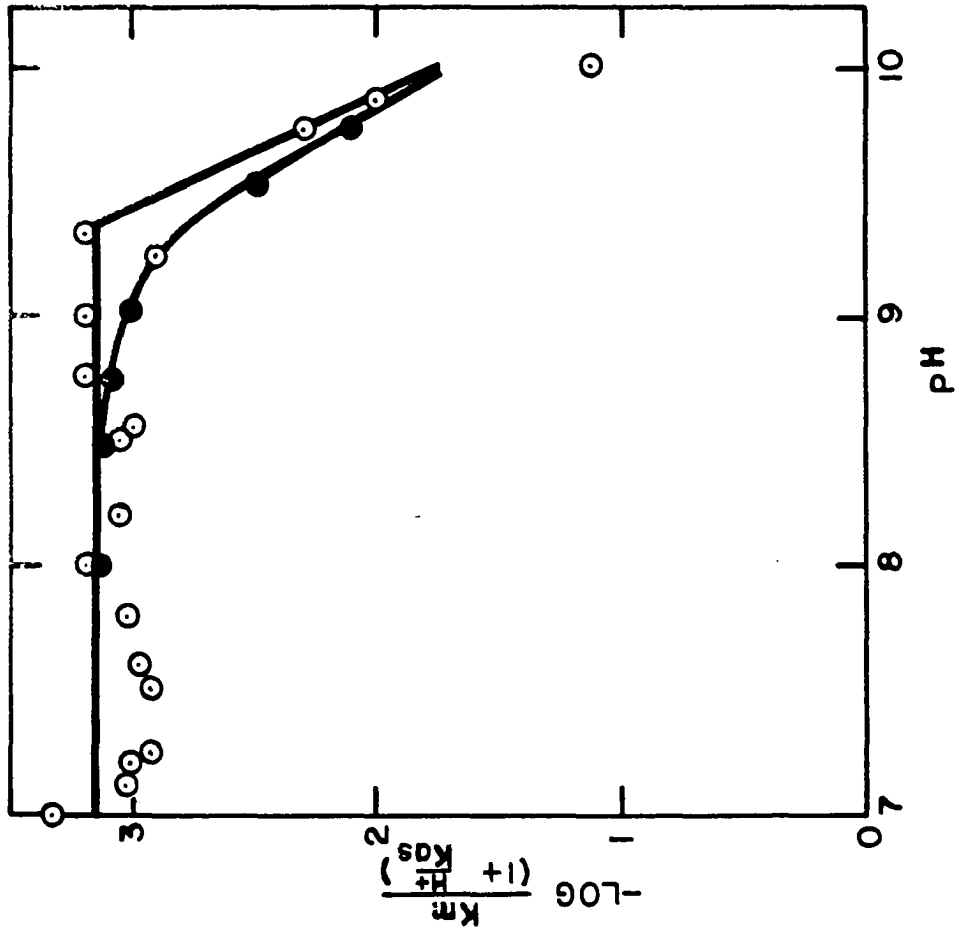
has been used to calculate the theoretical lines.

Since K_{ae} and K_{be} appear as one break in the graph of $-\log \frac{K_m}{(1+ H/K_{as})}$ vs. pH, in calculating the theoretical lines in (Figure 16) it is assumed that they differ at least by the statistical difference of 1:4. In Table 5 a summary

Figure 16. Negative $\log_{10}$ of the Michaelis constant / $(1+\frac{H^+}{K_{as}})$ as a function of pH

Experimental —⊙—

Theoretical —●—


pH
10
9
8
7
0
1
2
3
$-\log \frac{Km}{(1+\frac{[H^+]}{Kas})}$

of the values of K_m, $\frac{K_m}{1 + \frac{H+}{K_{as}}}$ and V_{max} as a function of pH is presented.

7. Michaelis constant for serine

The method of Lineweaver and Burk (44) was used to determine K_m (Michaelis constant) and V_{max} (maximum velocity).

Due to the fact that the product vs. time curve for serine levels off after about 10 minutes the velocity had to be measured before this leveling off occurred. Therefore, a single measurement at 1 minute was made and taken as the velocity of the reaction.

When 1/v was plotted against 1/S, (Figure 17), a straight line was obtained. From the intercept we obtained a maximum velocity of .25. The velocities v were converted to the base of 1 unit of enzyme as defined by the reaction of the enzyme on L-threonine. By doing this the serine data was placed on the same basis as the threonine data and allowing a comparison between the two substrates.

The values of V_{max} were found to be identical within experimental error. This can be taken to mean that the reactions occurring, once the substrates are bound, proceed at the same rates.

The differences in the K_m (Michaelis constant) is explained by a difference in the case of binding of the

Figure 17. Reciprocal 1/s vs. 1/v for threonine dehydrase with L-serine as substrate

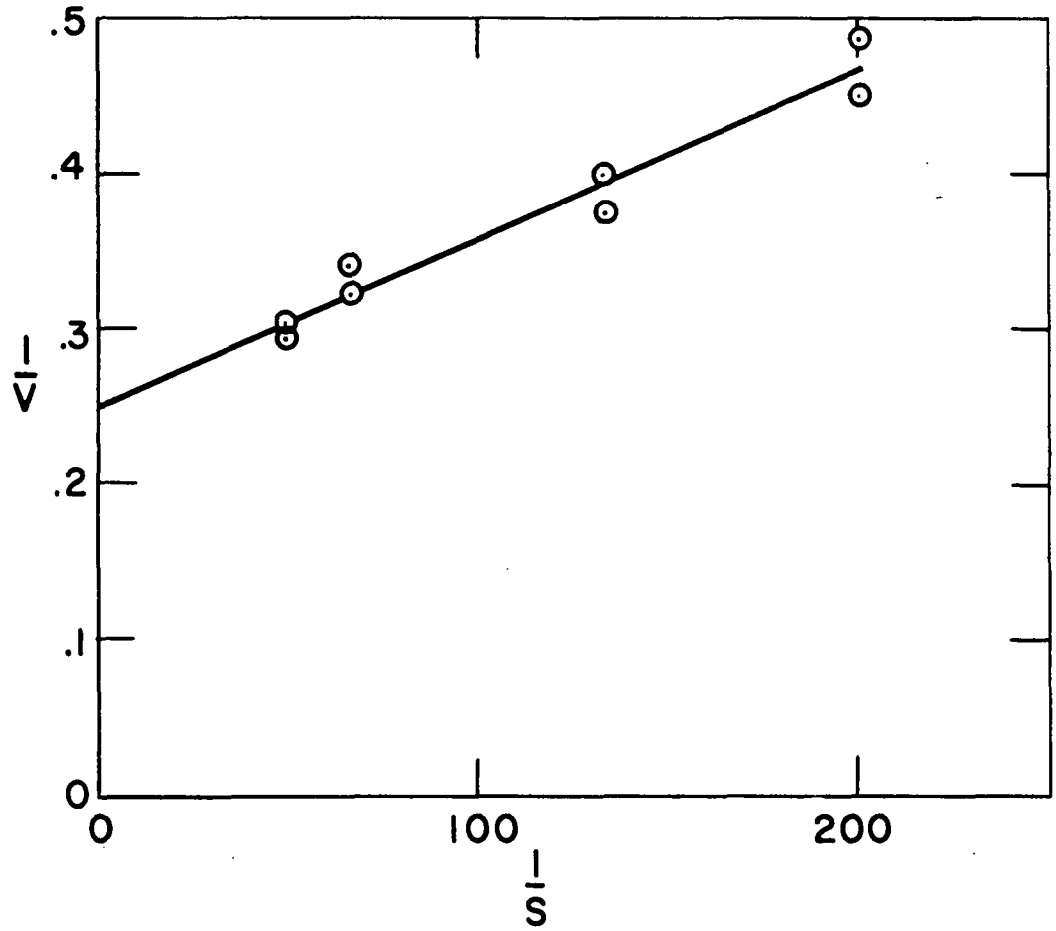

.5
.4
.3
.2
.1
0
1/V
0
100
200
1/S

Table 6. Kinetic functions as a function of pH

pH	V_{max}[a]	$-\log_{10} K_m$	$K_m \times 10^3$	$\frac{K_m}{(1+\frac{H+}{K_{as}})} \times 10^3$	$\frac{-\log_{10} K_m}{(1+\frac{H+}{K_{as}})}$
5.46	.28	0.914	1.22	---	---
5.60	---	1.45	35.6	---	---
5.90	---	1.23	58.5	---	---
5.94	.28	1.34	45.5	---	---
6.25	---	1.35	45.4	---	---
6.37	---	1.49	32.0	---	---
6.50	.28	1.84	14.3	---	---
7.03	.27	1.85	14.3	0.47	3.33
7.12	.28	1.64	23.2	0.93	3.03
7.17	.23	1.66	21.8	0.97	3.01
7.25	---	1.55	28.0	1.49	2.83
7.50	.23	1.80	15.7	1.43	2.85
7.58	---	1.97	10.8	1.16	2.94
7.80	.23	2.23	5.90	0.99	3.01
8.07	.26	2.68	2.21	0.60	3.22
8.18	---	2.55	2.78	0.90	3.05
8.50	.26	2.75	1.80	0.90	3.05
8.55	---	2.71	1.90	1.00	3.00
8.75	.27	3.00	1.00	0.64	3.20
8.97	---	3.13	.74	0.56	3.26
9.00	.26	3.28	.53	0.40	3.40
9.32	.27	3.18	.65	0.63	3.20
9.73	---	2.26	5.46	5.15	2.29
9.84	.25	2.00	10.00	9.7	2.01
10.00	---	1.13	74.5	72.5	1.14
10.12	.25	.76	174.0	171	0.76

[a] V_{max} in arbitrary units. A unit is defined as the amount of enzyme which produces an optical density of one by the 2,4-dinitrophenylhydrazone method when incubated with 30 μ moles of L-threonine at 37° in 0.1 M tris-carbonate buffer for 30 minutes. Total volume of reaction mixture 1.5 ml. One ml. taken for assay.

threonine to the enzyme over that of the serine only if $\frac{1}{K_m}$ is an indication of the binding of substrate to enzyme. Another point to be taken into consideration is the fact that the serine may be effecting the resolution of the enzyme into apo and co-enzyme.

8. Inhibition studies

The action of threonine dehydrase is inhibited by a number of organic compounds. The effectiveness of these compounds is presented in Table 7.

It was of interest to note the effects of carbonyl reagents upon the activity of threonine dehydrase for the following reason. The postulated role of PPal in the action of threonine dehydrase involves combination of the 4-formyl group of PPal with the amino group of threonine (Figure 2). Therefore compounds which specifically react with carbonyl groups should inhibit threonine dehydrase if PPal is at the active center.

Hydroxylamine at a concentration of 5×10^{-3} M completely inhibits threonine dehydrase. At lower concentrations as shown in Table 7 the inhibition is less. The inhibition constant (K_i) was determined from the following relationship:

$$K_i = \frac{K_m(I)}{K_{app} - K_m}$$

Table 7. Inhibitors of threonine dehydrase

Additive	Concentration	Relative activity[a]
Ethanolamine	2×10^{-4}	1
Ethanolamine	2×10^{-5}	1
Ethanolamine	2×10^{-2}	.7
Ethanolamine	2×10^{-3}	1
Pyruvate	4×10^{-4}	1
Cysteine	2×10^{-2}	.23
Sodium cyanide	1×10^{-3}	.97
Hydroxylamine	1×10^{-5}	.97
Hydroxylamine	1×10^{-4}	.22
Hydroxylamine	1×10^{-2}	0
Hydroxylamine	1×10^{-3}	0
Hydroxylamine	5×10^{-3}	0
Semicarbazide	1×10^{-2}	.96
Semicarbazide	1×10^{-3}	1
Semicarbazide	2×10^{-2}	.6
Dipotassium diethylstilbesterol disulfate[b]		

[a] Ratio of O.D. at 520 mμ in presence of additive to that of reaction mixture containing no additive. Reaction mixture contained .6 ml. enzyme Sp. Ac. 40 dil. 1/25, 30 μ moles threonine, 0.3 ml. 0.1 M pH 8.95 tris carbonate buffer and various additives. Total volume 1.5 ml. One ml. alliquotes were taken for assay. Samples were run in duplicate.

[b] 0.3 ml. of saturated solution added.

Table 7. (Continued)

Additive	Concentration	Relative activity[a]
Dipotassium α-diphenyl disulfate[b]		1
Dipotassium estradiol -17 β disulfate	1×10^{-2}	1
DL-(Threo) hydroxyaspartic	2×10^{-1}	1
DL-(Allo) hydroxyaspartic	2×10^{-1}	1
Isoleucine	2×10^{-1}	1
Glycine	1×10^{-1}	.72
Serine methyl ester	2×10^{-2}	.50
Phospho serine	2×10^{-2}	.92

K_i = inhibition constant. The concentration of inhibitor at which the enzyme is half saturated with inhibitor.

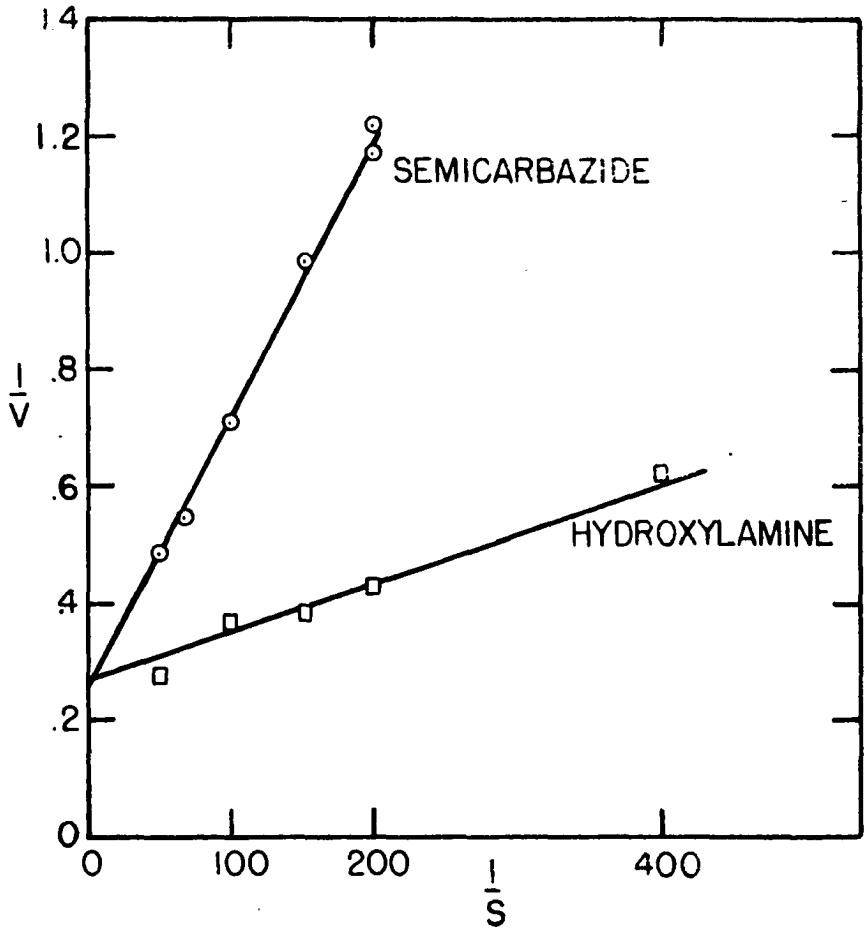
K_m = Michaelis constant.

(I) = Concentration of inhibitor

K_{app} = K_m obtained from reciprocal plot of $\frac{1}{v}$ vs. 1/s using methods previously described.

The reciprocal 1/v vs. 1/s graph for threonine dehydrase in the presence of 2×10^{-6} M hydroxylamine is presented in (Figure 18). From this graph we were able to calculate an inhibitor constant of 5.3×10^{-7} at pH 9. The maximum velocity is unchanged by the presence of the inhibitor while the Michaelis constant (K_m) is affected. This behavior

Figure 18. Reciprocal 1/s vs. 1/v for threonine dehydrase in the presence of 2×10^{-6} M hydroxylamine -□-□- and 2×10^{-2} M semicarbazide -⊙-⊙- . Number on graph indicate pH at 25° of reaction mixture


1.4
1.2
1.0
.8
.6
.4
.2
0
1/V
SEMICARBAZIDE
HYDROXYLAMINE
0
100
200
400
1/S

corresponds to competative inhibition in which the inhibitor competes with the substrate (threonine) for the available active sites.

Semicarbazide was not as effective as hydroxylamine as an inhibitor. Its inhibition like that of hydroxlamine was competative. The inhibitor constant was determined at pH 9 from the reciprocal 1/s vs. 1/v graph (Figure 18) and found to be 7.7×10^{-4}.

The difference observed between hydroxylamine and semicarbazide as inhibitors probably lies in the specificity of the enzyme. However, it may be due to the stability of the adduct formed with PPal. Therefore, it should be of interest to investigate the stability of these adduct compounds between PPal with semicarbazide and hydroxylamine and try to correlate it to the effectiveness with which these compounds inhibit threonine dehydrase. Cyanide, a third carbonyl reagent, was less effective than hydroxylamine and semicarbazide as an inhibitor for threonine dehydrase.

The steroids and dipotassium diphenyl disulfonate were investigated as potential inhibitors because Mason and Gullekson (49) had reported that these compounds inhibit a number of PPal requiring enzymes. However, as seen in Table 7 these compounds had no effect on threonine dehydrase.

Glycine acts as a competative inhibitor. Its inhibitor constant has been determined at several pH values. The pH dependence of the inhibitor constant for glycine is analogous to that found for the Michaelis constant (Figure 15). This behavior of the inhibitor constant with pH confirms the conclusions made regarding the active site from the pH dependence of the Michaelis constant.

9. Metal Requirement

Threonine dehydrase of sheep liver was most active in the presence of potassium and ammonium ions. A slight activation was observed in the presence of sodium ions also. This activation is greatest at high substrate concentration and high pH. The effect of pH and substrate concentration on the activation of threonine dehydrase is summarized in Table 8. The relative activity is the ratio of the keto-acid produced in the presence of added salts to that in the absence of any salts.

In Table 9 the results of increasing amounts of sodium chloride and potassium chloride on the activity of threonine dehydrase are presented. The per cent activation is seen to increase with increasing salt concentration.

To find out if the monovalent ion effect was operative on the formation of the enzyme substrate complex, the break-

Table 8. Effect of pH and substrate concentration on sodium and potassium activation of threonine dehydrase[a]

Additive	Salt concentration	pH	Substrate concentration	Relative activity[b]
NH_4Cl	0.02 M	7.0	0.02 M	1
NH_4NO_3	0.02 M	7.0	0.02 M	1
KCl	0.02 M	7.0	0.02 M	1
KNO_3	0.02 M	7.0	0.02 M	1
NaCl	0.02 M	7.0	0.02 M	1
$NaNO_3$	0.02 M	7.0	0.02 M	1
KCl	0.2 M	9.0	0.02 M	1.30
KCl	0.2 M	8.6	0.0014 M	1
NaCl	0.2 M	8.6	0.02 M	1.12
NH_4Cl	0.2 M	9.0	0.02 M	1.40

[a]Reaction mixture contained 0.6 ml. enzyme (specific acitivity 40) 0.3 ml. buffer, 30 u moles L-threonine and salts. Total volume of reaction mixture 1.5 ml. One ml. sample taken for assay.

[b]The ratio of the optical density at 520 mμ of the 2,4-dinitrophenylhydrazone of the ketoacid formed in the presence of added salt to that produced in the absence of any salt.

Table 9. Activity of threonine dehydrase in the presence of sodium and potassium ions[a]

Moles/liter	Potassium Relative activity[b]	% Activation	Moles/liter	Sodium Relative Activity	% Activation
0.2	1.30	30	0.2	1.12	12
0.1	1.27	27	0.1	1.10	10
0.02	1.25	25	0.02	1.09	9
0.01	1.23	23	0.01	1.03	3
0.002	1.20	20	0.002	1.03	3
0.00002	1.00	0	0.00002	1.00	0
-----	1.00	--	-----	1.00	--

[a]The enzyme was precipitated with 60% saturated $(NH_4)_2SO_4$ dissolved in 0.1 M pH 8.6 tris buffer. The enzyme was then dialyzed against two charges of the same buffer for 24 hours. Reaction mixture contained 0.6 ml. of enzyme (specific activity 0.3 ml. pH 8.6 tris carbonate buffer, 30 μ moles L-threonine and salts. Total volume of reaction mixture was 1.5 ml.

[b]The ratio of the optical density at 520 mμ of the 2,4-dinitrophenylhydrazone of the ketoacid formed in the presence of added salt to that produced in the absence of any salt.

down of the complex, or on the number of available active sites, the Michaelis constant and maximum velocity were determined. In the presence of .002 M KCl the Michaelis constant was 8.3×10^{-4} M compared with 1.9×10^{-3} M in the absence of added KCl at pH 8.6. The maximum velocity was not changed.

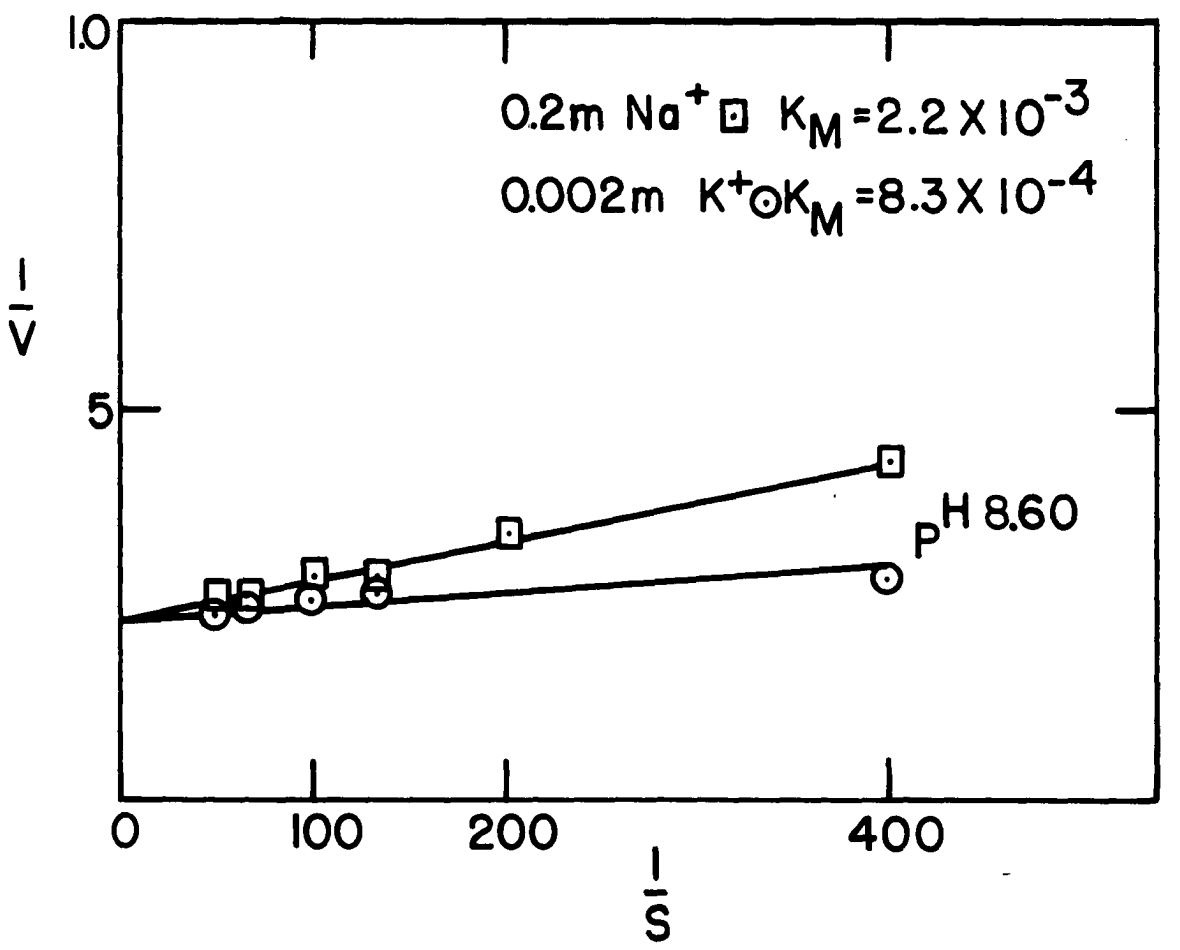
In almost all the reported cases of activation by potassium and ammonium ions inactivation by sodium occur. This was not the case with threonine dehydrase in fact a slight activation was observed. This activation is not an ionic strength effect since several other salts gave no effect and there was a difference in the degree of activation (Table 9) by the same concentration of KCl and NaCl. The Michaelis constant was determined at pH 8.6 in the presence of .02 M NaCl and did not change (Figure 19).

Since potassium acts by increasing the Michaelis constant it probably aids in the formation of the enzyme substrate complex. This is all that can be said at this time. However, future studies on this ion effect should be quite rewarding.

D. Discussion

Although a crystalline enzyme has not been obtained a 4000 fold purification of sheep liver threonine dehydrase has been achieved. One of the major problems in the purification has been the variation in enzyme concentration from liver to

Figure 19. 1/s vs. 1/v for threonine dehydrase in the presence of sodium and potassium ions


1.0
5
0
100
200
400
1/V
1/S
0.2m Na+ ⊡ KM=2.2X10-3
0.002m K+⊙KM=8.3X10-4
pH 8.60

liver. This variation could be the result of genetic differences. Another possibility is that the presence of substrate triggers the production of the enzyme either *de novo* of from some precussor. This would be consistent with the behavior of serine and threonine dehydrases from microorganism. McFall *et al.* (50) were able to demonstrate the induction of D-serine dehydrase in *E. coli.* Pardee and Prestidge (51) demonstrated that the L-serine dehydrase was also substrate inducable. Umbarger and Brown (33) demonstrated that of the two L-threonine dehydrases isolated from *E. coli*, the one which is activated by AMP, glutathione and PPal is induced by substrate. L-Serine dehydrase from rat liver is also inducable (52).

If the sheep liver threonine dehydrase is indeed inducable then it may be profitable to use this as a method of obtaining starting material of higher activity than the livers now available. The induction of threonine dehydrase may represent a control mechanism. Elliott and Neuberger (53) observed that the feeding of N^{15} glycine to rabbits and rats resulted in no incorporation of N^{15} in threonine. They concluded that threonine does not enter in reversible N transfer. Since threonine is a required amino acid, some biological systems e.g. protein synthesis, utilize the molecule *in toto*. The induction of threonine dehydrase may represent a means of getting rid of the threonine after it

reaches a certain level unfavorable for operation of protein synthesis and other similar systems.

A number of observations were made during the purification of threonine dehydrase which may be of value in future work. For example, indications have been obtained that threonine dehydrase is more stable at temperatures of 70° C and above in the presence of substrate than in the absence of it. This may be useful as a purification step to remove contaiminating protein at temperatures above 70° C. It may be profitable in the future to replace the centrifugation after the first heat treatment with a filtration through some strong cotton material, since this is one of the most time consuming steps in the isolation. The author observed that if the pH of the preparation was adjusted to 7.2 according to the procedure of Sayre and Greenberg before ammonium sulfate (18) often all the enzyme activity was lost. When no pH precipitations adjustment was made about 30% of the activity was lost as the pH became more acidic after the ammonium sulfate was added. Since this relatively constant percentage of activity was lost, perhaps at the lower pH destruction of some other enzyme which has threonine activity occurred. This was not a slow denaturation or inactivation, for allowing the sample to stand for long periods of time did not result in increase loss of activity. This may mean

that there are more than one enzyme in sheep liver which act on threonine.

It has been much more difficult to show a requirement for PPal for the mammalian threonine dehydrase than for the microbial enzyme. Methods used in the past to establish the presence of PPal consisted of looking for increased activity of the enzyme in the presence of PPal, for decreased activity in the presence of inhibitors and PPal analogues and reversal of the latter two by PPal.

No increase in activity of threonine dehydrase is observed in the presence of PPal. However, serine rapidly inactivates the enzyme. Nishimara (37) claims to be able to reverse this inactivation by the addition of PPal. This author has been unable to repeat these experiments with his enzyme. The co-enzyme picture has been complicated also by reports that biotin (54-55) and AMP and glutathione (56) activate other threonine dehydrases. However, the almost universal occurrence of PPal as a co-enzyme for enzymes which act on amino acids makes it very likely that sheep liver threonine dehydrase is an enzyme of this type.

It was hoped that threonine dehydrase could be purified to the degree that the spectrum of PPal could be examined.

Jenkins (57) working with pig liver transaminase has purified it to the point where the spectrum of PPal shows up clearly over that of the protein. The pyridoxal enzyme

exist in a bright yellow acidic form λ max 430 mμ and in a colorless basic form λ max 362 mμ. These forms change over around a pK of 6.2 (58).

The change from yellow to colorless is a property of a PPal imine rather than the free PPal. Because of this and other evidence (59) Jenkins and Sizer suggested that the PPal is bound through its 4-formyl group to an amino acid group on the enzyme.

Matsuo and Greenberg have obtained a crystalline enzyme that cleaves homoserine and cystathionine and which requires PPal (43). In neutral solutions the enzyme has in addition to the protein peak, a peak at 427 mμ. This peak represents PPal. Between pH 4 and 8.4 the height of the peak does not change, but the maximum wavelength shifts from 430 to 424 mμ. Somewhere between pH 9.5 and 10.5 the peak shifts to 388 mμ (60). The yellow color of this enzyme like that of transaminase is a property of a PPal imine rather than of free PPal.

Shukuya and Schwert (61) have purified a glutamic acid decarboxylase from E. coli. The purified enzyme is yellow in acid solution and colorless at neutral pH values. This behavior is quite similar to that exhibited by the transaminase (58). A pH change from 6.5 to 5 is accompanied by an increase in optical density at 415 to 420 mμ and by a diminution at 340 mμ. An isosbestic point occurs at 360 mμ.

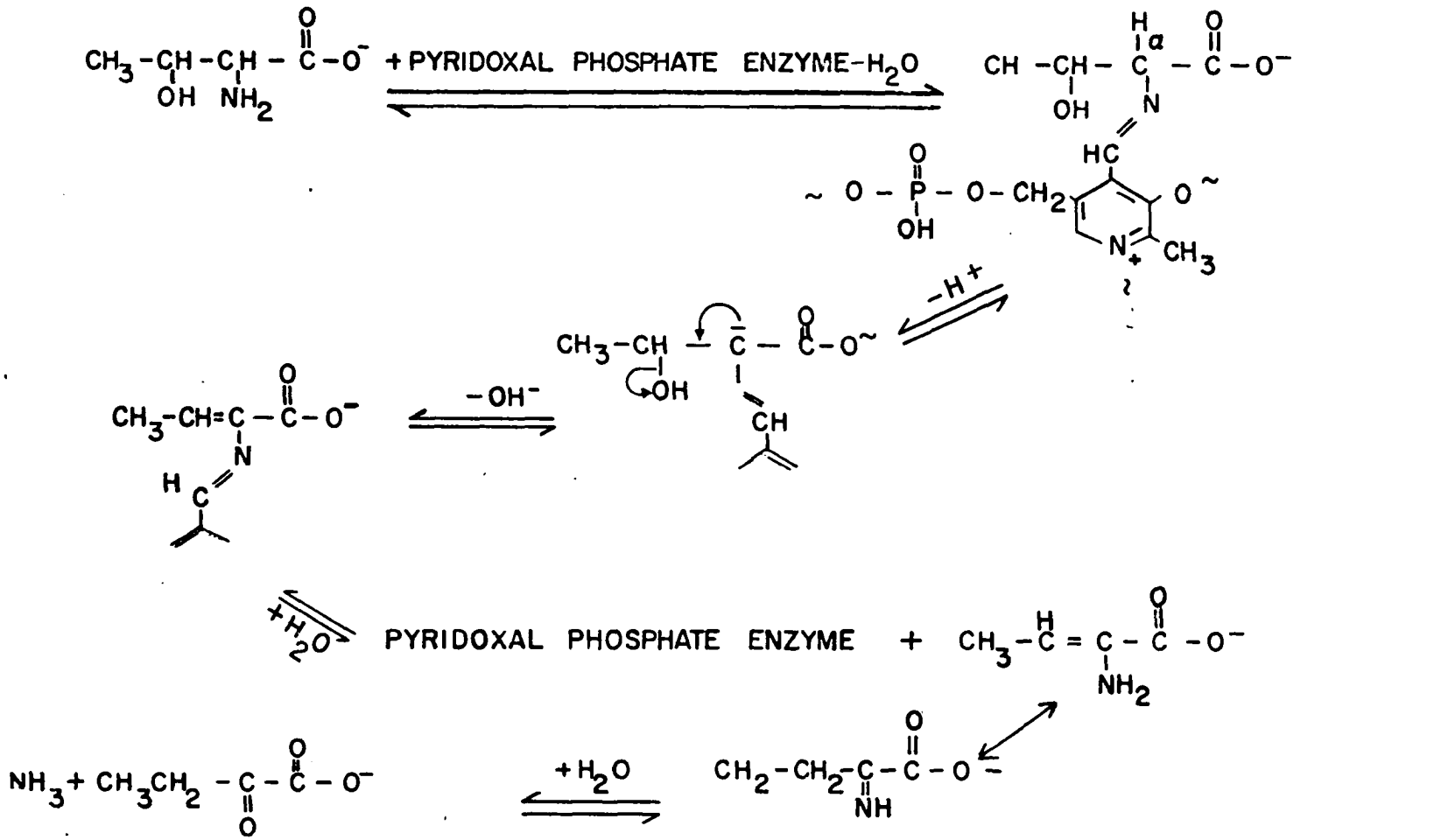
The change of optical density with pH indicates that several protons are involved in the changes observed in the absorption spectrum (62).

These spectral observations and the apparent tenacity with which PPal is bound raises the very interesting question as to the means by which PPal is bound. Undoubtedly the 5 phosphate is involved in this binding, evidence has been obtained that the 4-formyl group may be involved also. Fischer and Krebs (63) have isolated, after reduction, ϵ N pyridoxyllysine from phosphorylase, transaminase, and homoserine crystathionine dehydrase. This would indicate that in these three cases the PPal is bound to the ϵ-amino group of lysine through imine formation with its 4-formyl group.

When a 4000 fold purified sample of threonine dehydrase was examined spectrophotometrically some residual absorption, but no maximum, appeared in the region of 350 to 420 mμ. This is the region where the PPal if present would be expected to show up. Probably there is still contaminating protein present in this preparation which obscures the PPal spectrum.

A proposed mechanism for the conversion of threonine to α-ketobutyrate and ammonia is shown in (Figure 19). The dehydration probably takes place on the enzyme by the action of PPal. Once the dehydration takes place the enolic form of the imine which results is freed from the PPal. The rearrangement and hydrolysis of the enolic form of the imine probably takes place away from the active center.

Figure 19. Proposed mechanism for action of threonine dehydrase


+ PYRIDOXAL PHOSPHATE ENZYME-H_2O
$-H^+$
$-OH^-$
$+H_2O$
PYRIDOXAL PHOSPHATE ENZYME +
$+H_2O$

Evidence for a positive role of metal ions in the action of threonine dehydrase is quite inconclusive. However, monovalent cations (K^+, Na^+, NH_4^+) do play a role. Theories concerning the mechanism of activation by cations have been discussed by Happold and Beechey (64). However, no evidence has been obtained working with threonine to prove any of the possibilities mentioned.

The activity of threonine dehydrase varies with pH. A maximum activity is observed in the pH range 8.5 to 9. This maximum is due to a composite of effects. However, a variation in the maximum velocity as a function of pH is not one of these effects (see Figure 14). The pH dependence of threonine dehydrase results from ionization of groups either on the enzyme or the substrate, it is expected that these groups are few. There are numerous ionizable groups on the enzyme but it is not very likely that the ionization of groups other than those located at or near the active center have any effect on the rate of the reaction, or the binding of the substrate.

The Michaelis constant, in contrast to the maximum velocity, is highly pH dependent, with a minimum value in the pH range 8.5 to 9. An analysis of the pH vs. -log Michaelis constant (see section 6) implies that there are a minimum of three ionizable groups involved in formation of

the enzyme substrate complex. Furthermore, the analysis is consistent with a binding of the threonine anion to the enzyme.

The inhibitor constant for glycine also shows a minimum in the pH range 8.5 to 9 (Figure 15). This pH behavior of the inhibitor constant for glycine is consistent with the results and conclusions made regarding the pH dependence of the Michaelis constant.

The fact that the Michaelis constant and inhibitor constant for glycine in (Figure 15) change from a slope of zero to a minimum slope of 2 around pH 9.2 implies the titration of two groups. The complexity of situations which result in such phenomena do not allow much more than speculation about the actual identity of the groups involved.

One possibility is that the titration of one of the groups with a normal pK in this range causes secondary and tertiary changes in the protein structure which releases the second group and allows it to be titrated. This change around the pH of 9.2 is reminiscent of the spectral changes Matsuo and Greenberg (60) observed in their homoserine dehydrase. The possibility exist that this pH effect around pH 9.2 observed by the author and the spectral changes of the PPal in homoserine dehydrase around the same pH range may be related. Proof of this will be forthcoming when threonine

dehydrase is purified to the point where the spectrum of PPal can be seen over that of the protein.

E. Summary

1. Sheep liver threonine dehydrase has been obtained in a highly purified form using heat treatments, ammonium sulfate fractionations and diethylaminoethyl cellulose chromatography. A specific activity of 200 micro moles ketoacid formed per hour per mg. protein has been obtained.

2. Threonine dehydrase is stable over the pH range 5.5 to 9.

3. The production of α-ketobutyrate is linear with time at substrate saturation. The production of ketobutyrate is directly proportional to enzyme concentration in 0.02 M threonine.

4. Threonine dehydrase has activity towards L-serine, L-threonine and DL-(allo) threonine.

5. The activity of threonine dehydrase is destroyed in about 10 minutes when serine is used as a substrate at pH 7. When the pH of the reaction mixture is 9 the production of pyruvate does not cease at the end of 10 minutes but is produced at a different rate for up to 2 hours.

6. Carbonyl reagents (hydroxylamine and semicarbazide) and glycine competitively inhibit threonine dehydrase.

The carbonyl reagents inhibit the enzyme more effectively at pH 9 and above than at lower pH values. The inhibitor constants are 5.2×10^{-7} M and 7.7×10^{-4} M at pH 9 for hydroxylamine and semicarbazide respectively. These values can be compared with a value of 5.3×10^{-4} M for the Michaelis constant at this pH.

7. Threonine dehydrase shows a pH optimum over the range 8.5 to 9. This pH optimum is due to ionizable groups on the enzyme and on the substrate. The maximum velocity of threonine dehydrase does not vary with pH over the range 5.5 to 9. In contrast, the Michaelis constant is highly pH dependent, with a minimum value in the pH range 8.5 to 9. The pH dependence of the Michaelis constant is consistent with a binding of the threonine anion to the enzyme in a form containing two protonated groups at the active site.

8. The inhibitor constant for glycine as a function of pH is consistent with the behavior of the Michaelis constant as a function of pH.

9. Threonine dehydrase of sheep liver is most active in the presence of potassium, sodium and ammonium ions. This effect is greatest at high substrate concentration and high pH.

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