

This dissertation has been 65-4589
microfilmed exactly as received

ALBERT, Jerry David, 1937-
SOME PYRIDOXAL ANALOGS AND THEIR TRANS-
AMINATION WITH AMINO ACIDS.

Iowa State University of Science and Technology
Ph.D., 1964
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan

SOME PYRIDOXAL ANALOGS AND THEIR TRANSAMINATION
WITH AMINO ACIDS

by

Jerry David Albert

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1964

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF PERTINENT LITERATURE	5
Vitamin B ₆ Chemistry and Function	5
Absorption Spectra and Ionic Equilibria of Some Pyridoxine Analogs	6
Equilibria of Imines of Pyridoxal Analogs	20
Function of Vitamin B ₆ in Transamination	47
Nonenzymic Transamination in Model Systems	55
EXPERIMENTAL	74
Materials	74
Spectrophotometric Measurements of the Ionic Equilibrium of Pyridoxal-N-methochloride	77
Spectrophotometric Determination of the For- mation Constant for the Hydrogen-bonded Aldimine of Pyridoxal-N-methochloride and Valine	79
General Procedure for Nonenzymic Transamination in Model Systems	83
Analytical Methods	84
Kinetic Methods	97
RESULTS AND DISCUSSION	107
Absorption Spectrum and Ionic Equilibrium of Pyridoxal-N-methochloride	107
Equilibrium Constant for the Formation of the Hydrogen-bonded Aldimine of Pyridoxal-N-metho- chloride and Valine	116
Equilibrium Constants for the Formation of Aldimines of Leucine and Deoxypyridoxal, Leucine and Pyridoxal Phosphate as a Function of pH	125

	Page
Nonenzymic Transamination of Leucine with Deoxypyridoxal	131b
Nonenzymic Transamination of Leucine with Pyridoxal Phosphate	167
Intramolecular Acid-Base Catalysis of Non-enzymic Transamination of Leucine with Pyridoxal Phosphate	171
General Acid-Base Catalysis of Nonenzymic Transaminations of Leucine with Pyridoxal Analogs	182
Metal Ion Catalysis of Nonenzymic Trans- amination of Amino Acids with Pyridoxal Analogues	210
Nonenzymic Transamination of Leucine with 5- Carboxylate Analogs of Pyridoxal	228
Nonenzymic Transamination of Alanine Methyl Ester with Pyridoxal Analogs	240
Deuterium Isotope Effect on the Rate-Limiting Step in Nonenzymic Transamination	257
Future Pyridoxal Analogs Possibly Capable of Enhanced Intramolecular Catalysis of Non- enzymic Transamination	266
SUMMARY OF CONCLUSIONS	277
LITERATURE CITED	281
ACKNOWLEDGEMENTS	297
DEDICATION	299
VITA	300

INTRODUCTION

One of the many functions of vitamin B₆, in its coenzyme forms, is the catalysis of the transamination of alpha-amino acids and alpha-keto acids, a key reaction in nitrogen metabolism. An object of this study has been to learn more about the detailed action of vitamin B₆-dependent enzymes on amino acids by inference from studies of nonenzymic reactions of pyridoxal analogs and amino acids.

From previous studies of these nonenzymic reactions it is known that the rates of both the formation of amino acid imines of pyridoxal and the tautomeric interconversion of these imines (the rate-determining step in transamination) are several orders of magnitude lower than the corresponding steps in enzymic catalysis. Since general acid-base catalysis of the reaction is known to occur in model transamination systems, it is reasonable to ask whether certain catalytic groups at the active site of a transaminase play a similar role in enhancement of the enzymic reaction.

This situation seems likely because the available evidence supports the catalytic role of acidic and basic groups at the active sites of enzymes. Such functional groups of amino acids which might be available to enzymes for this purpose include the imidazolyl group of histidine, the phenolic group of tyrosine, the guanidino group of arginine, the epsilon-amino group of lysine, the hydroxyl groups of serine and

threonine, and the terminal carboxyl groups of aspartic and glutamic acids.

But the great efficiency of the enzymic catalysis is still not fully understood; other factors must be considered. Could the dissociation constants of these acidic and basic groups be important factors in effecting the efficiency of the catalysis? If so, then how does the stereochemical arrangement of these groups in relation to the coenzyme-substrate complex account for the catalysis? Furthermore, how important are dynamic conformational effects of the protein to the process of bringing the catalytic groups and the coenzyme-substrate complex into a critical configuration for polarizing the appropriate chemical bonds required in the transamination of alpha-amino acids and alpha-keto acids?

However, the problem to be dealt with here is how many of these enzymic features of catalysis can be duplicated in a model system? The model transamination system of pyridoxal (vitamin B₆ aldehyde) and an amino acid or of pyridoxamine (vitamin B₆ amine) and a keto acid has been improved by adding intermolecular catalysts such as metal ions and acid-base buffers. This model transamination system might be further improved with intramolecular catalysis by substituting certain functional groups in the pyridoxal structure and by altering the amino acid structure, such as by esterification, in order to simulate effects of the enzyme on its substrate.

An approach which has added and could add further to our

understanding of the mechanisms of reactions on enzyme surfaces is the spectral measurement of pyridoxal analogs and of their reactions with amino acids. These model systems are also studied with the intent of finding suitable conditions for conveniently comparing reactivities of pyridoxal analogs and measuring catalytic effects of the incorporated substituents, as well as of various intermolecular catalysts. Such experimental conditions include those of temperature, pH, and concentration of reactants and catalysts.

Analytical methods, such as chemical, chromatographic, and spectrophotometric techniques, and appropriate graphical methods for analyzing equilibria and kinetic data need to be adapted in order to measure these catalytic effects in these nonenzymic reactions. The transamination reaction is complex in that imine intermediates are involved in equilibria with reactants and products, and the amounts and reactivities of each of their ionic species are important considerations in determining their contributions to the overall reaction rates. Measurements of, not only reaction rates, but also ionic equilibria of each pyridoxal analog and its amino acid imines, as well as the formation constants of these imines, need to be made. Thus, in studying the transamination reaction in a model system many factors need to be controlled or quantitatively taken into account in any accurate interpretation of kinetic results.

In particular, intermolecular catalytic effects of metal

ions, such as aluminum (III), copper (II), and zinc (II), of acid-base buffers, such as acetate, phosphate, and imidazole, are to be measured. Intramolecular catalytic effects of a positive charge on the pyridine nitrogen atom of pyridoxal and of substituent groups at the 5-position of pyridoxal are to be determined. The latter substituents include hydroxymethyl, methylol phosphate, propionate and carboxylate groups, with reference to a methyl group. The deuterium isotope effect in deuterio-leucine and the effect of an esterified amino acid on the rates of these nonenzymic transamination reactions are to be measured.

The purpose of these investigations is to add to the understanding of the mechanism of pyridoxal-mediated transamination of amino acids and of the increased reactivity of pyridoxal phosphate in the enzymic reactions, which possibly is due to the nature of catalytic groups in the protein at the active site and to the mode of attachment of the coenzyme to apoenzyme. It is further hoped that this experimental approach will lead to the design and synthesis of pyridoxal analogs capable of carrying out the intramolecular acid-base catalysis of the intermediate imines with amino acids more efficiently than the presently available analogs, and perhaps as efficiently as the enzymes found in nature. Such an investigation of the chemical action of vitamin B₆ coenzyme (or of pyridoxal analogs) constitutes an important part of the basic research on a fundamental problem of biochemistry, namely, the elucidation of the mechanisms of enzymic reactions.

REVIEW OF PERTINENT LITERATURE

Vitamin B₆ Chemistry and Function

Thirty years ago vitamin B₆ was discovered and defined as a discrete factor in the vitamin B-complex (György, 1934). During the subsequent three decades a wealth of information has been gained about the chemistry and biological role of this vitamin. This information has pointed to the central role of vitamin B₆ in nitrogen metabolism. In his review entitled "Pyridoxal Phosphate," a vitamin B₆ coenzyme, Braunstein (1960, p. 180), the discoverer of biological transamination, concludes: "Pyridoxal catalysis is clearly one of the most important and versatile among the fundamental enzymic mechanisms constituting the chemical basis of life. Its very ancient origin in the evolution of living matter is evident from the many strategic functions of pyridoxal phosphate enzymes in the biosynthesis and transformations of virtually all biologically important nitrogen compounds." A recent symposium on the Chemical and Biological Aspects of Pyridoxal Catalysis has reviewed the current knowledge and has indicated the directions of research in this field (Snell et al., 1963). Another review on the chemistry of the vitamin itself (pyridoxine) has recently appeared (Wagner and Folkers, 1964).

In its coenzyme forms vitamin B₆ participates in a variety of important enzyme-catalyzed reactions (Braunstein, 1960;

Gonnard, 1962; Snell, 1958, 1962). Our present understanding of the roles of vitamin B₆ coenzymes in enzymic reactions has developed from several different types of experimental approaches. The approaches which will be emphasized in this review include investigations on nonenzymic model systems and studies of enzyme systems reacting with antagonists (carbonyl reagents and structural analogs) of vitamin B₆.

Absorption Spectra and Ionic Equilibria of Some Pyridoxine Analogs

The electronic absorption spectra of pyridoxine and other hydroxypyridine derivatives change markedly with pH when dissociation of the phenolic or pyridinium groups occurs. These spectra and the ionic equilibria of these compounds in aqueous solution have been compared and correlated with those of a number of other pyridine and benzene derivatives. Effects of molecular structure on these spectra and on the observed ionization constants have been discussed (Williams and Neilands, 1954; Metzler and Snell, 1955; Nakamoto and Martell, 1959 a,b). A summary of the current knowledge in this area of vitamin B₆ chemistry has been presented in a recent review (Martell, 1963).

Pyridoxol, PO-Figure 1

The spectrum of pyridoxol may be compared with that of 3-hydroxypyridine. The absorption maximum at 291 mμ for an acid solution of pyridoxol has been assigned to the cationic form

(as a-Figure 2). In neutral solution the absorption maximum at 324 mμ has been assigned to the dipolar ionic form (as b-Figure 2), in which the phenolic group has dissociated. Spectrophotometric and potentiometric titration data have been used to calculate the ionization constants for the phenolic and pyridinium groups of pyridoxine analogs. The first apparent constant has been observed in the range of pK 3.3-5.0 (pK₁-Table 1) for these analogs. The second apparent constant has been observed in the range of pK 6.8-9.0 (pK₂-Table 1), and the absorption maximum at 310 mμ for a basic solution of pyridoxol has been assigned to the anionic form (as c-Figure 2).

5-Deoxypyridoxal (DPL) and 3-hydroxypyridine-4-aldehyde (HPA)-Figure 1

The introduction of an aldehyde substituent in the 3-hydroxypyridine nucleus results in changes both in absorption spectrum and in ionization constants. In general, both apparent ionization constants decrease somewhat from their values in pyridoxol. The electronic absorption bands have been assigned to two sets of ionic structures. Those for the hydrated aldehydes (as C-Figure 2) correspond with those of pyridoxol (alcohol structures). The unhydrated aldehyde forms (as B-Figure 2) of deoxypyridoxal have absorption maxima at 342 mμ for the cation, 383 mμ for the dipolar ion, and 391 mμ for the anion, which correspond closely with the absorption bands for 3-hydroxypyridine-4-aldehyde.

Figure 1. Structures and nomenclature of some pyridoxal analogs

(PO) 3-Hydroxy-2-methyl-4,5-dihydroxymethylpyridine: pyridoxol, vitamin B₆ alcohol

Structure with R = R' = H

(HPA) 3-Hydroxy-4-formylpyridine or 3-hydroxypyridine-4-aldehyde

Structures with R = methyl and with 5-substituents

(PL) R' = hydroxymethyl: Pyridoxal, vitamin B₆ aldehyde

(DPL) R' = methyl: 5-Deoxypyridoxal, a vitamin B₆ anti-metabolite

(PLP) R' = methylol phosphoric acid ester: Pyridoxal-5-phosphate, a vitamin B₆ coenzyme

(CPL) R' = carboxy: "5-Pyridoxalyllic acid" or "5-carboxypyridoxal"

(FPL or PLF) R' = carboxymethyl: "alpha⁵-Pyridoxalylformic acid"

(APL or PLA) R' = carboxyethyl: "alpha⁵-Pyridoxalylacetic acid"

Structures with an N-methyl substituent

(PLM) R' = hydroxymethyl: Pyridoxal-N-methochloride

(MPLP) R' = methylol phosphoric acid ester: 5-Phosphopyridoxal-N-methochloride or N-methopyridoxal-5-phosphate

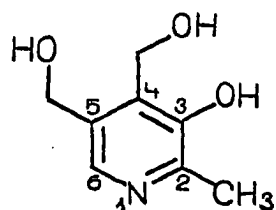
(DPLM) R' = methyl: 5-Deoxypyridoxal-N-methochloride

Pyridoxamine analogs

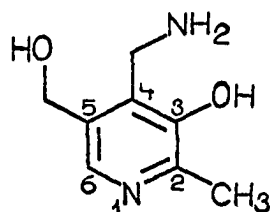
(PM) 3-hydroxy-2-methyl-4-aminomethyl-5-hydroxymethylpyridine: Pyridoxamine, vitamin B₆ amine

(PMP) Pyridoxamine-5-phosphate, a vitamin B₆ coenzyme

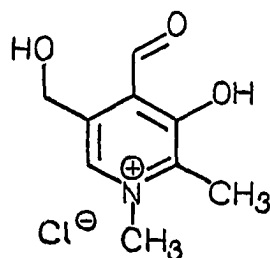
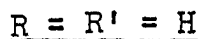
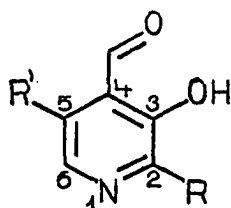
(DPM) 5-Deoxypyridoxamine, a vitamin B₆ antimetabolite



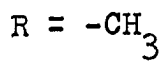
(PO) Pyridoxol



(PM) Pyridoxamine



(HPA) 3-Hydroxypyridine-4-aldehyde (PLM) Pyridoxal-N-methochloride



(PL) $R' = -CH_2OH$, Pyridoxal

(DPL) $R' = -CH_3$, Deoxypyridoxal

(PLP) $R' = -CH_2OPO_3H_2$, Pyridoxal phosphate

(CPL) $R' = -CO_2H$, "5-Pyridoxalylic acid" or "5-carboxypyridoxal" (3-hydroxy-2-methyl-5-carboxypyridine-4-aldehyde)

(FPL) $R' = -CH_2CO_2H$, "alpha⁵-Pyridoxalylformic acid" (3-hydroxy-2-methyl-5-carboxymethylpyridine-4-aldehyde)

(APL) $R' = -(CH_2)_2CO_2H$, "alpha⁵-Pyridoxalylacetic acid" (3-hydroxy-2-methyl-5-carboxyethylpyridine-4-aldehyde)

Figure 2. Aqueous solution equilibria of ionic forms of pyridoxal analogs in addition to those for deoxypyridoxal reported by Metzler and Snell (1955) and for hydroxypyridine aldehydes reviewed by Martell (1963)

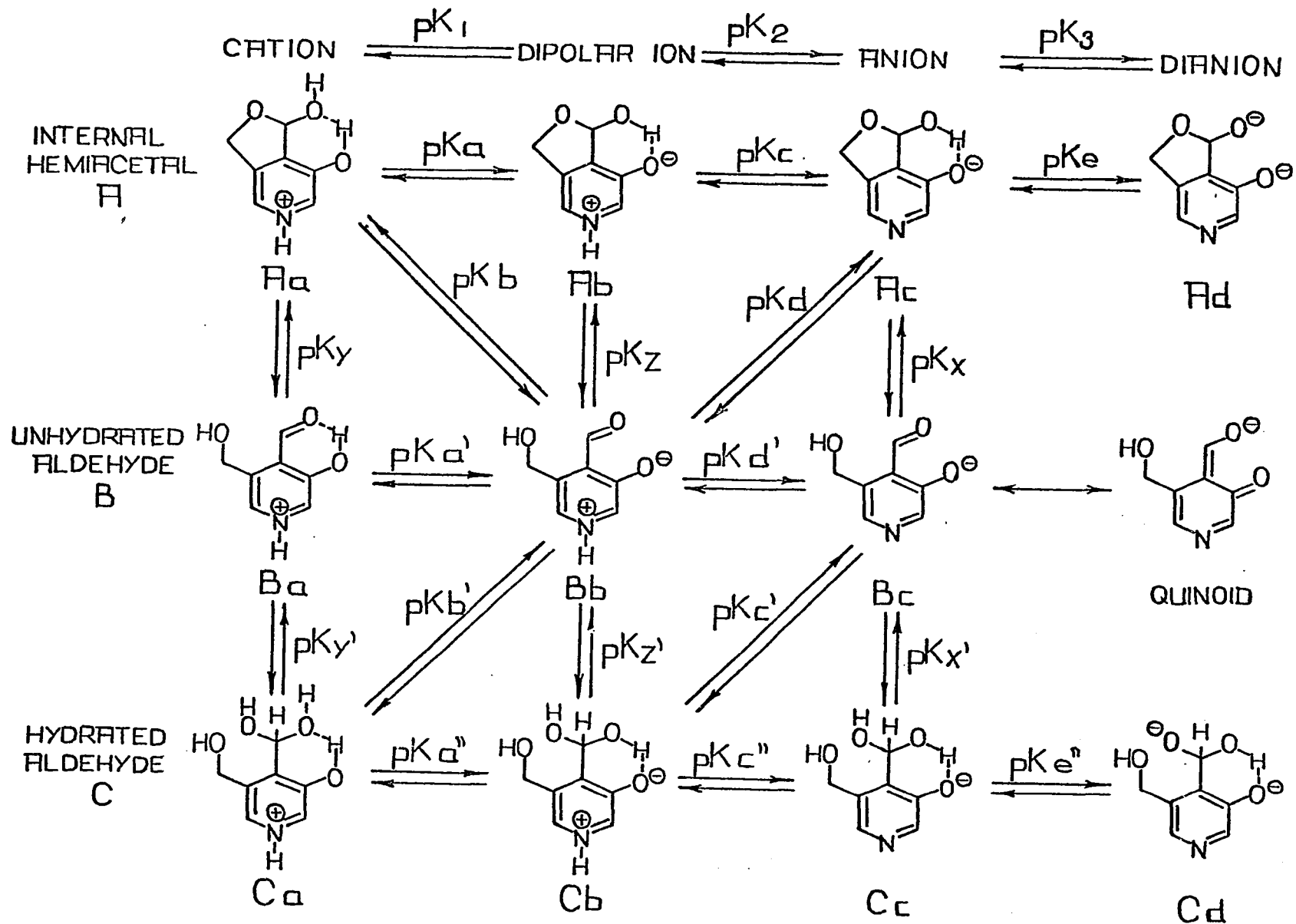


Table 1. Ionization constants and assignments of observed electronic absorption bands with molar absorbancy indices of ionic forms of some pyridoxine analogs at 25°C, 0.1 M ionic strength^a

A	B	C	D	E	pK ₁	C	D	E	pK ₂	C	D	E	pK ₃	C
PO ^b	pK _a 5.00	a	291	8.6	5.00	b	324	7.2	8.97	c	310	6.8		
	pK _c 8.97		(232)s	2.1			254	3.9			245	6.3		
PL ^c	pK _a 4.3	Aa	288	9.0	4.20	Ab	317	8.9	8.66	Ac	302	5.71	13	Ad ^d
	pK _b 5.3		(230)s	3.3	± 0.02		252	5.8	± 0.06		240	8.4		
	pK _c 8.6					Bb	390	0.14		Bc	390	0.2		
	pK _d 7.6						$A_b/B_b = 80$				$A_c/B_c = 2.8$			
	pK _z 1.06						$A_b/C_b = 120$				$A_c/C_c = 500$			
							$C_b/B_b = 0.66$				$C_c/B_c = 0.006$			

^aThroughout this table the following letters are substituted for these headings: A = PL analog (Figure 1); B = Microscopic (molecular) constants (except for phosphoryl and amino pK's which do not affect spectra); C = Ionic form (Figure 2); D = Wavelength of maximum absorbancy, mμ (s = shoulder); E = Molar absorbancy index, $a_m \times 10^{-3}$

^bSource: Lunn and Morton, 1952; Williams and Neillands, 1954; Peterson and Sober, 1954; Metzler and Snell, 1955; Martell, 1963

^cSource: von Viscontini et al., 1951; Lunn and Morton, 1952; Williams and Neillands, 1954; Peterson and Sober, 1954; Metzler and Snell, 1955; Nakamoto and Martell, 1959b; Martell, 1963

^dAd has $a_m = 5.8 \times 10^3$ at 300 mμ

Table 1. (continued)

A	B	C	D	E	pK ₁	C	D	E	pK ₂	C	D	E	pK ₃	C
PLM ^e	pK _a 4.05	Aa 293.5 (220) _s	7.22	4.05 ±0.03	Ab 323 254.5 Bb 400 A _b /B _b = 46	7.80 4.8 0.17								
HPA ^f	pK _b 4.2	Ba 324 (220) _s	0.3 4.2	4.05	Bb	6.77	Bc 384 Cc (245) _s	6.9 6.6						
	pK _c 6.6	Ca 285	6.2											
	pK _d 6.4													
	pK _z 0.22													
DPL ^g	pK _a 3.8	Ba 342 (255) _s	1.9 1.6	4.17 ±0.04	Bb 383 (250) _s	4.26 4.2	8.14 ±0.08	Bc 391 (265) _s	6.2 3.4					
	pK _a " 4.5	Ca 294.5	6.4											
	pK _b 4.3													
	pK _c 7.8													
	pK _d 8.0													
	pK _z 0.22													

^eSource: Johnston et al., 1963^fSource: Nakamoto and Martell, 1959b; Martell, 1963^gSource: Heyl et al., 1953; Metzler and Snell, 1955; Nakamoto and Martell, 1959b; Martell, 1963

Table 1. (continued)

A	B	C	D	E	pK ₁	C	D	E	pK ₂	C	D	E	pK ₃	C
PLP ^h	pK _a , 5.9	Ba	335	1.8	3.44	Bb	384	5.00	8.45	Bc	388	6.6		
	pK _d , 7.96				4.00		388	4.90	+0.04		(270) _s	3.0		
					4.14		325	2.56	8.4	Cc	305	1.1		
		Ca	295	6.7	phosphoryl	Cb	330	2.50	8.69					
					1.4		(250) _s	4.00						
					+0.4									
					+0.2									
		$C_a/B_a = C_b/B_b = C_c/B_c = 0.36 \pm 0.07$												
APL ⁱ		Ba	340		3.96	Bb	390		8.4	Bc	390			
		Ca	290			Cb				Cc				
PM ^j		a	293	8.5	3.54	b	325	7.70	8.21	c	308	7.30		
					3.31		253	4.60	7.90		245	5.90		
					3.4									
					amino pK									
					10.63									
					10.4									

^hSource: Umbreit et al., 1948; Heyl et al., 1951; Lunn and Morton, 1952; Peterson et al., 1953; Williams and Neillands, 1954; Peterson and Sober, 1954; Metzler and Snell, 1955; Christensen, 1958; Bonavita and Scardi, 1959; Martell, 1963; Anderson and Martell, 1964

ⁱSource: Tomita and Metzler, 1964; F. Scott Furbish, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. 1964. Spectrophotometric determination of ionization constants of "alpha⁵-pyridoxalylacetic acid." Private communication.

^jLunn and Morton, 1952; Williams and Neillands, 1954; Metzler and Snell, 1955

Table 1. (continued)

A	B	C	D	E	pK ₁	C	D	E	pK ₂	C	D	E	pK ₃	C
PMP ^k		a	293	9.00	3.69 3.25 +0.07 phosphoryl pK ₁ < 2.5 amino pK 10.92	b	325 253	8.3 4.7	5.76	c	308 245	8.0 6.7		

^kSource: Williams and Neilands, 1954; Metzler and Snell, 1955

The relative ratios of amounts of hydrated to unhydrated aldehydes have been estimated from the molar absorbancy indices (C/B-Table 1). The decrease in the relative concentration of hydrated aldehyde with increasing pH is due to a decrease in electron-withdrawing power of the pyridine ring as a result of the step-wise dissociation of protons. In decreasing the positive character of the charge on the formyl carbon atom, the addition of water across this carbonyl bond will be less likely.

Pyridoxal-5-phosphate (PLP) - Figure 1

The spectrum of the vitamin B₆ coenzyme, pyridoxal phosphate, is almost identical with that of deoxypyridoxal, a potent vitamin B₆ antimetabolite, over the whole pH range (von Viscontini et al., 1951; Heyl et al., 1953), and the results of the interpretation of the spectrum of the latter can also be applied to that of the coenzyme (Metzler and Snell, 1955). Ionizations of the phosphoric acid ester group (phosphoryl pK's Table 1) cause little difference in the spectrum of pyridoxal phosphate from that of deoxypyridoxal (Williams and Neillands, 1954).

Recently, molecular species of pyridoxal phosphate have been studied in D₂O solution by infrared spectra at various pD values, and the results have been compared with those obtained, in the same manner, from pyridoxal, pyridoxamine, pyridine, and hydroxypyridine derivatives (Martell, 1963; Anderson and

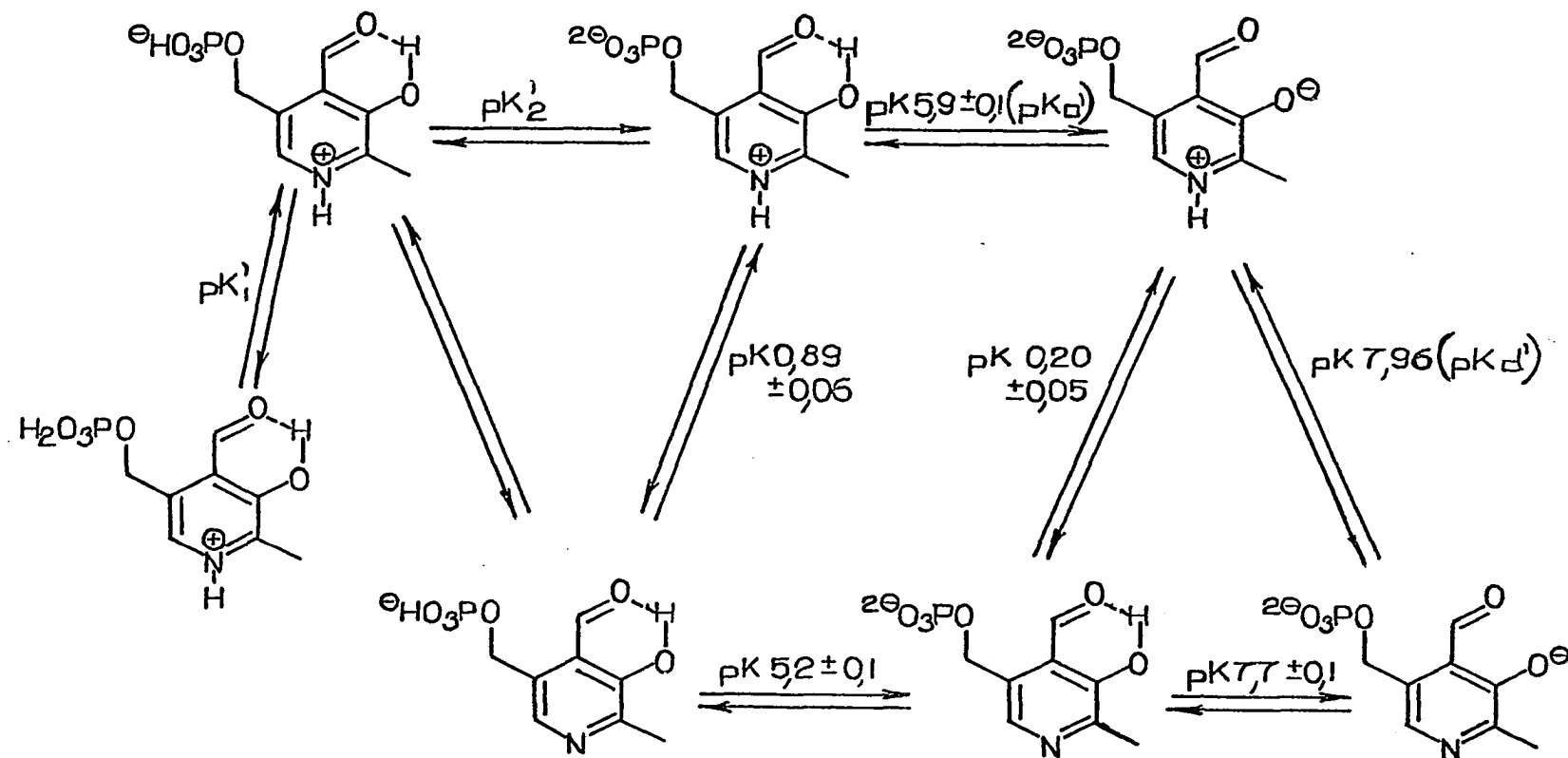
Martell, 1964). Spectral band assignments have been made and pK'_a values have been obtained. The ionization constants have been used to calculate some microscopic (molecular) equilibrium constants for pyridoxal phosphate in aqueous solution. These pK_a values (Figure 3) are for individual group ionizations, in contrast to the apparent pK'_a values (Table 1) reported previously for pyridoxal phosphate. The apparent pK'_a values agreed reasonably well with the previously reported values except that pK_1 (Figure 2, Table 1) for dissociation of the phenolic group was 3.44, in contrast to the earlier values of 4.14 and 4.0 (Christensen, 1958; Williams and Neilands, 1954).

Also, the ratio of hydrated to unhydrated aldehyde forms was constant, within experimental error (C/B-Table 1), over all pD values from 4.7 to 11.0, in contrast to the increasing proportion of the unhydrated aldehyde form in deoxypyridoxal and pyridoxal, with increasing pH (Metzler and Snell, 1955). The pK 's also were reported for two different ionic strengths (0.1 and 2.0 M), and an appreciable difference was noted (Anderson and Martell, 1964).

Pyridoxal (PL) - Figure 1

The introduction of a hydroxymethyl substituent at the 5-position of 3-hydroxypyridine-4-aldehyde causes considerable changes in the spectrum due to the predominance of the cyclic hemiacetal form (A-Figure 2). The hemiacetal forms predominate

Figure 3. Aqueous solution equilibria of some ionic forms of unhydrated pyridoxal phosphate showing microscopic or molecular dissociation constants (pKa's); from infrared spectra of D₂O solutions (Anderson and Martell, 1964; Martell, 1963)



over the free aldehyde forms over the whole pH range because of the stability of the 5-membered ring (Metzler and Snell, 1955; Nakamoto and Martell, 1959b). However, the ratio of hydrated to unhydrated aldehyde (C/B-Table 1) is about the same as that for deoxypyridoxal at similar pH values (Metzler and Snell, 1955; Table 1). In general, the electronic absorption spectrum of pyridoxal is similar to that of pyridoxol, except for the small absorption bands due to the free aldehyde forms (Table 1).

Pyridoxal-N-methochloride (PLM) - Figure 1

The absorption spectra and acid dissociation constants (pK_a 's) of the phenolic groups of pyridoxal and pyridoxal-N-methochloride have been compared (Johnston et al., 1963). Like the pyridoxal hemiacetal cation (Aa-Figure 2), pyridoxal methochloride can dissociate to form the dipolar ion (Ab-Figure 2). However, unlike pyridoxal, the N-methyl analog can not form an anion (Ac-Figure 2), and this was reflected in the same absorption spectrum at high pH as at neutral pH. A small amount of free aldehyde should also be present in equilibrium with the hemiacetal form, as in the case of pyridoxal. (See Results and Discussion for further details of this study.)

Equilibria of Imines of Pyridoxal Analogs

"The B₆ vitamins, and coenzymes, their imines (Schiff bases), and the corresponding metal chelates exist in many

equilibrium states, including various tautomers in neutral and ionic forms. Considerable information is currently available on the structure, absorption spectra and ionization and stability constants of these forms and on the effects of pH and other factors on their equilibria. These data provided a basis for successful application of spectrophotometric and other methods in investigations on the linkage and structure of the coenzyme moiety of highly purified B₆ enzymes in aqueous solutions at different hydrogen ion-concentrations and in the presence of specific substrates or inhibitors" (Braunstein, 1960, p. 182).

Absorption spectra and ionic equilibria of imines

When solutions of pyridoxal or pyridoxal phosphate and an amino acid are mixed characteristic electronic absorption spectral changes result from rapid formation of aldimines (Eichorn and Dawes, 1954; Metzler, 1957; Matsuo, 1957a; Christensen, 1958; Cattaneo et al., 1960; Lucas et al., 1962; Heinert and Martell, 1963a). A recent review has summarized and interpreted the spectral changes associated with the formation of various ionic species of these aldimines (Martell, 1963).

In general, the hydrogen-bonded aldimine forms of pyridoxylidene amino acids have been compared with those of salicylaldehyde imines. The 404-25 mμ absorption (π - π_1^* transition) and the 265-80 mμ absorption (π - π_2^* transition) bands have been assigned to the hydrogen-bonded aldimine

Figure 4. General ionic equilibria of pyridoxylidene amino acids

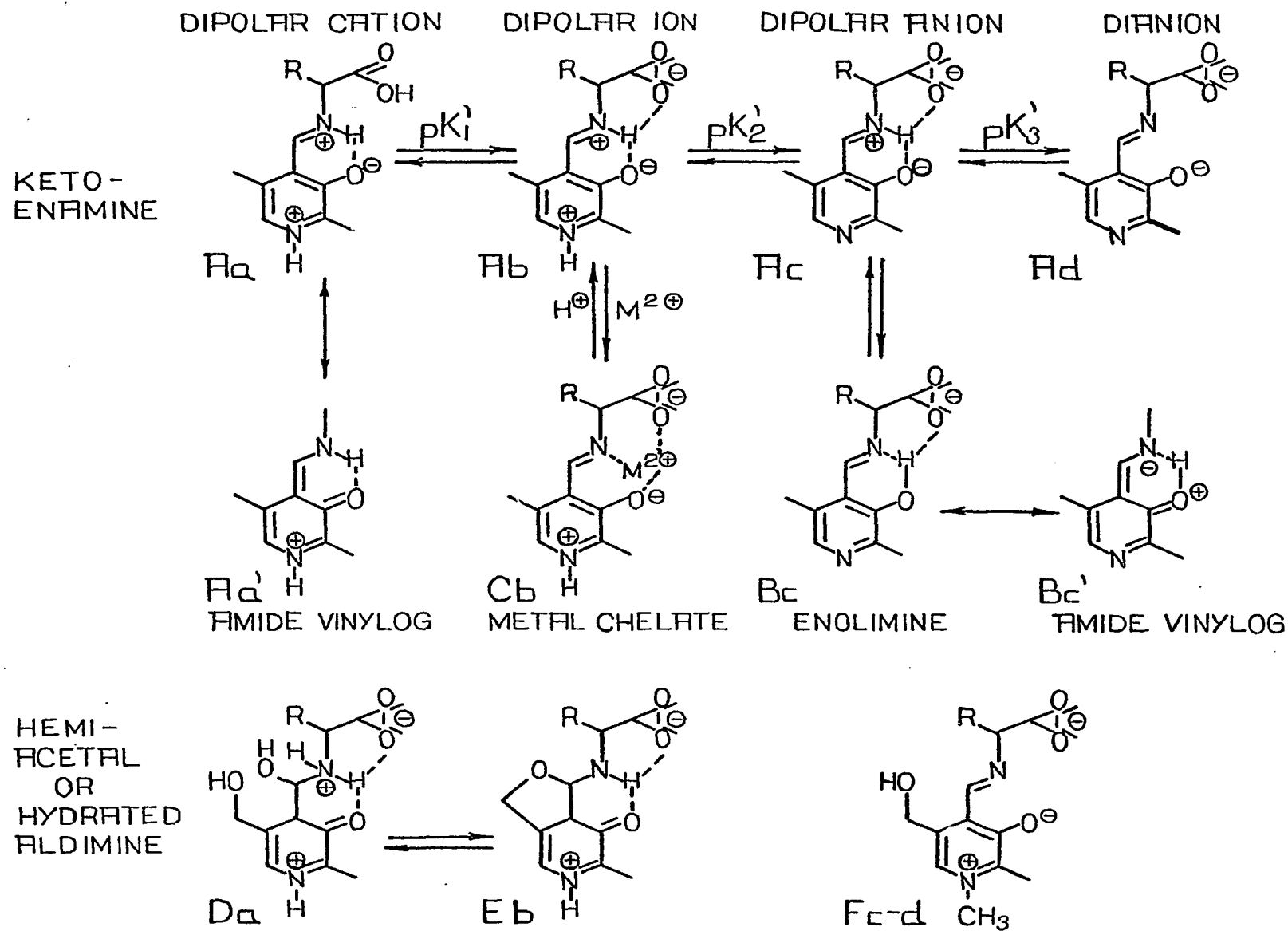


Table 2. Ionization constants and assignments of observed electronic absorption bands of ionic forms of some pyridoxylidene amino acids at 25°C

Imine	Ionic forms (Figure 4)												
	Aa-c			Ad			Bc		Cb-Zn ²⁺			Da or Eb	
	pK' ₂	λ_{\max} m μ	a _m x10 ⁻³	pK' ₃	λ_{\max} m μ	a _m x10 ⁻³	λ_{\max} m μ	m μ	λ_{\max} m μ	a _m x10 ⁻³	pK' ₂	λ_{\max} m μ	m μ
PL-Val ^a	5.9	414 280	6.24 7.23	10.49 ± 0.03	367-70	7.88	316-30 238-60		377 277	8.7 4.8	6.5	320-30 250-60	
pK' ₁ (2.3) ^b													
PLP-Val ^c	(6.3)	415 280		{10.8} {11.5}	367							278-85	
DPL-Leu ^d	(6.1)	414 294	(9)				234 (217)					323	
PLM-Val ^e	8.0	Aa-b 419 288	7.5 4.7		Fc-d 378	8.4			383 280	7.4 5.1			

^aSource: Metzler, 1957; Davis et al., 1961; Heinert and Martell, 1963a; Martell, 1963

^bThroughout, numbers in () = value uncertain

^cSource: Christensen, 1958

^dSource: Rommel, Erika, and David E. Metzler, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. 1964. Spectrophotometric determination of formation constants of imines of leucine and deoxypyridoxal. Private communication

^eSource: Johnston et al., 1963

(Metzler, 1957; Lucas et al., 1962; Heinert and Martell, 1963a). Protonations of the pyridine or imine nitrogen atoms are not expected to result in more than a slight shift in these wavelengths of maximum absorbancy (Martell, 1963). From infrared spectral studies it was found that the ketoenamine form (A-Figure 4), in which the proton of the hydrogen bond is closer to the imine nitrogen atom than to the phenolic oxygen atom, predominates over the enolimine form (Bc-Figure 4), the other tautomer, in aqueous solution (Heinert and Martell, 1962). The enolimine tautomer (Bc-Figure 4) has absorption bands similar to the hemiacetal (Eb) or hydrated imines (Da-Figure 4): 316-30 m μ for π_1 -transitions and 238-60 m μ for π_2 -transitions (Table 2).

Removal of the hydrogen-bonded proton, forming a dianion (Ad-Figure 4), does result in a shift of the absorption maximum to lower wavelengths (Table 2), as does replacement of this proton with a metal ion (Cb-Figure 4; Table 2; Eichorn and Dawes, 1954). The ionic equilibria of these imine-metal chelates have been studied spectrophotometrically. The effect of chelation by metal ions on the pK'_a of the pyridinium group depended on the nature of the metal ion. Values have been reported for pK'_a from about 5.5 to 7.9 (Christensen, 1959; Davis et al., 1961).

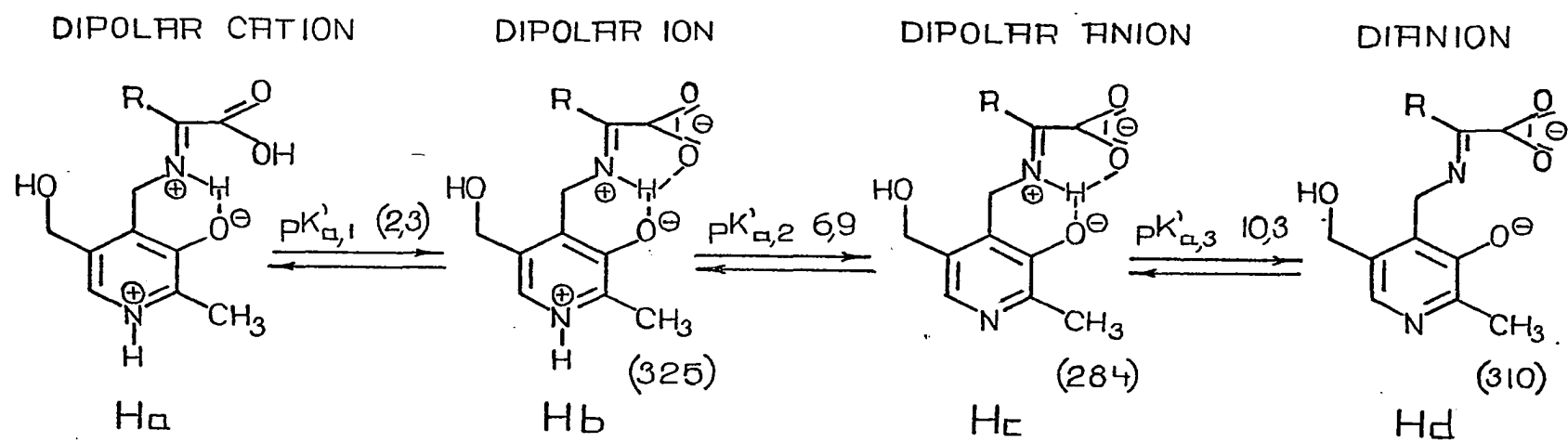
Ionic equilibria of N-(3-hydroxy-4-pyridylmethylene)-valine were studied quantitatively as a model system for pyridoxal imines (Heinert and Martell, 1963b). The ionization

constants are reversed in going from pyridoxal to its imine forms. The pK'_a of 8.6 for the pyridinium group in pyridoxal changes to 5.9 in the imine, and the pK'_a of 4.2 for the phenolic group in pyridoxal changes to 10.5 in the imine, as a result of the formation of a rather stable hydrogen bond between the phenolic oxygen atom, the imine nitrogen atom, and perhaps the carboxylate group of the amino acid residue (Ac-Figure 4; Metzler, 1957; Christensen, 1958). In addition, the ionic equilibria of ketimines from pyridoxamine and keto acids have been determined spectrophotometrically (Figure 5; Banks et al., 1961).

The elimination of the absorption band at 278 m μ upon the addition of acid or base to a neutral solution of pyridoxal phosphate and valine was followed to find apparent pK values of 6.3 and 11.5, respectively. Also, a pK value of 11.5 was found for the elimination of the absorption band at 415 m μ upon the addition of alkali. This was compared with a pK value of 10.8 from a potentiometric titration, but the ionic strength was 0.42 M, whereas it was only 0.10 M in the spectrophotometric titrations. Because tri- and tetravalent ions are involved in equilibria of pyridoxal phosphate imines, high sensitivity to ionic strength was anticipated (Christensen, 1958).

The existence of imines of pyridoxal and amino acids (and their metal chelates) has been proven by their isolation, identification, structure elucidation, and syntheses, which

Figure 5. Absorption spectra and ionic equilibria of some ionic forms of ketimines of pyridoxamine and pyruvate ($R = CH_3$) (Banks et al., 1961)



have been accomplished repeatedly in a number of different laboratories in the past twenty years. Some of the more recent accomplishments in this area also include reviews of previous findings (Christensen, 1958, 1959; Fasella et al., 1958; Davis et al., 1961; Heinert and Martell, 1962, 1963c; Cennamo, 1963). Furthermore, the stereochemistry of the manganese (II)-pyridoxylidene valine chelate has recently been established by x-ray diffraction (Willstadter et al., 1963), which indicated an essential planarity of the fused ring systems (from the measured bond angles and lengths) and a strong phenolic oxygen anion-metal cation bonding.

Mechanism of imine formation

The mechanism of imine formation has been described as initiating with a nucleophilic attack of the uncharged amino acid nitrogen atom on the electrophilic carbonyl carbon atom of the 4-formyl group of pyridoxal. Tautomerization of the dipolar ion intermediate followed by dehydration of the carbinolamine (tetrahedral addition intermediate) leads to the imine, a readily observed and a relatively more stable intermediate in the reaction between pyridoxal and an amino acid. All of these steps leading to the imine are equilibria which are rate-determining at certain pH values (Figure 6). This general mechanism of imine formation and hydrolysis has recently been substantiated with kinetic data (Cordes and Jencks, 1962a, 1963; Martin, 1964), which have been reviewed

Figure 6. Mechanism of imine formation; schematic comparison of aldimine formation (Snell, 1962)

- A. From an amino acid and pyridoxal
- B. From an amino acid and a preformed aldimine of pyridoxal by transaldimination

Step 1. Nucleophilic attack of amino-N on carbonyl-C

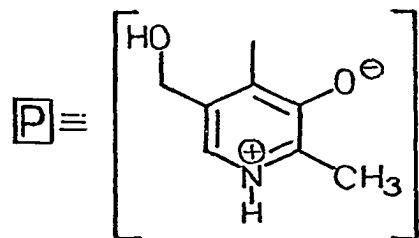
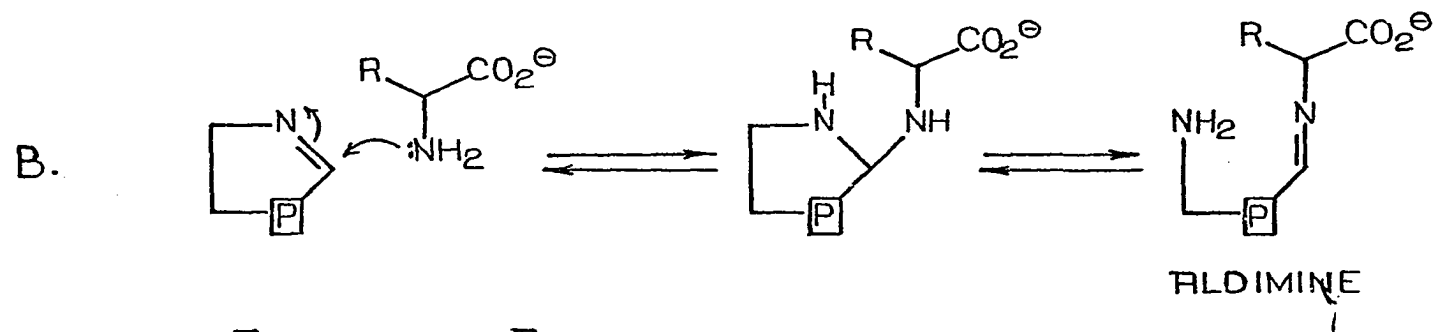
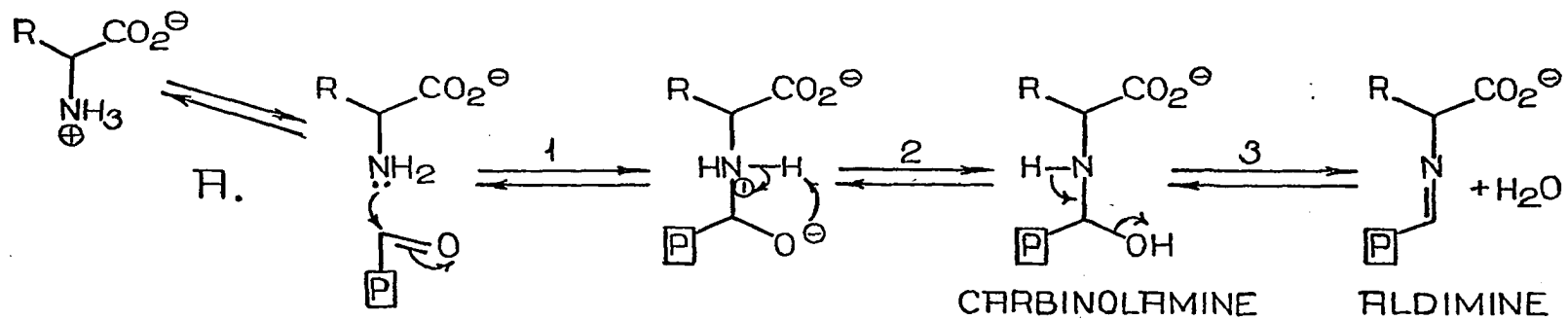
(For semicarbazone formation: rate-determining for pH less than 7)

Step 2. Tautomerization

Step 3. Dehydration

(For semicarbazone formation: rate-determining for pH above 7 and general acid-catalyzed for pH less than 4)

(Jencks and Cordes, 1963; Martin, 1964)



recently (Jencks and Cordes, 1963; Jencks, 1963; Martin, 1964). It is interesting that the dehydration step is subject to general acid catalysis (Cordes and Jencks, 1963; Jencks and Cordes, 1963; Jencks, 1963).

Rates of imine formation

Formation and hydrolysis of imines occur rapidly and reversibly in aqueous solution at room temperature. A non-aqueous medium, such as ethanol, enhanced the formation of imines of pyridoxal or pyridoxal phosphate and amino acids (Matsuo, 1957b). In the system of pyridoxal and valine equilibrium was achieved within ten minutes (Metzler, 1957). Several investigators have reported absorbancy changes with half-lives of a few minutes when pyridoxal and certain amines were mixed or when pH values of the resulting solutions were changed (Williams and Neillands, 1954; Metzler, 1957; Christensen, 1958; Fleck and Alberty, 1962).

Rate constants for the formation of carbinol amine (the tetrahedral addition intermediate - Figure 6) and aldimine were calculated from spectral changes in solutions of pyridoxal and alanine (Fleck and Alberty, 1962). Depending upon the conditions of pH and concentration, the former was $0.11 \pm 0.04/\text{sec.}$ at a maximum (half-time of about 0.7 sec.) and the latter was from 0.003 to 0.065/sec. (half-times of 10-200 sec.) at 25°C. From the rate constant reported for the hydrolysis of the aldimine of pyridoxal and gamma-aminobutyrate (Olivo et al., 1963), the half-time can be estimated as five minutes.

From the observed rate and equilibrium constants reported for formation of imines of pyridine-4-aldehyde and eleven amino acids (Bruice and French, 1964), half-times could be calculated. They ranged from about 47 seconds for leucine to 187 seconds for phenylglycine. From a similar quantitative analysis of the formation of imine of pyridoxal phosphate and cysteine (and its S-derivatives) (Mackay, 1962), half-times of 40 to 230 seconds, depending upon the conditions of pH and concentration, can be calculated. A slow imine-cupric chelate formation was observed over a period of 24 hours to equilibrium (not transamination) (Christensen, 1959). Other metal ions, such as zinc and sodium, replace the proton in hydrogen-bonded aldimines about as fast as imine formation itself (Davis et al., 1961).

The reactive form of pyridoxal for imine formation requires the free formyl group, and the limiting factor in the rate of imine formation from pyridoxal is the unfavorable equilibrium between the hemiacetal and free aldehyde forms, which also affects the final imine concentration or equilibrium constant (Snell, 1958). The higher rate of semicarbazone formation from pyridoxal phosphate, compared to pyridoxal, is the result of pyridoxal existing in aqueous solution principally as the unreactive internal hemiacetal (Cordes and Jencks, 1962b; Jencks and Cordes, 1963). The half-time for semicarbazone formation from pyridoxal is about $3\frac{1}{2}$ minutes ($k_{\text{obs}} = 0.2/\text{min.}$). Also, imine from gamma-amino-

butyrate and pyridoxal phosphate forms almost immediately, as compared to a slightly slower imine formation from pyridoxal (Olivo et al., 1963).

Equilibrium constants for formation of imines

Extent of imine formation depends upon pH, temperature, nature of the solvent and presence of metal ions. More imine is formed from pyridoxal and gamma-aminobutyrate with increasing temperature (Olivo et al., 1963). Imine formation is also favored by non-polar solvents, such as ethanol (Matsuo, 1957b), and by metal ions which form chelates (Longenecker and Snell, 1957; Davis et al., 1961; Cennamo, 1963).

The equilibrium constants for formation of imine anions from pyridoxal and amino acid anions approach maximum values in the alkaline pH range. Calculated values from spectral data at 25°C agreed with a theoretical curve of pH versus the logarithms of the equilibrium constants for formation of imine anion ($\log K_i$) from pyridoxal and valine (Metzler, 1957). This theoretical curve was based on pK'_a values of pyridoxal, amino acid and imine, and is compared with similar curves for imines of leucine with deoxypyridoxal and with pyridoxal phosphate (Figure 15; Results and Discussion).

In addition, formation constants of ketimines of pyridoxamine and pyruvate have been determined spectrophotometrically as a function of pH. These experimental values agreed with predicted formation constants calculated from pK'_a values

of the dissociable ketimines and reactants (Banks et al., 1961). A maximum formation constant of 9.8 was obtained at about pH 10, and this may be compared with the formation constant of 9 obtained for the aldimine anion of pyridoxal and alanine (Metzler, 1957).

The imine formation constant depends also somewhat on the structures of the amino acid and pyridoxal analog. Leucine was shown to have a favorable imine formation constant with pyridoxal, compared to other amino acids, and its value was about three times greater than that for alanine (Metzler, 1957), although reasons for this difference are unknown. Equilibrium constants for imine formation from eleven amino acids with pyridine-4-aldehyde were compared recently (Bruce and French, 1964). In the graphical determination of these constants between pH 7 and 10 it was concluded that only the free amino group is essential to the over-all equilibrium. The highest value (leucine) was three times that for the lowest (phenylglycine). These values are between ten and thirty times higher than those reported for pyridoxal and amino acids, due to hemiacetal formation in the case of pyridoxal. Also, imine formation takes place to a lesser extent from pyridoxal and amino acid esters than from pyridoxal, metal ions and amino acids (Cennamo, 1963, 1964).

Early imine formation constants for pyridoxal phosphate and amino acids ranged from 10^3 to 10^4 in neutral, aqueous solution, but the calculations from spectral data at 278 m μ

took into account the water (55.5 M) formed along with imine (Matsuo, 1957a). The formation constants for the sum of products from pyridoxal phosphate reacting with amino acids, peptides and proteins were estimated from both decreasing and increasing spectral changes (Christensen, 1958). These values ranged from 150 to 8000 at pH 7.5 (Christensen, 1958). More recently, the imine formation constant for pyridoxal phosphate and S-methylcysteine was 52/M at pH 6.3 and 450/M at pH 7.9, 21°C (Mackay, 1962). The values for pyridoxal phosphate and gamma-aminobutyrate were 15.5/M at pH 6.3 and 111/M at pH 7.4, 25°C, and were 25 times greater than those for pyridoxal and gamma-aminobutyrate under the same conditions (Olivo et al., 1963).

In summary, equilibrium constants for imine formation are 10 to 100 times greater with pyridoxal phosphate than with pyridoxal (Matsuo, 1957a; Metzler, 1957; Christensen, 1958). Also, pyridoxal phosphate is 10 to 100 times more reactive than pyridoxal toward semicarbazide (Cordes and Jencks, 1962b), and toward a variety of carbohydrazides (Wiegand, 1956). Acid dissociation constants of the hydrazides were linearly related to specific reaction rates for formation of pyridoxal phosphate hydrazones. The greater reactivity of pyridoxal phosphate than of pyridoxal toward nucleophilic reagents, because of the existence of the latter compound largely in the form of the unreactive internal hemiacetal, provides a further chemical advantage for

pyridoxal phosphate as a coenzyme (Cordes and Jencks, 1962b).

Spectral methods for determining imine formation constants

Consider the reaction, $A + P \rightleftharpoons I$, where A is amino acid, P is pyridoxal analog, I is aldimine. The observed constant, $K_O = I_e/A_e P_e$, where I_e , A_e , P_e are the sums of concentrations of all ionic species of I, A, and P, respectively, at equilibrium.

Early estimates of imine formation constants were based on molar absorbancy indices of solutions of imines, using the equation: $K_O = (a - a_p)(a_i - a_p)$, where a , a_i , a_p are apparent molar absorbancy indices of the solution, the imine, and the pyridoxal analog, respectively, at the same wavelength. Direct extrapolation of the spectral data was used to obtain a_i for imines from pyridoxal phosphate (Matsuo, 1957a; Christensen, 1958). Calculations of a_i and formation constants by successive approximations were carried out for imines from pyridoxal (Metzler, 1957) and for metal chelates of imines from pyridoxal (Davis *et al.*, 1961).

The double reciprocal plot of Lucas *et al.* (1962) was used to obtain absorbancies for imines of pyridine-4-aldehyde (Bruice and French, 1964), and a slightly different equation was used to calculate K_O : $K_O = (D - D_p)/A_o(D_i - D)$, where D is absorbancy at 270 mμ for imine (i), total solution (D), and pyridoxal analog in the solution (p), A_o is the initial amino acid concentration in excess over that of

pyridoxal analog. "Affinity constants" (K_M values) for imines of pyridoxal phosphate and amino acids were measured by the same graphical method at various pH values (King and Lucas, 1959; Lucas *et al.*, 1962).

The equilibrium constant for the formation of aldimine from pyridoxal phosphate and cysteine was determined from kinetic rate constants and from an equation relating absorbancies at a particular wavelength, initially (D_o) and at equilibrium (D_e), with different amino acid concentrations (Mackay, 1962). In the former case, $K_o = k_1/k_2$, where k_1 , and k_2 are rate constants for imine formation and hydrolysis, respectively.

The direct spectrophotometric determination was based upon the following relationships: $I_e = (D_e - D_o)/(a_1 - a_p) = P_o/K_o \cdot (1 + 1/K_o A_e)$. At two different concentrations of A, A' and A'' :

$$I'_e/I''_e = (D'_e - D'_o)/(D''_e - D''_o) = \frac{D'_o (1 + 1/K_o A''_e)}{D''_o (1 + 1/K_o A'_e)} \quad \text{And}$$

$$\text{solving for } K_o = \frac{D''_o (D'_e - D'_o)/A'_e - D'_o (D''_e - D''_o)/A''_e}{D'_o (D''_e - D''_o) - D''_o (D'_e - D'_o)} \quad \text{If}$$

A'_e and A''_e are equal to the concentrations of A added (pseudo first-order conditions), then K_o can be directly determined from the spectrophotometric measurements. A method of successive approximations, less time consuming than the method of Matsuo (1957a), was suggested if pseudo first-order conditions did not prevail. In general, accuracy in this method increases as K_o increases (Mackay, 1962).

A similar equation was derived for measuring apparent

equilibrium constants for ketimine formation from pyridoxamine and pyruvate (Banks et al., 1961). Consider the above reaction where A is now keto acid, P = pyridoxamine, I = ketimine. The derived equation adapted to this situation follows:

$1/(D_0 - D_i) = (1 + 1/K_0 A) \cdot 1/(a_p P_0 - a_i P_0)$, where D_0 is absorbancy of P when $A = 0$, D_i is absorbancy of a solution of P + I at some concentration A, P_0 is initial concentration, $a_i = (2/P_0) (D_i - D_0)/K_0 A$. Plotting $1/(D_0 - D_i)$ vs. $1/A$ yielded a straight line, and K_0 was found from the ratio, intercept/slope. The precision of measuring K_0 increased as $(D_0 - D_i)$ increased.

Molar absorbancy indices and apparent equilibrium constants for imine formation from gamma-aminobutyric acid and pyridoxal or pyridoxal phosphate were determined by the graphical methods of Ketelaar et al. (1951, 1952) and of Isenberg and Szent-Györgi (1958) (Olivo et al., 1963). These methods are suitable for molecular complexes in which each component participates with only one molecule, provided that all reactants and reaction products obey Beer's law. As in the previously described methods, all spectral data were corrected for absorbancy of amino acid, but since the amino acid concentration is high compared to imine concentration at equilibrium, small decreases in amino acid concentration were neglected in applying the correction. Molar absorbancy indices (a_p) of pyridoxal and pyridoxal phosphate were determined at the desired wavelengths, temperature and pH, because

the contributions of various ionic forms of these compounds to their total absorbancies change with the experimental conditions.

Ketelaar's equation, $1/(a - a_p) = 1/K_o (a_i - a_p) \cdot 1/A + 1/(a_i - a_p)$ is derived in the Experimental, since it was used for determining formation constants for imines from pyridoxal methochloride and valine, from pyridoxal phosphate and leucine, and from deoxypyridoxal and leucine. A plot of $1/(a - a_p)$ vs. $1/A$ yields a straight line, if pseudo first-order conditions prevail. The K_o value is the ratio of intercept, $1/(a_i - a_p)$, to slope, $1/K_o (a_i - a_p)$.

An analogous method for determining equilibrium constants for formation of complexes uses the following equation adapted to this situation: $1/(D_p - D_i) = 1/(a_i - a_p) \cdot 1/P_o + K_o/(a_i - a_p) \cdot 1/P_o \cdot 1/A_o$. A plot of $1/(D_p - D_i)$ vs. $1/A_o$ gives a straight line with slope of $K_o/(a_i - a_p) \cdot 1/P_o$ and intercept of $1/(a_i - a_p) \cdot 1/P_o$, and $K_o = \text{slope}/\text{intercept}$ (Harbury and Foley, 1958).

The method of Isenberg and Szent-Györgi (1958) uses differential spectral data and the equation: $1/A = K_o (a_i - a_p) P/(D_i - D_p) - K_o$. The absorbancy of a solution of imine is measured against a blank solution of pyridoxal analog at the same concentration as in the imine solution initially. A plot of $1/A$ vs. $1/(D_i - D_p)$ gives a straight line with slope of $K_o (a_i - a_p)$, ordinate intercept of $-K_o$, and abscissa intercept of $1/(a_i - a_p) P$.

Calculations of K_o at different wavelengths yielded concordant results, which was further evidence for correctness of the assumption that observed spectral changes represented imine formation and for proper application of Ketelaar's and Isenberg's methods for measures of K_o and a_i (Olivo et al., 1963). The fact that almost identical K_o values were obtained by the two methods was evidence that K_o may be calculated from observed spectral changes. Although K_o increased with temperature, a_i was independent of temperature (about 5×10^3 for pyridoxal and gamma-aminobutyrate, 20-40°C).

Pyridoxal-N-methochloride imines

The spectral changes resulting from the addition of an amino acid to pyridoxal methochloride have been interpreted and compared to that resulting from formation of pyridoxal imines (Johnston et al., 1963). The imine equilibrium (or formation) constant was directly calculated from the spectrophotometric data at pH 11, where the imine dipolar anion (Fc-d, Figure 4; Table 2) is formed from an amino acid anion and the pyridoxal methochloride dipolar ion. But at pH 6, incomplete formation of the imine dipolar ion complicated the direct determination of the equilibrium constant. At pH 6, decrease in the pyridoxal methochloride absorption band at 323 mμ corresponds with the loss of the hemiacetal dipolar ion, and the simultaneous appearance of an absorption band at 419 mμ, upon the addition of valine, corresponds with the hydro-

gen-bonded imine, from comparison with spectral studies of the equilibria between pyridoxal and amino acids and their imines (Metzler, 1957).

At equilibrium in a solution with initial concentrations of 10^{-4} M pyridoxal methochloride and 0.5 M valine the absorbancy at 419 m μ is about 0.7 as much as the absorbancy at 323 m μ . This was interpreted as a 30% conversion of the pyridoxal analog to the hydrogen-bonded valine imine. The remaining absorption band at 323 m μ was assumed to represent the 70% unreacted pyridoxal methochloride. From these assumptions the extent of imine formation was estimated (Johnston et al., 1963) by solving for the fractions of total pyridoxal methochloride in the free dipolar ion, imine dipolar ion and imine anion forms by a successive-approximation method and from molar absorbancy indices measured at wavelengths of maximum absorbancy for each of these three forms.

The absorption maximum of 367 m μ at pH 12 for the pyridoxal-valine imine dianion is shifted to 377 m μ upon methylation of the pyridine ring nitrogen atom which thereby gains a positive charge. This is in the same direction but of a different magnitude than the 15 m μ shift of the absorption band of the free pyridoxal anion upon protonation to form the dipolar ion (Metzler and Snell, 1955). In general, the effect of methylation of pyridoxal-amino acid imines on the absorption maxima for other ionic forms is to shift these maxima about 5 m μ to longer wavelengths (Johnston et al., 1963). Although

the dipolar-ionic valine imines of pyridoxal and of pyridoxal methochloride do not differ appreciably in stability, the hydrogen-bonded anionic imine of pyridoxal is approximately 100 times more stable than the non-hydrogen-bonded imine of pyridoxal methochloride (Johnston et al., 1963).

Biological significance of imines of pyridoxal analogs

The pK for loss of a proton from the imine of pyridoxal methochloride and valine was estimated to be 8.0, compared with values of 6.2 for glutamic-aspartic transaminase and 7.3 for glutamic-alanine transaminase. These differences and spectral differences between the enzymes and pyridoxal methochloride imines suggest that if the enzyme-bound coenzymes exist as a dipolar anion, their spectral behavior must be profoundly affected by groups in the proteins (Johnston et al., 1963).

Present knowledge of pyridoxal phosphate-enzyme spectra has been recently reviewed (Velick and Vavra, 1962a; Jenkins, 1963; Guirard and Snell, 1964). Spectra of imines of pyridoxal analogs or of coenzyme analogs bound to apoenzymes may help in the assignment of spectral absorption bands to the various structural forms of pyridoxal phosphate enzymes.

Imines resulting from interaction of pyridoxal, deoxypyridoxal and pyridoxal phosphate with proteins, as well as with amino acids and peptides, have been studied as to their electronic absorption and infrared spectra, ionization and formation constants (Table 3; Christensen, 1958; Dempsey and

Christensen, 1962).

Table 3. Spectra of imines of albumin and pyridoxal analogs

	pH 7.5	
	λ_{\max} m μ	$a_m \times 10^{-3}$
PL	317	6.80
	415	1.45
DPL	339	2.22
	426	2.81
PLP	332	3.71
	413	0.94

Imine stability constants fell into three classes depending upon the nature and specificity of binding groups. The highest constants ranged from 10^5 to 10^6 . Hydrogen-bonded imines (at 415 m μ) were stabilized by phosphate ester linkages to the protein, but were not specific for phosphate esters. Deoxypyridoxal, for example, reacted at non-specific sites. Pyridoxylamines (substituted imines) were seen at 332 m μ (Dempsey and Christensen, 1962).

Affinity constants for the formation of imines between pyridoxal phosphate and amino acids have been calculated from changes in the absorption spectrum of pyridoxal phosphate upon the addition of graded concentrations of amino acid and compared to Michaelis constants of pyridoxal phosphate-

enzymes with their amino acid substrates (King and Lucas, 1959; Lucas et al., 1962; King, 1963). A close correlation was found for the complex formation of leucine with leucine decarboxylase and with pyridoxal phosphate, which was interpreted to mean that the coenzyme may be the binding site which largely determines substrate affinity, and that the apoenzyme does not greatly alter the stability of the coenzyme-substrate link. The apparent pH optimum of 6.5 to 7, which was found for the model imine, agreed with that of the enzyme, and was considered due to a decrease in substrate affinity at lower pH values (Lucas et al., 1962). Also, inhibition of homoserine deaminase (a pyridoxal phosphate enzyme) by amino acids paralleled the stability constants of their imines with pyridoxal phosphate (Matsuo, 1957a).

The calculated rate of imine formation for aspartate in glutamate-aspartate transaminase is at least $7.7 \times 10^6/\text{M}/\text{min.}$ at pH 8.5, and may be compared with a value of $30/\text{M}/\text{min.}$ for nonenzymic imine formation between aspartate and pyridoxal phosphate (Cordes and Jencks, 1962b). Only a slightly higher value would be expected for the reaction of aspartate with a pyridoxal phosphate imine. This strong catalytic influence of the enzyme even on imine formation takes place perhaps through intramolecular formation of cationic imine which is more susceptible than an uncharged imine toward nucleophilic amino groups in transaldimination.

In Figure 6, which compares (A) aldimine formation from pyridoxal and an amino acid with (B) formation from a pre-formed aldimine of pyridoxal (as in enzymic catalysis) by transaldimination, the latter process is more efficient. It is thus advantageous for pyridoxal phosphate-enzymes to have the coenzyme bound as an imine, since imines of pyridoxal and pyridoxal phosphate have been found to be at least thirty times more reactive toward nucleophiles, such as semicarbazide in semicarbazone formation, than the free aldehydes (Cordes and Jencks, 1962b). Therefore, the first covalent bond-forming reaction with pyridoxal phosphate enzymes and amino acids is transaldimination, since enzyme coenzyme linkage has been shown to be an azomethine of the aldehyde group of pyridoxal phosphate and the epsilon amino group of a lysine residue in the protein (Jencks and Cordes, 1963; Snell, 1962; Guirard and Snell, 1964).

Synthetic imines (hydrazones, hydrazides and oximes) of pyridoxal phosphate have been found, not only to behave as coenzymes, for some pyridoxal phosphate enzymes, but in some cases to be more active than pyridoxal phosphate itself, and in others, to be more resistant to inhibition by strong pyridoxal phosphate inhibitors, such as penicillamine (Gonnard, 1963; Gonnard et al., 1964; Makino et al., 1962, 1963; Ooi, 1964). Evidence for the biological roles of imines of pyridoxal phosphate (their roles in enzymic reactions) has been reviewed (Braunstein, 1960; Snell, 1958, 1962; Guirard and

Snell, 1964). However, only the role of these imines in transamination reactions with amino acids will be considered in this review.

Function of Vitamin B₆ in Transamination

"Transamination may be defined as the transfer of an amino group from one molecule to another without the intermediate formation of ammonia." (Guirard and Snell, 1964, p. 138). Metabolic significance of the transamination reaction, including its central importance in intermediary nitrogen metabolism, has recently been reviewed (Braunstein, 1957; Meister, 1962; Guirard and Snell, 1964). Its widespread occurrence and role in biosynthesis of many amino acids are among its important features.

Early nonenzymic models of transamination ignored the coenzyme role of vitamin B₆ (Herbst, 1944), and it was not until 15 years after the discovery of the vitamin that its coenzymic function was proposed (Snell, 1945b). This proposal was based upon observed changes in specificity of lactic acid bacteria for pyridoxal, pyridoxamine and pyridoxol, after autoclaving pyridoxol (previously treated with potassium permanganate) and glutamic acid (Snell, 1944; Snell and Rannefeld, 1945), as well as with other amino acids; and upon the demonstrated natural occurrence of pyridoxal and pyridoxamine (Snell, 1945a; Rabinowitz and Snell, 1947). The evidence at present for the coenzymic role of vitamin B₆

phosphate in transamination is quite substantial and has been reviewed a number of times recently (Meister, 1962; Snell, 1958; Snell and Jenkins, 1959; Braunstein, 1960; Sizer and Jenkins, 1963; Guirard and Snell, 1964).

Mechanism of transamination

Early suggestions that imine intermediates were involved in transamination (Braunstein and Kritzmman, 1937; Herbst, 1944), influenced the independent proposals for the classical mechanisms for enzymic transamination (Braunstein and Shemyakin, 1952, 1953), and for nonenzymic transamination (Metzler et al., 1954a). "Although the conclusions of these two groups of investigators do not always coincide in details, it seems justified to label their propositions with the general denomination of the theory of Braunstein, Metzler and Snell." (Perault et al., 1961, p. 555).

The similarity of these enzymic and nonenzymic mechanisms, in which amino acid imines of pyridoxal phosphate or of pyridoxal (or their metal chelates) are intermediates, is in direct contrast to the different mechanisms proposed in an early review of enzymic and nonenzymic transamination, neither of which involved vitamin B₆ (Herbst, 1944). The generally accepted mechanism for enzymic transamination (sometimes called the binary mechanism or pyridoxal phosphate-pyridoxamine phosphate "shuttle mechanism") takes into account the established nature of coenzyme binding and resolution, recent

kinetic studies of transaminases, and results from investigations on nonenzymic model systems, all of which have been adequately reviewed recently (Lis et al., 1960; Jenkins and Sizer, 1960; Velick and Vavra, 1962a, b; Snell et al., 1963; Hammes and Fasella, 1963b; Guirard and Snell, 1964). The descriptive details of this mechanism have been clearly presented recently (Snell and Jenkins, 1959; Snell, 1962; Jenkins, 1963; Jencks, 1963; Pullman, 1963; Guirard and Snell, 1964) and summarized in Figure 7.

Initial activation of the amino acid is postulated to occur through formation of an aldimine between the amino acid and pyridoxal (or a pyridoxal-containing enzyme). Labilization of the bonds to the alpha-carbon atom of the amino acid leads to the several reactions, including transamination, that are catalyzed by the versatile vitamin, or more efficiently by enzymes that contain it (Guirard and Snell, 1964). However, the only differences in the enzymic and nonenzymic mechanisms are: 1) imine formation and hydrolysis in the nonenzymic mechanism is replaced by the more efficient transamination process in the enzymic mechanism, and 2) the nature of the catalytic groups effecting the aldimine-ketimine tautomerization and the stereospecificity in the enzymic reaction on the substrate are difficult to duplicate in a model system.

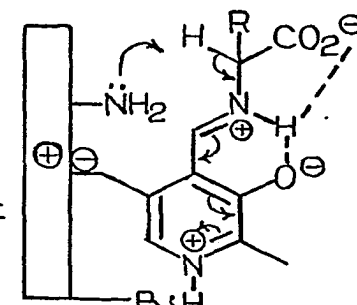
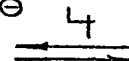
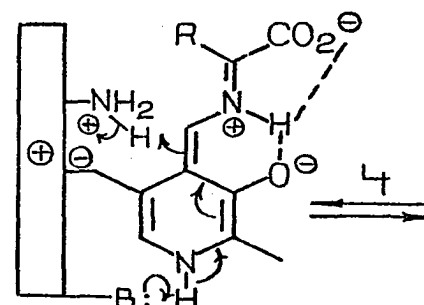
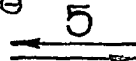
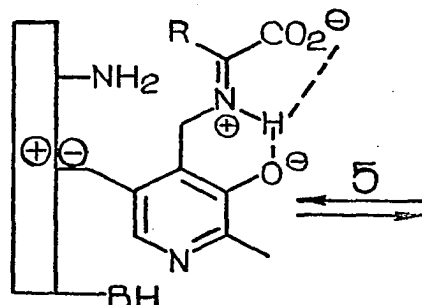
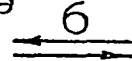
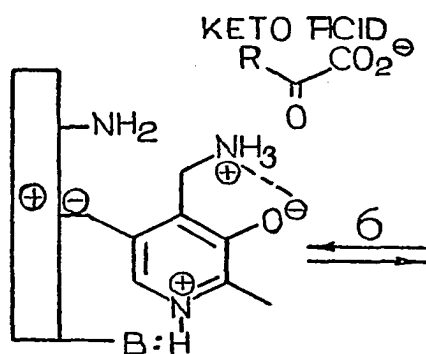
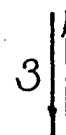
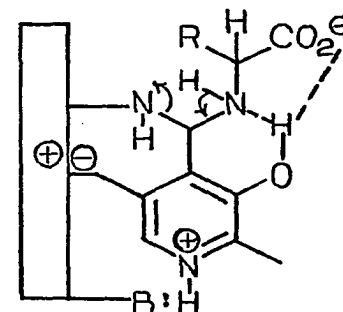
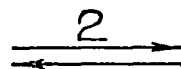
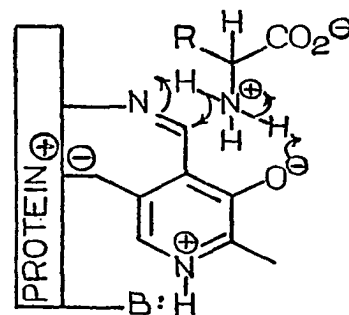
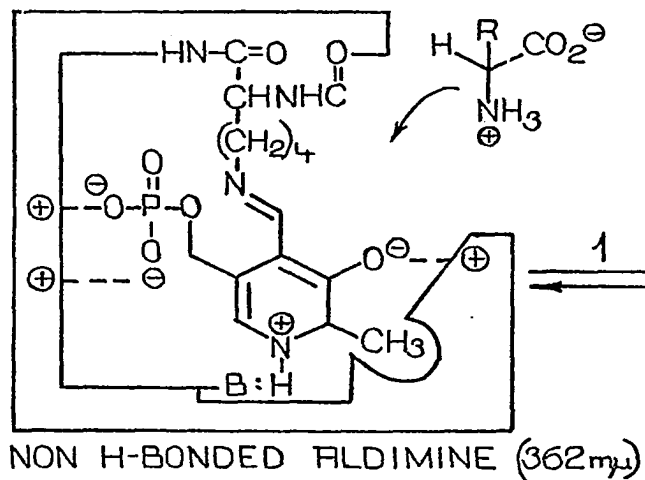
After transaldimination the liberated epsilon-amino group of lysine may act as a general acid-base catalyst for the electron shifts initiated by the pyridoxal coenzyme (Snell,

Figure 7. Classical binary or shuttle mechanism of pyridoxal phosphate function in transamination (Snell, 1962; Guirard and Snell, 1964)

- Steps: 1. Approach of non-hydrogen bonded aldimine form of enzyme to amino acid
- 2,3. Transaldimination
- 4,5. Tautomerization (rate-determining of aldimine to ketimine)
6. Hydrolysis

Other suggested absorption bands for enzyme-substrate intermediates for glutamic-aspartic transaminase (Sizer and Jenkins, 1963; Jenkins, 1963; Hammes and Fasella, 1963a; Guirard and Snell, 1964):

Non-H-bonded aldimine (upper left)	362 m μ
X (upper right)	330 m μ
H-bonded aldimine (lower right)	425 - 35 m μ
Y (lower center)	492 m μ
Ketimine	325 m μ
Pyridoxamine form (lower left)	332 m μ



1962). This suggestion of the role of the epsilon-amino group of lysine was meant to be symbolic of the way in which one or more proximal nucleophilic groups on the protein may facilitate the labilization and transfer of protons. "At our present level of knowledge, such schemes ignore the unknown factors responsible for substrate and reaction specificity of individual pyridoxal phosphate-proteins, and undoubtedly oversimplify as well the catalytic events occurring on the coenzyme." (Guirard and Snell, 1964, p. 158).

The prototropic shift (or removal of the alpha-hydrogen), converting the aldimine to the ketimine form, has been shown to be the rate-determining step in enzymic, as well as in nonenzymic transamination systems, as predicted (Metzler, 1957) and recently substantiated (Velick and Vavra, 1962a,b; Hammes and Fasella, 1963a,b; Jenkins, 1963; Banks et al., 1961, 1963; Blake et al., 1963; Bruice and Topping, 1963a,d; Junk and Svec, 1964; Vernon, 1964). General base-catalysis facilitates the removal of the proton from the alpha-carbon atom of the amino acid residue in the aldimine form, and general acid catalysis may enhance protonation of the formyl carbon atom, as well as the pyridine nitrogen atom. Hydrolysis of this resulting ketimine leads to formation of an alpha-keto acid corresponding to the amino acid substrate. Or, another keto acid may exchange with this coenzyme-bound keto acid in a process of transketimination, followed by the prototropic shift, and resulting in a new amino acid after

hydrolysis of this second aldimine, thus completing a transamination between two amino and two keto acids in a consecutive manner by way of binary complexes with the coenzyme on the enzyme surface.

A positively-charged nitrogen atom of the pyridine ring acts as an "electron sink" to facilitate electron withdrawal from the alpha-carbon atom of the amino acid in the aldimine (Cordes and Jencks, 1963). The original Metzler-Snell-Braunstein hypothesis proposed that the inductive effect of this electronegative group weakens the bonds around the alpha-carbon atom in the aldimine through a reduction of electron density in these bonds. Rupture of one of these bonds with formation of a conjugated system of double bonds extending from the alpha-carbon atom to the electronegative group (pyridine ring nitrogen atom) gives an intermediate in which the "extra" electron pair can be localized in any of a number of possible ways (Metzler et al., 1954a; Braunstein, 1960; Guirard and Snell, 1964).

However, a modification in the interpretation of the driving force of this mechanism has been made on the basis of molecular orbital (LCAO) approximations (Perault et al., 1961; Pullman, 1963). These approximations indicate that the electron density about the alpha-carbon atom in the aldimine is not reduced, but is actually increased. Furthermore, this carbon atom is not a part of, but outside of, or next to the conjugated system, since it is a saturated carbon atom in the

aldimine. The driving force for prototropic shift in the interconversion of alpha-amino acid aldimines of pyridoxal is then the stabilization of an intermediate carbanion owing to a gain in resonance energy by creating a particularly favorable conjugated system. That is, resonance stabilization of an intermediate carbanion drives the tautomerization of imines in transamination.

Since appreciable quantities of ketoenamine exist in solutions of pyridoxal imines, the participation of the protonated imine nitrogen atom as a strong electrophilic center has been suggested as an additional driving force in the mechanism of pyridoxal-catalyzed reactions (Bruice and Topping, 1963d; Jencks, 1963). The most reactive species of pyridoxal phosphate imines towards transamination is protonated at the imine nitrogen atom (Cordes and Jencks, 1962b; Jencks and Cordes, 1963). This protonated nitrogen atom, adjacent to the two carbon atoms involved in proton exchange during transamination, may facilitate the rate-determining loss of the proton from the adjacent saturated carbon atom to form the transition state. The electronic influence of a proton or metal ion attached to this imine nitrogen atom facilitates formation of such a transition state with a positive imine nitrogen atom (Martell, 1963). The inductive effect of the pyridine ring nitrogen atom assists in the formation of the transition state which is favored by the positively-charged nitrogen atom in the enamine. This inter-

pretation of the mechanism also applies to the metal ion-catalyzed transamination reactions (Metzler and Snell, 1952b) and to the transamination reactions in ethanol (Matsuo, 1957b), since significant amounts of the ketoenamine form are expected in both model systems.

Kinetics of the transaminase reaction has supported a Ping Pong Bi Bi mechanism (i.e., Binary complexes between amino acid or keto acid and coenzyme are formed and exchanges of the amino and keto groups take place between coenzyme and substrate.), and has directly contradicted the suggestions (Evangelopoulous and Sizer, 1963) that the enzyme has only a pyridoxal phosphate coenzyme form and that the enzyme forms a ternary complex with both amino and keto acids prior to exchange of amino and keto groups (Henson and Cleland, 1964). Although the modified classical mechanism of coenzyme action in transamination is unquestionably insufficient to explain the way in which the protein so greatly enhances the catalytic effectiveness of the coenzyme, this theory has most of the evidence in its favor, has correlated a large body of data, and has suggested many further experiments, thus proving its usefulness.

Nonenzymic Transamination in Model Systems

The purpose of a model system is to simulate the real system found in nature, at least in the particular aspects to be studied, and to provide a simpler subject for convenience

of measurement. Model transamination systems have consisted of either pyridoxal phosphate, pyridoxal or one of its structural analogs reacting with an amino acid, peptide or other amino acid derivative, or pyridoxamine or its phosphate coenzyme reacting with a keto acid, under suitable conditions, which may include various additional catalysts. These non-enzymic systems have provided simple and suitable media for detailed studies of the mechanism of the enzymic transamination, of the role of the coenzyme and of its structural requirements in the reaction. However, these chemical models are related to the enzymic reaction only if it is known how readily pyridoxal phosphate or pyridoxal combines with amino acids, how chelating metal ions influence the reaction, and what relation, if any, exists between the affinity of pyridoxal phosphate and an amino acid under physiological conditions and the affinity of pyridoxal phosphate enzymes for amino acids (Lucas et al., 1962). For recent reviews on non-enzymic reactions of pyridoxal with amino acids and their significance see the following: Snell, 1963; Snell, 1962; Westheimer, 1960; Bruice and Topping, 1963a; Cennamo, 1963; Guirard and Snell, 1964.

When pyridoxal and pyridoxamine became available in pure form, Snell first demonstrated the nonenzymic transamination between these vitamin B₆ compounds and glutamate and alpha-ketoglutarate at physiological pH values in dilute aqueous solutions at autoclave temperatures (Snell, 1945b). The

reaction occurred with other amino and keto acids, with pyridoxal and pyridoxamine, acting as an amino group acceptor and an amino group donor, respectively, in the reversible transformation of an amino acid to a keto acid. The rate of the reaction was increased by as much as 20-fold by the addition of appropriate metal ions, of which the most effective were Cu (II), Al (III), and Fe (III) (Metzler and Snell, 1952b; Greggerman and Christensen, 1956; Longenecker and Snell, 1957), and by as much as 100-fold by Cu (II) at pH 10, room temperature (Banks et al., 1961). Nonenzymic, metal ion-catalyzed transamination between peptides and pyridoxal has also been demonstrated (Cennamo, 1954, 1958). The activity of metal ions in catalyzing these reactions increased in approximately the order of increasing stability of the pyridoxamine chelates with the corresponding metal ions (Longenecker and Snell, 1957; Gustafson and Martell, 1957).

The roles of metal ions in imine chelate formation and in the catalysis of nonenzymic transamination have been described (Metzler et al., 1954; Snell, 1958, 1962; Bruice and Topping, 1963a; Guirard and Snell, 1964). Similarity between reactions catalyzed by pyridoxal phosphate enzymes and those catalyzed by pyridoxal and metal salts has been stressed (Metzler, et al., 1954a). Metal ions have been considered as playing inefficiently in nonenzymic systems the functional role played by acidic and basic groups in the protein in enzymic systems

(Metzler et al., 1954a; Snell, 1958, 1962; Snell and Jenkins, 1959; Perault et al., 1961; Guirard and Snell, 1964).

Evidence that such nonenzymic transamination reactions proceed through imine intermediates has included spectrophotometric (Eichorn and Dawes, 1954; Metzler, 1957, Matsuo, 1957a; Fasella et al., 1957), electrophoretic or chromatographic detection of reactions, aldimine and ketimine intermediates, and products (Fasella et al., 1957; Matsuo, 1957b; Banks et al., 1961; Olivo et al., 1963; Cennamo, 1963). The nonenzymic transamination of pyridoxal phosphate by an excess of amino acid to pyridoxamine phosphate at physiological temperature (37°C), in the presence of metal ions, was observed chromatographically and spectrophotometrically. At pH 5 there was good reaction because chelated imines were unstable, but at pH 7 there was poor reaction because chelated imines were more stable (Cattaneo et al., 1960).

Although at least a portion of the nonenzymic reaction proceeds through the metal chelated imines when metal ions are present, as shown by several semi-quantitative kinetic studies (Fasella et al., 1957; Cattaneo et al., 1960), the reaction proceeds slowly in water (Metzler, 1957; Metzler and Snell, 1952b; Banks et al., 1961; Fleck and Alberty, 1962) and rapidly in ethanol (Matsuo, 1957b; Bruice and Topping, 1963a) and in highly alkaline medium (Gustafson and Martell, 1957) without the addition of metal ions. In fact, metal ions inhibit reaction in ethanol (Matsuo, 1957b) and do not

seem to affect the reaction rate in alkaline solution in the presence of high buffer concentration, such as 1.8 M imidazole (Bruice and Topping, 1962, 1963a).

Another metal ion-independent nonenzymic transamination has recently been established for pyridoxal and leucine ethyl ester (Cennamo, 1964). This reaction proceeded at a rate comparable to the alum-catalyzed reaction between pyridoxal and leucine. Although the transamination of amino acid esters with pyridoxal proceeded to greater than 90% decrease in pyridoxal after 40 minutes at 100°C, pH 5, the reverse reaction was also uncatalyzed with metal ions; nor was it enhanced by keto acid esters (Cennamo, 1961, 1964). Reactions of pyridoxal analogs with leucine ethyl ester at 25°C have indicated that deoxypyridoxal and pyridoxal phosphate are much better amino group acceptors than pyridoxal and that favorable conditions are in slightly acidic solution (Johnson, 1964).

Furthermore, less imine formation was observed for the amino acid esters than for free amino acids and metal ions, which suggests that the former imines are more reactive (less stable) than the latter (Cennamo, 1962). Although metal chelation allows formation of high concentration of imines, most of these chelates may be poor reactive intermediates (Cennamo, 1963). These reactions of pyridoxal with amino acid esters have provided experimental evidence for the concept that one role of the metal ion in imine chelates is to increase the electronegativity of the carboxyl group by suppressing its

ionization (Snell, 1958), which is what esterification does. An essential feature of enzymic transamination is the binding of the carboxyl group of amino acids to the apoenzyme (Jenkins et al., 1959). On the other hand, lack of an inductive effect of the carboxyl group in gamma-aminobutyric acid caused inadequate activation of protons bound to the gamma-carbon atom of this amino acid in an imine with pyridoxal, and resulted in a very slow transamination reaction (Olivo et al., 1963).

Imidazole catalyzed a transamination between phenylglycine and pyridoxal in aqueous solution that was even more rapid than the metal-ion-catalyzed reaction (at least at pH 8.6) (Bruice and Topping, 1962, 1963a,b,d). This finding is of potential significance because of the presence of imidazolyl and other polar groups of proteins, perhaps acting as catalysts in reactions of the imines of substrates and pyridoxal phosphate enzymes, and because of the lack of metal ions in purified transaminases and the lack of their effect in enzymic transamination (Fasella et al., 1962; Snell and Jenkins, 1959; Jenkins et al., 1959).

This model for the pyridoxal-requiring transamination reaction has other features of enzymological interest: 1) The ability of imidazole to form a catalyst-substrate complex resulted in a virtual specificity for imidazole at low reactant concentrations compared with other general bases investigated, such as carbonate and morpholine. The formation constants for

the complexes of imidazole with phenylglycine were found to be constant over the pH range 7 to 10, although the over-all rate of reaction with pyridoxal increased with an increase in pH (Bruice and Topping, 1963b). The pK_a of 7.78 for the maximum velocity (V_m) as a function of pH approaches the pK_a of 8.3 for pyridoxal (Metzler, 1957). 2) The reaction operated in aqueous media at physiological pH values (7 to 10) and temperatures (30°).

3) The transamination reaction in this model system was equally effective if preceded by a transamination reaction, the transamination of phenylglycine and pyridoxal proceeded at the same rate if phenylglycine was added to the imine of pyridoxal and morpholine (Bruice and Topping, 1963c). The catalysis of pyridoxal reactions by morpholine, which must involve the intermediate with a protonated imine nitrogen atom, was highly effective in the case of pyridoxal phosphate but less effective and levelled off in concentrated morpholine solutions in the case of pyridoxal. This levelling off, and the spectral changes observed upon the addition of morpholine to pyridoxal, suggested that an unreactive cyclic aminoacetal was formed (Cordes and Jencks, 1962b).

4) Catalysis was by weakly basic imidazole and weakly acidic imidazolium ion, in which the proton of the alpha-carbon atom of the amino acid residue in the aldimine was abstracted by the general base and a proton was donated to the azomethine bond by the general acid species of imidazole, to complete the

concerted general acid, general base catalysis of the intra-complex prototropic shift (Bruice and Topping, 1963d). In the enzymic reaction the abstraction of the proton must be carried out by the weakly basic groups available to the protein at pH values near neutrality, the most effective of which would be the imidazolyl group of histidine. If it was assumed that dissociation constants of all the complexes with imidazole and imidazolium ion are similar, that formation constants for aldimines are in the usual range of 1 to 100 (Metzler, 1957), and that the acid dissociation constants of the imine intermediates are similar, then it was calculated that the rate of prototropic shift leading to conversion of aldimine to ketimine was only about 100 to 1000 times slower than the corresponding step for glutamate-aspartate transaminase (Bruice and Topping, 1963c). The rate constant for this prototropic shift was calculated to be 4 to 200/min. (Bruice and Topping, 1963d).

Other comparisons of enzymic and model transamination reactions

The imine formation constant for pyridoxal and valine is about 10^7 times less than that for transaminase and an amino acid and the rate constants for the reaction of these imines differ by a factor of 10^8 in favor of the enzyme. This indicates that the enzymes catalyzes both the transamination step and the tautomerization step (Cordes and Jencks, 1962b; Bruice and Topping, 1963d; Hammes and Fasella, 1963a). Even

though the gross aspects of model systems are similar to the enzymic reaction, the details are obviously quite different.

The role of the protein molecule is unknown; there may be static and dynamic conformational effects which bring the substrate into critical configuration for polarizing appropriate chemical bonds. If acid-base catalysis is involved the reaction rate is limited by the rate of dissociation of acidic or basic groups. If the pK_a for such a critical group is between 6 and 7, the maximum rate of acid-base catalysis is about $10^3/\text{sec.}$ assuming a diffusion-controlled rate of protonation. And the interconversion of imines is slow enough for acid-base catalysis to be involved.

Dynamic conformational changes may cause physical straining of bonds and the rate of such a change could be kinetically important. It is known that the basic form of the enzyme is necessary for the catalytic interconversion of intermediates, but not for imine formation. This catalytic interconversion is of the order of $10^7/\text{M}/\text{sec.}$ and the rate of aldimine to ketimine transformation was calculated to be between 10 and 100/sec. (Hammes and Fasella, 1963a).

The reaction of free aspartate and pyridoxal phosphate imines is too slow to account for the over-all rate of enzymic reaction, and consequently, the transamination between aspartate and enzyme-bound pyridoxal phosphate must be catalyzed by glutamic-aspartic transaminase, possibly by conversion of the pyridoxal phosphate-enzyme imine to the much more reactive,

protonated species (Cordes and Jencks, 1962b). However, this enzyme reacts with alanine at pH 8 with a first-order rate constant of 0.20/min., a value similar to that for the non-enzymic reaction of pyridoxal phosphate with aspartate (Jenkins, 1961). Also, glutamic-aspartic transaminase lost its ability to catalyze the transamination reaction to the extent of its resolution to coenzyme and apoenzyme. The apotransaminase formed a holoenzyme analog with pyridoxal and catalyzed the reversible transamination of pyridoxamine and alpha-ketoglutarate or oxaloacetate, the half-reactions of the over-all enzymic transamination. But the efficiency of catalysis by this holoenzyme analog was only about 0.1% of the rate of the corresponding reaction catalyzed by the natural holoenzyme, a finding which emphasizes the importance of firm and sterically-determined coenzyme-apoenzyme binding. This markedly enhanced efficiency of the enzymic process was made possible by eliminating the several additional reactions involved in binding and releasing free pyridoxal or pyridoxamine after each interaction with substrate (Wada and Snell, 1962).

In summary, the catalytic effects of the enzyme on the transamination reaction has recently been estimated as 10^9 times faster than the tautomeric interconversion of aldimine and ketimine in free solution, at zero buffer concentration and in the absence of other intermolecular catalysts (Vernon, 1964).

Structural requirements in pyridoxal for nonenzymic transamination

In addition to providing simple systems permitting the study of aldimine and ketimine intermediates in the transamination reaction, the nonenzymic reaction also provides a convenient experimental system in which the structural requirements for participation in amino group exchange reactions and hence for catalysis of the over-all transamination reaction can be studied. By studying many aldehydes as amino group acceptors in such model systems the importance of each of the functional groups of pyridoxal in the over-all reaction was assessed (Ikawa and Snell, 1954a; Metzler et al., 1954a,b). The essential functional group requirements are the following:

1) a formyl group, since it is converted during transamination to the aminomethyl group of pyridoxamine and since compounds lacking a carbonyl group do not accept amino groups from amino acids. Its function is to form imines with the amino acids.

2) A heterocyclic nitrogen atom provides a strongly electrophilic grouping which aids the catalysis of reactions of the imines. The nitrogen atom needs to be in an ortho- or para-position to the formyl group on the pyridine ring for maximum effectiveness.

3) A phenolic group must be unsubstituted, since substitution, or lack of a phenolic group resulted in greatly decreased reaction rates. The free phenolic group should be

in an ortho-position to the formyl group, since 2- or 4-hydroxypyridine possessed the minimum structural requirements for catalysis of nonenzymic transamination and similar reactions by substituted pyridines (Metzler et al., 1954a). A possible role of the phenolic group is stabilization of the imine intermediate through hydrogen-bonding or metal ion-chelation, thus tending to maintain the system of conjugated bonds in a planar configuration and favoring the requisite electron shifts.

The hydroxymethyl group at the 5-position of pyridoxal is not necessary for nonenzymic reactions; in fact it causes a decrease in reaction rate, as compared to analogs lacking the hydroxyl group (as in deoxypyridoxal) or having it substituted (as in pyridoxal phosphate), since it permits internal hemiacetal formation with the adjacent formyl group, thus reducing the concentration of the reactive aldehyde in solution. The hydroxymethyl group is essential, however, for formation of the coenzyme, and therefore, for enzymic reactions. The phosphate ester group not only prevents hemiacetal formation and maintains a high level of free aldehyde, but also has an important role of contributing to the binding of coenzyme to apoenzyme (Banks et al., 1963; Sizer and Jenkins, 1963; Wada and Snell, 1962).

The methyl group in position 2 appears to be non-essential, however, through its inductive effects, it may alter the reaction rate (Marvel and Tarköy, 1957). Also, omega-methyl-

pyridoxal-5-phosphate (PLP with an ethyl instead of a methyl group at position 2) provided coenzymic activity for some, but not for other apoenzymes, indicating wide differences in the nature of binding sites of individual apoenzymes (Olivard and Snell, 1955a,b). A correlation of inhibitory potencies of various coenzyme analogs for different PLP-enzymes has revealed that every substituent grouping of PLP affects the firmness of binding of coenzyme to apoenzyme (Snell, 1958). Finally, a recent suggestion has been made for the role of the electrophilic site around position 6 and the nitrogen atom of the heterocyclic ring (Makino et al., 1963).

Analytical methods

Quantitative analytical methods used in following non-enzymic transamination reactions between pyridoxal and amino acids have included spectrophotometric measurements of electronic absorption bands associated with reactants, imine intermediates, and products; measurements of optical rotation changes resulting from racemization of the amino acid, and chemical methods for reacting with remaining reactant or forming product, followed by a colorimetric or spectrophotometric determination. Qualitative methods for detecting the presence of products, intermediates, and reactants have included paper chromatography and electrophoresis.

Spectral changes have been followed during model transamination reactions with increasing absorbancy at 246 mμ,

corresponding to ketimine formation, and with increasing absorbancy at 395 m μ , corresponding to pyridoxal loss (Bruice and Topping, 1962, 1963a); with decreasing absorbancies at 440, 410, 380, 320 m μ , corresponding to loss of pyridoxal or its aldimine intermediate (Fleck and Alberty, 1962); with decreasing absorbancies at 414 and 280 m μ , corresponding to aldimine-metal chelates of pyridoxal phosphate, and with increasing absorbancy at 355 m μ , corresponding to ketimine-metal chelates of pyridoxamine phosphate (Cattaneo, et al., 1960); and with changing absorbancy at 370 m μ , corresponding to aldimine-metal chelate of pyridoxal, and with decreasing absorbancy at 317 m μ , corresponding to pyridoxal hemiacetal (Cennamo, 1963). Optical rotation changes were measured at 546 m μ by the symmetrical-angle method (Fleck and Alberty, 1962).

Chemical methods have included analysis of pyridoxal by forming an ethanolimine with maximum absorbancy at 365 m μ (Metzler and Snell, 1952b; Cennamo, 1961, 1962, 1963, 1964; Banks et al., 1961; Gregerman and Christensen, 1956) and by forming a red condensation product from two moles of pyridoxal and one mole of acetone in alkaline solution (Siegel and Blake, 1962; Blake et al., 1963). Other methods which could be used for analysis of pyridoxal analogs in nonenzymic systems include condensation with cyanide (pyridoxal phosphate was determined as a cyanohydrin derivative at its maximum absorbancy of 385 m μ by Scardi and Bonavita, 1957), or with

phenylhydrazine (phenylhydrazones of pyridoxal and pyridoxal phosphate had identical maximum absorbancy indices at 410 mμ, as measured by Wada and Snell, 1962). Analysis of pyridoxamine has been made spectrophotometrically after separation of the 2, 4-dinitrophenylhydrazones of pyridoxal and keto acid in a complex procedure (Metzler and Snell, 1952b; Banks et al., 1961).

Analysis of keto acid has been carried out by methods using a nonspecific chemical reaction with dinitrophenylhydrazine, followed by spectrophotometric measurement after separation of the pyridoxal hydrazone derivative (Metzler and Snell, 1952b; Greggerman and Christensen, 1956; Banks et al., 1961). Recent reviews have evaluated the relative advantages and disadvantages of analytical methods for keto acids (Neish, 1957; Spikner and Towne, 1962; Robins et al., 1956). Chemical reagents superior to the outmoded phenylhydrazone methods because of their much higher degree of specificity for alpha-keto acids include 3-quinolyldiazine (Robins et al., 1956) and ortho-phenylenediamine (Bruice and Topping, 1963a; Spikner and Towne, 1962).

Qualitative identifications of intermediates and products have been made by electrophoresis (Fasella et al., 1957) and by paper and column chromatography (Metzler and Snell, 1952a; Metzler et al., 1954b; Fasella et al., 1957; Kalyankar and Snell, 1957; Cattenéo et al., 1960; Cennamo, 1954, 1962, 1963, 1964). Detection was made by fluorescence techniques and by

chemical reagents, such as dinitrophenylhydrazine yielding yellow products with keto acids, ninhydrin yielding an orange product with pyridoxamine and indigo or violet products with amino acids, and ammonia or ethanolamine yielding a yellow product with pyridoxal. Recently, thin-layer chromatography and spectrophotometry of alpha-keto acid hydrazones have been studies in detail (Dancis et al., 1963).

Kinetic methods

Earliest rate measurements of nonenzymic transamination reactions were merely comparisons of changes in concentrations of pyridoxal, pyridoxamine, or keto acid in a certain period of time under different conditions (e. g., Metzler and Snell, 1952b). Although many of these qualitative rate studies were made, quantitative rate constants were not reported until 1961 by Banks et al. Their derived rate expression included the imine formation constant: $1/v = 1/kA_0 \cdot (1/K_0 P_0 + 1) + 1/kP_0$, where v is the initial reaction velocity, k is the pseudo first-order rate constant for conversion of the ketimine to pyridoxal and alanine, in the reverse reaction between pyridoxamine (P_0) and an excess amount of pyruvate (A_0). The formation constant for ketimine was found from a plot of $1/v$ vs. $1/A_0 K_0 = 1/(\text{slope/intercept} - P_0)$.

However, the complexities of their system and the slowness of the reactions prevented a determination of order, etc., and only allowed a determination of initial rates,

because the reactions could be followed to a maximum of only 6% completion, according to Bruice and Topping (1963a).

These reactions were not simple since both rapid and slow spectral changes occurred on mixing the reactants (Matsuo, 1957; Christensen, 1958; Blake et al., 1963). These changes were functions of pH, temperature, concentrations of reactants, buffer or metal ion catalysts, and indicated a number of intermediates, as well as different species from reactants (e. g., hemiacetal, hydrated and unhydrated aldehyde forms in various ionic states) (Banks et al., 1961; Blake et al., 1963). At relatively low amino acid concentrations the reaction rate obeyed pseudo first-order kinetics, but at high amino acid concentrations, zero-order kinetics were followed, as in enzymic catalysis (Banks et al., 1961). By increasing the temperature 75°, to 100°C, it was found that the reaction went practically to completion and was first-order in pyridoxal and in alanine (Blake et al., 1963). (Kinetics of buffer and metal ion catalyses effects of pH, concentrations of reactants, and ionic strength; stoichiometry, and comparisons of rate constants under various conditions for these model systems are discussed later: see Results and Discussion).

Guggenheim plots were used to obtain pseudo first-order rate constants for the nonenzymic transaminations of pyridoxal with alanine (Fleck and Alberty, 1962) and with phenylglycine (Bruice and Topping, 1963a). (See Experimental for further discussion of the Guggenheim method, its advantages and

suitability for these reactions.) In the former reaction three spectral relaxation times were found. These reaction steps supposedly corresponded to formation of carbinolamine, aldimine and ketimine from alanine and pyridoxal. When the initial alanine concentration was much greater than the initial pyridoxal concentration, the absorbancy, A_t , due to pyridoxal, intermediates and products, is given as a function of time by $A_t = B_0 + B_1 e^{-m_1 t} + B_2 e^{-m_2 t} + B_3 e^{-m_3 t}$, where m_1 , m_2 , and m_3 are pseudo first-order rate constants in the order of decreasing magnitude.

Kinetics for the latter imidazole-catalyzed reaction (Bruice and Topping, 1963a) followed a rate equation which depended upon the square of the imidazole concentration. This form of the equation is of the Michaelis-Menten type for enzyme kinetics and represents saturation of an intermediate with two molecules of an imidazole species, or alternatively like saturation of a rate-determining step:

$k_{\text{obs}} = 0.0095 (\text{Im})^2 / 0.20 + (\text{Im})^2$, where k_{obs} is the observed or pseudo first-order rate constant for ketimine formation and (Im) is the total imidazole buffer concentration. The equation derived for the observed first-order rate constant for attainment of equilibrium was the following: $k_{\text{obs}} = k_1 K_1 (2c/2.303) = 1/t \log \left(\frac{c-u}{c+u} \right) + B$, where k is the first-order rate constant for the rate-determining, imidazole-intra-complex, aldimine-ketimine tautomerization, K_1 and K_2 are equilibrium constants for formation of aldimine and its imidazole complex,

$$c = \left[a^2 + b^2 + \frac{2(a+b - ab) + 1/(K_1 K_2)^2}{4} \right]^{1/2}$$
 , a is the pyridoxal concentration and b is the amino acid concentration at t_0 and at any time t, ketimine concentration is x, pyridoxal concentration = a - x, amino acid concentration = b - x, since aldimine was at a low steady state concentration, was rapidly established and couldn't be measured spectrophotometrically; $u = x - \frac{(a + b + 1/K_1 K_2)}{2}$, $B = -\frac{1}{2}c \ln \left(\frac{c - u + x}{c + u - x} \right)$,

$(c - u) = 0$ at t_e .

These two equations for k_{obs} were combined to provide the following expression for the second-order rate constant, k_2 , for initial equal reactant concentrations ($a = b = 10^{-4}$ M):
 $k_2 = k_1 K_1 = 1.15/ct \log \left(\frac{c - u}{c + u} \right)$ $B = 0.0218 K_1 K_2 (Im)^2 / (0.20 + (Im)^2) (0.0004 K_1 K_2 + 1)^{1/2}$. At higher imidazole buffer concentrations than about 1.8 M the rate constant merely depended upon K_1 and K_2 : $k_2 = 0.0218 K_1 K_2 / (0.0004 K_1 K_2 + 1)^{1/2}$.

EXPERIMENTAL

Materials

Pyridoxal analogs

Pyridoxal-N-methochloride (PLM-Figure 1) was synthesized and found to be of good quality by elemental analysis (Johnston et al., 1963). Deoxypyridoxal (DPL-Figure 1) was synthesized by an improved method and purified by recrystallization from water by Dr. Isao Tomita in this laboratory. Deoxypyridoxal was also purified by sublimation at about 40°C, atmospheric pressure ($a_m = 6.45 \times 10^3$ at 294.5 μ , 0.1 N HCl; lit. value: $a_m = 6.32 \times 10^3$, pH 1; Metzler and Snell, 1955; Heyl et al., 1953). Dr. Tomita also supplied samples of 5-"carboxypyridoxal" or "5-pyridoxalylic acid" (CPL-Figure 1) and "alpha⁵-pyridoxalylacetic acid" (APL-Figure 1; Tomita and Metzler, 1964). These pyridoxal analogs were synthesized according to the methods presently unpublished and outlined in Figures 35, 36 (Results and Discussion).

Pyridoxal phosphate monohydrate (98% by assay) was obtained from Sigma Chemical Co. (observed $a_m = 7.18 \times 10^3$ at 295 μ , pH 1; lit. value: 6.70×10^3 ; $a_m = 5.27 \times 10^3$ at 388 μ , pH 7; lit. value: 4.90×10^3 ; $a_m = 2.47 \times 10^3$ at 330 μ , pH 7; lit. values: 2.45 to 2.50×10^3 ; refer to Table 1, Review of Pertinent Literature for sources. These spectral

data indicated that this source of pyridoxal phosphate was of high quality. Pyridoxal hydrochloride (PL) was also obtained from Sigma Chemical Co. In general, pyridoxal analogs were stored in a freezer at -20°C and warmed to room temperature in a desiccator.

Amino acids, keto acids, buffers, and other chemicals

Amino acids, buffers and other chemicals were obtained from commercial sources. Leucine was recrystallized from ethanol and water after treatment with activated charcoal prior to use in measurements of imine formation constants, in order to remove impurities which have a small absorbancy below 350 m μ . However, no noticeable effect of these impurities on the over-all rate constants for nonenzymic transamination reactions with pyridoxal analogs was observed. Especially when a high concentration of leucine was desired, such as 0.1 M, the L-isomer was used because of its greater solubility (0.171 M) than the D, L-racemate (0.071 M). Imidazole was also recrystallized prior to use.

Tri-deuterio -D, L-leucine, which had been prepared by Larry Levine (1963) by racemization of leucine in D_2O catalyzed by pyridoxal and alum, was analyzed by mass spectroscopy (Junk and Svec, 1964) as 97% alpha-deuterated, including 92% alpha-D-beta, beta'- D_2 -leucine (or D_3 -Leu).

Sodium alpha-ketoisocaproate (KIC) was prepared from the commercially-available keto acid, recrystallized, and char-

acterized by melting points of KIC and its 2, 4-dinitro-phenylhydrazone, according to the procedures of Meister (1953). It was used for standard curves in the analysis of KIC by the quinoxaline, dinitrophenylhydrazone, and quinolyldhydrazone methods.

Preparation of solutions

Reaction solutions and stock solutions of amino acids and pyridoxal analogs were prepared with boiled, redistilled water. Amino acid stock solutions, 0.05 to 0.167 M for leucine, for example, usually had to be heated or dissolved in acid or base. Amino acid was carefully weighed into a volumetric flask and dissolved by heating on a steam cone. After cooling to room temperature the solution was diluted to final volume. Solutions were filtered to remove any insoluble matter and usually stored in a freezer at -20°C to keep for an extended period of time. Stock solutions of pyridoxal analogs were quite stable when stored frozen, as determined by spectral analyses. It was found convenient to store these solutions in small vials, to enable thawing of a portion and warming to room temperature before use.

Concentrations of pyridoxal analogs were checked spectrophotometrically by comparing observed molar absorbancy indices to values reported in the literature. (Refer to Table 1, Review of Pertinent Literature, for spectral data of cationic, dipolar ionic and anionic forms of pyridoxal analogs.) Solutions for spectral analysis were 10^{-4} M PL, PLM, DPL, PLP,

APL, CPL in 0.1 N HCl; PL, PLM, DPL, PLP in 0.1 M phosphate buffer -pH7, and in 0.1 N NaOH.

Spectrophotometric Measurements of the Ionic
Equilibrium of Pyridoxal-N-methochloride

Absorbancies of prepared solutions of varying pH

Solutions were prepared by 1 to 5 dilutions of an aqueous stock solution of 4.84×10^{-4} M pyridoxal-N-methochloride (PLM) with acetate buffers of varying pH near the pK_a included. These solutions were 0.10 M in ionic strength, assumed mainly due to buffer ions. Solutions of the same concentration of PLM were prepared to be 0.01 N in HCl, pH 2.3, and 0.1 N in carbonate buffer, pH 10.1. Absorbancies of each of these solutions were determined and blank corrections were made at the three wavelengths of maximum absorbancy from the recorded spectra (See Table 4, Figures 11 and 12 - Results and Discussion). All measurements were made at 25°C.

Calculation of pK'_a

Calculation of the apparent pK'_a value was based on the following assumptions: The spectrum of the cation form of PLM is that obtained from the pH 2.3 solution (in agreement with that of a pH 1 solution; compare a_m values in Table 4, Results and Discussion); the spectrum of the dipolar ion form is that of the pH 10.1 solution. The fractions, f_x , of PLM

in the conjugate base or dissociated form in solutions x, of pH near the pK'_a were related to absorbancies of solutions x and of solutions of 100% cation form, $f = 0$, and of 100% dipolar ion form, $f = 1$, by equation (1).

$$(1) \quad f_x = (A_0 - A_x) / (A_0 - A_1)$$

The apparent pK'_a was then calculated from equation (2), relating the equilibrium constant for dissociation of the cation form with experimental pH values of solutions and calculated fractions of dipolar ion form in these same solutions.

$$(2) \quad pK'_a = pH - \log_{10} (f/1 - f)$$

If the absorbancy of the 293.5 mμ band is proportional to the concentration of cation form, the pH at which the absorbancy becomes an average value of the limiting values (at pH 2.3 and at pH 10.1) is the pK'_a . The same assumption was made for the absorbancies of the 323 and 254.5 mμ bands for the dipolar ion form. This method for calculation of ionization constants from spectrophotometric data has been described (Irvin and Irvin, 1947; Lunn and Morton, 1952; Williams and Neillands, 1954; Metzler and Snell, 1955; Albert and Phillips, 1956; Mason, 1958; Nakamoto and Martell, 1959b).

In general, for spectrophotometric measurements, the direct proportionality between absorbancy (A) of a solution with respect to the solvent at a certain wavelength and the molar concentration (c) of the absorbing species was assumed to hold: $A = a_m bc$, where a_m is the molar absorbancy index and

and b is the optical path length of the solution in cm.

Spectrophotometric Determination of the Formation
Constant for the Hydrogen-bonded Aldimine of
Pyridoxal-N-methochloride and Valine

Preparation of stock solutions

A stock phosphate buffer solution was prepared by dissolving a calculated amount of dry potassium dihydrogen phosphate in a calculated volume of standard 0.1 N NaOH in a volumetric flask and diluting to the mark. Ten stock solutions (50 ml. each) were prepared by dissolving carefully weighed amounts of dry D, L-valine in calculated volumes of standard 0.1 N NaOH and adding 25 ml. of stock phosphate buffer. (The dissolving of valine was hastened by heating on a steam cone and shaking.) These solutions contained amounts of phosphate, base and valine that were calculated so that the final solutions for study of imine equilibria would be 0.5 M in ionic strength, 0.1 M in phosphate, pH 6.0 and in ten graduated concentrations of valine convenient for plotting reciprocal valine concentrations.

The concentration of stock pyridoxal methochloride solution, prepared as 4.165 mg./ml., was determined spectrophotometrically at 293 m μ to be 4.053 mg./ml. First, the concentration was calculated as 0.0186 M from the absorbancy of a 1 to 500 dilution of this stock solution in 0.1 N HCl, using 7.22×10^3 for the molar absorbancy index of the hemiacetal

cation at this wavelength (Table 4, Results and Discussion). The latter concentration of the pyridoxal methochloride determined as the hemiacetal cation was used in calculations of molar absorbancy indices involved in the measurement of imine equilibria.

Procedure for measuring imine equilibria

Solutions for measuring varying extents of imine formation were prepared by adding 0.5 ml. of pyridoxal methochloride (0.0186 M) to each of ten 25 ml.-volumetric flasks and diluting to the marks with each of the ten respective phosphate-buffered solutions of graduated valine concentrations. The average pH measured for the ten solutions was 6.02 ± 0.01 . These solutions were allowed to reach a state of equilibrium in the formation of the hydrogen-bonded aldimine of valine and pyridoxal methochloride. Thirty minutes was found to be a sufficient period of time for this equilibration at 25°C , and the solutions were protected from light to prevent loss of pyridoxal methochloride. The absorbancy of each solution was read immediately after its thirty-minute equilibration period against an appropriate blank solution at 419 m μ , a wavelength of maximum absorbancy of the hydrogen-bonded pyridoxal methochloride-valine imine (Table 2). Each blank solution had the same composition as its corresponding imine solution, except that an equal volume of water was substituted for the pyridoxal methochloride

solution.

Determination of molar absorbandy index of pyridoxal methochloride dipolar ion (Figure 14, P[±]) at 419 mμ

The concentration of a pyridoxal methochloride solution (10 mg./ml.) was determined spectrophotometrically to be 0.00473 M (from the absorbancy at 293 mμ of a 1 to 10 dilution of this solution in 0.1 N HCl). From spectrophotometric measurements at 419 mμ of a 2 to 5 dilution (in 0.5 M phosphate, pH 6.00, 0.5 M ionic strength) the molar absorbandy index, a_p , of the PLM dipolar ion under these conditions was calculated to be 98.8.

Derivation of an equation relating the equilibrium constant with observed molar absorbandy indices

Consider the following net equilibrium for the formation of the hydrogen-bonded aldimine of pyridoxal methochloride and valine:

$$P^{\pm} + V^{\pm} \xrightleftharpoons{K_1'} PV^{\pm}$$
 where P^{\pm} , V^{\pm} , PV^{\pm} are dipolar ions of PLM, val, and their imine, respectively. Assume that all of PLM, val and their imine are of dipolar ionic forms at pH 6.0. This assumption may be justified since pK'_a of PLM is 4.05, pK'_1 of val is about 2.3, pK'_2 of val is about 9.6 and pK'_a of PLM-val is 8.0 (Johnston et al., 1963). Let (P), (V), (PV) represent equilibrium concentrations of PLM, val and their imine, respectively. The experimental conditions are such that $(V) \gg (P)$ or (PV) .

The equilibrium constant for formation of the hydrogen-

bonded imine may then be expressed by equation (3).

$$(3) \quad K_1' = (PV)/(P)(V) = 1/(V) \cdot (PV)/(P)$$

Let the fraction of free (unreacted) PLM be expressed as f_p
 $= (P)/[(P) + (PV)]$, and the fraction of PLM reacted be expressed
 as $f_{pv} = (PV)/[(P) + (PV)]$. Then $K_1' = 1/(V) \cdot f_{pv}/f_p$ or from
 $f_p + f_{pv} = 1$,

$$(4) \quad K_1' = 1/(V) \cdot (1 - f_p)/f_p.$$

These fractions may also be related to molar absorbancy indices, as in equation

(5) $a_o = a_p f_p + a_{pv} f_{pv}$, where a_o is the observed molar absorbancy index at 419 mμ of a solution of P, V, and PV at equilibrium; a_{pv} and a_p are molar absorbancy indices of PV and P, respectively, at 419 mμ. Equation (5) may be simplified, in the same manner as equation (4), to obtain equation

$$(6) \quad a_o = a_p f_p + a_{pv} (1 - f_p).$$

After subtracting a_p from both sides of equation (6), solving equation (4) for $(1 - f_p)$, and substituting its equivalent, $K_1' (V) f_p$ into equation (6) one obtains equation

$$(7) \quad a_o - a_p = (a_{pv} - a_p) K_1' (V) f_p.$$

Since $f_p = 1/[1 + (PV)/(P)]$, and $K_1' (V) = (PV)/(P)$, from equation (3), then $f_p = 1/[1 + K_1' (V)]$. Substituting this expression for f_p into equation (7), taking the reciprocal of both sides of the resulting expression, and rearranging terms, one obtains equation

$$(8) \quad 1/(a_o - a_p) = 1/(V) \cdot 1/K_1' (a_{pv} - a_p) + 1/(a_{pv} - a_p).$$

A graphical plot of $1/(a_o - a_p)$ vs. $1/(V)$, as ordinate and abscissa, respectively, yields a straight line with slope of $1/K_i' (a_{pv} - a_p)$ and intercept of $1/(a_{pv} - a_p)$. Thus, K_i' may be calculated as the ratio, intercept/slope. Equation (8) is entirely analogous to the form of the equation of Ketelaar et al. (1951, 1952), as expressed in Review of Pertinent Literature.

General Procedure for Nonenzymic Transamination in Model Systems

Appropriate amounts of stock solutions of amino acid and pyridoxal analog were pipetted into volumetric flasks, and buffer, metal salt solutions or other additional catalysts were added, if desired, before dilution. The time of dilution and mixing of reaction solutions was considered as time zero. Aliquots of the reaction solutions were analyzed for keto acid and pyridoxal analog or measured spectrally at appropriate times. Control solutions in which the amino acid was omitted were used to check stability of pyridoxal analogs under reaction conditions, so that corrections could be applied to any changes observed in the concentration of pyridoxal analog in the absence of amino acid if necessary.

The solutions were transferred to screw-capped test tubes and kept in a constant-temperature water bath at 25°C during the reactions; however, the method of Metzler and Snell (1952b) was used for the preliminary comparisons carried out near 100°C (Johnston et al., 1963). Protection of all solu-

tions of pyridoxal analogs from photodecomposition by laboratory (fluorescent) light was afforded by aluminum foil-wrapping or by the water bath made opaque to visible light of shorter wavelengths with a soluble dye (amaranth or aniline red). Water of the same temperature was circulated around the cell compartments of spectrophotometers, and all pH and absorbancy measurements were made at 25°C (in a room kept at relatively constant temperature and humidity).

Analytical Methods

Dinitrophenylhydrazone method for keto acids

A diluted aliquot containing from zero up to two micromoles of keto acid and less than one micromole of pyridine aldehyde were reacted with a reagent containing five micromoles of 2,4-dinitrophenylhydrazine and two milliequivalents of acid. After thirty minutes of formation of the hydrazones of keto acid and pyridine aldehyde, the pH was adjusted to neutrality with two milliequivalents of base contained in a phosphate buffer. At neutral pH the hydrazones of pyridine aldehydes have no net charge and were filtered off as red precipitates. The filtrate contained the anionic keto acid hydrazone. Extraction of this filtrate with toluene removed excess reagent and any unprecipitated neutral hydrazones or unreacted pyridine aldehyde. Equal volumes of the extracted filtrate and 2.5 N sodium hydroxide were mixed and an uncharacterized, red colored product, having an absorption maximum

at 520 m μ , was allowed to form for ten minutes before measuring the absorbancy against a reagent blank treated in the same manner. More specific details of reagents and procedure of this method developed by Metzler and Snell can be found in their 1952 article (1952b).

This method for the analysis of keto acid formed in model transamination systems of pyridoxal and amino acids was used for following reactions at 100°C, where keto acid was rapidly formed (within a few minutes of reaction) and pyridoxal was rapidly converted to pyridoxamine, and for preliminary studies of the same reactions at 25°C. However, this method was found inadequate for the sensitivity desired. In reactions followed at 25°C, a much slower rate of keto acid formation required a method specific for measuring small amounts of keto acid in the presence of a large excess (about twenty times) of pyridine aldehyde. In this situation analyses for keto acid were up to 4% too low because of co-precipitation of the dinitrophenylhydrazones of keto acid and pyridine aldehyde (Metzler and Snell, 1952b). Another disadvantage of the method was the cloudy suspension which sometimes formed in the final color-developed samples. These two disadvantages of this method resulted in unreliable reproducibility and undermined the precision required for analysis of small quantities of keto acid formed in these model systems.

The dinitrophenylhydrazone method using perchloric acid, in place of sodium hydroxide, was considered too complex to be

useful, although it was used for analysis of the nonenzymic transamination of alanine with pyridoxal at 25°C, which was followed up to about 6% completion of the reaction (Banks et al., 1961). Instead, further modification of the Metzler-Snell procedure was attempted. Aliquot size was reduced, dilution was eliminated and volumes were adjusted for convenience in calculation and for saving time. Since it was found that formation of dinitrophenylhydrazone of ketoisocaproate was complete in five minutes but slow and incomplete in the case of pyridoxal, it was reasoned that dinitrophenylhydrazone formation of pyridoxal could be held to a minimum if only five minutes were allowed for this reaction. The problem of co-precipitation of a small amount of keto acid-dinitrophenylhydrazone with a relatively large amount of pyridoxal-dinitrophenylhydrazone was solved, because unreacted pyridoxal could still be extracted from the neutralized solution with toluene. The cloudy suspension of toluene was avoided by allowing for complete separation of layers after the extraction, by carefully preventing toluene from getting into the sample pipetted from the lower layer, and by shaking the alkaline solution of keto acid-dinitrophenylhydrazone vigorously, to enable dissolved air to force suspended toluene out to the surface of the liquid.

The detailed procedure of this modified method is as follows: 1) Pipet sample containing less than four micromoles of keto acid and less than one micromole of pyridoxal

analog into one ml. of dinitrophenylhydrazine reagent in a 10 ml.-volumetric flask. Reagents are prepared as usual (Metzler and Snell, 1952b). 2) Allow just five minutes for reaction. 3) Add five ml. basic-buffer reagent, dilute to the mark and mix well. 4) Filter into a 30 ml.-or 1 oz.-bottle containing about 10 ml. toluene; cover with ground-glass stopper and shake well. 5) Allow layers to separate, before carefully taking five ml. of aqueous layer, avoiding toluene in pipetting and transferring the aliquot to a test tube. 6) Add five ml. 2.5 N NaOH; cover tube with screw-cap and shake contents vigorously. 7) Measure $A_{520 \text{ m}\mu}$ with spectrophotometer, after ten minutes of color development, in one-cm, calibrated, pyrex cells, against a reagent blank treated in the same manner.

Although this modified procedure was believed to have made several improvements, this method for keto acids was still lacking in the desired reproducibility and precision. Results depended too much upon the conditions of the reaction solution being analyzed, such as the nature and concentration of buffer, metal ion, pyridoxal analog and pH. It was concluded that the method was too complex, due to the lack of specificity of the reagent toward keto acids and that the colored product was too time-sensitive (faded gradually). Others have discussed the following difficulties and disadvantages of the method: possible loss of keto acid-dinitrophenylhydrazone by extraction, acid-dependency of the reversible reaction between

keto acid and dinitrophenylhydrazine, and instability of the colored product (Meister and Abendschein, 1956; Robins et al., 1956; Spikner and Towne, 1962).

Quinoxaline method for alpha-keto acids

A specific method for the fluorometric microdetermination of alpha-keto acids (Spikner and Towne, 1962), was tried. The procedure for reacting ortho-phenylenediamine in 50% sulfuric acid with an aliquot of alpha-keto acid in the presence of other carbonyl compounds was simple and convenient. However, a heating period was required for the condensation. The quinoxaline product was very stable. But the unavailability of a reliable photofluorometer prevented the use of this method.

A spectrophotometric method for the specific o-phenylenediamine reagent was used for analysis of keto acid produced by a model transamination system (Bruce and Topping, 1963a). But the procedure called for isolation of the crystalline derivative, after a 24-hour reaction, before making a solution for spectrophotometry. The procedure lasted two days before results could be obtained.

Ethanolimine method for pyridoxal analogs

Analysis of the remaining amount of pyridoxal analog in a model transamination system was made by a method developed previously (Metzler and Snell, 1952b). An aliquot containing up to four micromoles of pyridine aldehyde was added to five ml.

50% (by volume) ethanolamine (EOA), and the mixture was diluted to ten ml. The condensation product forms almost immediately, is stable for at least an hour, and can be spectrophotometrically measured at absorption maxima characteristic of the corresponding imine anions (pH 11). These maxima are 365 m μ ($a_m = 6.82 \times 10^3$) for pyridoxal, 375 m μ ($a_m = 5.38 \times 10^3$) for pyridoxal methochloride, 350 m μ ($a_m = 3.85 \times 10^3$) for pyridoxal phosphate, and 344 m μ ($a_m = 4.67 \times 10^3$) for deoxypyridoxal. A molar absorptancy index of 6.74×10^3 has been reported for the ethanolimine of pyridoxal at 362.5 m μ and was constant over a temperature range of 18 to 27°C (Banks et al., 1961).

Although this was a simple, convenient and accurate method for measuring amounts of pyridoxal analog plus its amino acid aldimine remaining in a reaction solution, no reaction could be detected with "5-carboxypyridoxal" or with "alpha⁵-pyridoxalylacetic acid," the carboxylate analogs of pyridoxal, and ethanolamine (0.2 ml. 1 mM analog/5 ml. 25% EOA gave no spectral change or peak above 330 m μ). In fact, the spectrum of "carboxypyridoxal" did not change much from an aqueous solution of pH 6 to an ethanolamine solution of about pH 11 (λ_{max} , from 319 to 315 m μ and a_m from 7.0 to 9.5×10^3).

Alkaline-acetone method for pyridoxal (Siegel and Blake, 1962)

A red condensation product from acetone and pyridoxal in

alkaline solution formed slowly, but this method specified the measurement of absorbancy at 420 m μ at the point of greatest change in absorbancy with respect to time: fifteen minutes after mixing. This absorbancy increased at a much slower rate after thirty minutes at room temperature. The claims for simplicity, convenience and accuracy of this method were greatly exaggerated (Siegel and Blake, 1962). It has been found that the instability of the colored product, the decreased sensitivity to pyridoxal, and other undesirable features make this method inferior to the ethanolicimine method for the analysis of pyridoxal.

Quinolylhydrazone (QH) method for both pyridoxal analogs and alpha-keto acids

A modified method for the microdetermination of alpha-keto acids by a quinolylhydrazine (QH) reagent, originally described by Robins et al., (1956), was found to be very simple, accurate, reproducible and suitable for not only detecting keto acid formation, but also for detecting the decrease in concentrations of pyridoxal analogs in a model transamination system. Alpha-keto acids react with 3-hydrazinoquinoline (QH) to form a specific hydrazone of unknown structure (Unknown, because beta-and gamma-keto acids, aldehydes and ketones form different or unstable products with absorbancies at 305 m μ which virtually disappear in time or upon dilution.), having a very high absorption maximum at 305 m μ in acid solution ($a_m = 24.3 \times 10^3$ for KIC). This QH

reagent has been shown to be effective for measurement of as little as 0.4 millimicromoles of 13 different alpha-keto acids (Robins et al., 1956).

Concentrations of both keto acid and pyridoxal analog may be determined in the same QH solution. Absorbancy changes at absorption maxima of 305 and 400 m μ are linearly related to concentrations of QH products of keto acid and pyridoxal analog, respectively, e. g., alpha-ketoisocaproate and pyridoxal phosphate (Figure 8). Pyridoxal phosphate and pyridoxamine have identical molar absorbancy indices at 305 m μ (4.48×10^3) in 0.01 N HCl. This would have to be true for other pyridoxal and pyridoxamine analogs in order for the QH method to be quantitatively useful in these analyses of reaction components. These standard curves are compared with the ethanolicimine method for pyridoxal phosphate, which is about 1/5 as sensitive as the QH method for PLP. Agreement of the EOA and QH methods in following the decrease in concentrations of pyridoxal phosphate in a nonenzymic transamination with leucine is substantiated in Results and Discussion. Roze (1964) has also adapted the QH method for the analysis of pyridoxal phosphate ($a_m = 23.2 \times 10^3$ at 400 m μ).

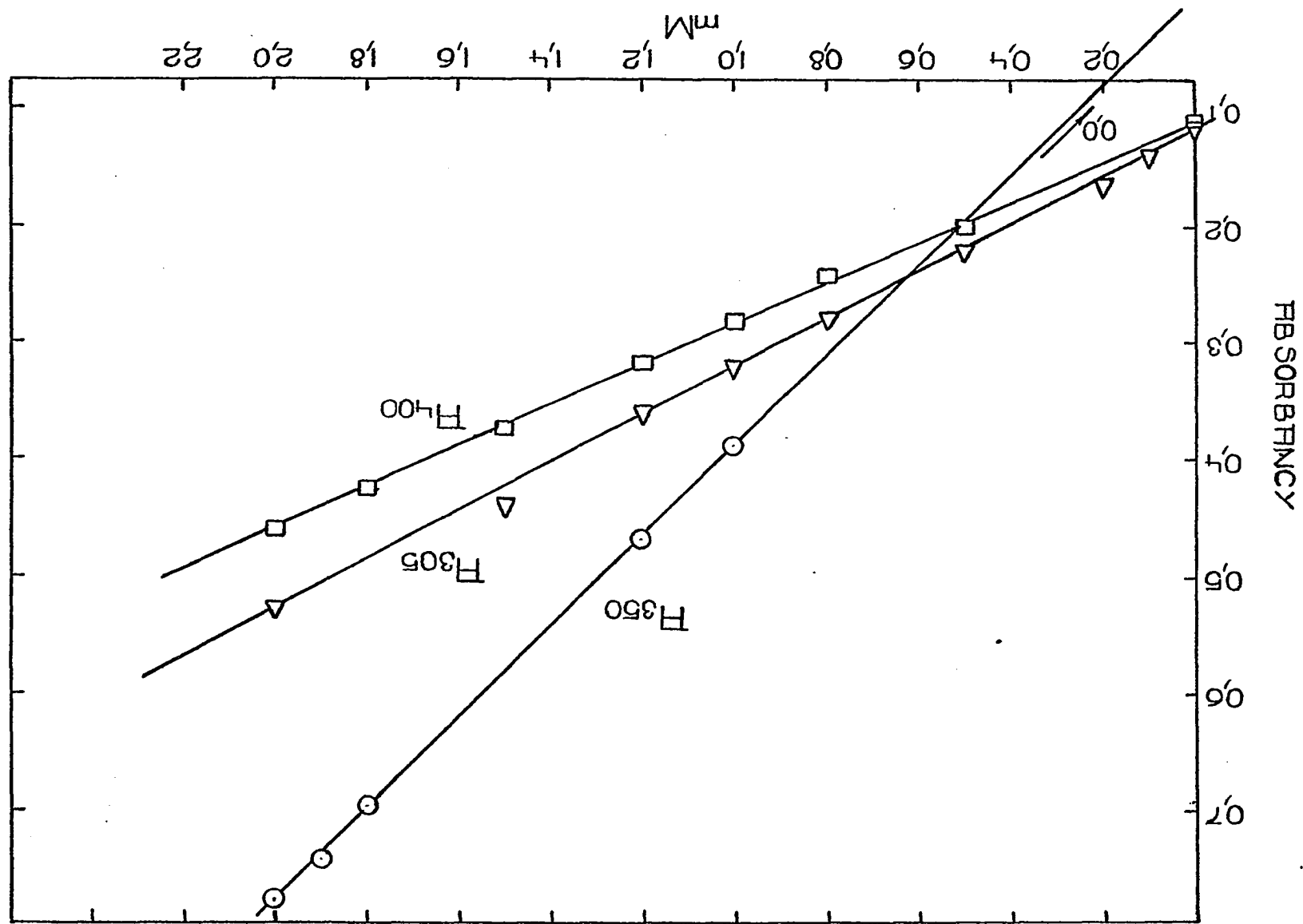
The original QH method of Robins et al., (1956) was modified for the analysis of model transamination reactions by decreasing the size of aliquots and reagent proportionately and by following decreases in absorbancy at 400 m μ corresponding to pyridoxal analog. An aliquot of model system solution

Figure 8. Standard curves for analysis of alpha-ketoglutarate (KIC) and pyridoxal phosphate (PLP) by the quinolyldiazotization (QH) method and of PLP by the ethanolimine (EOA) method

Absorbancies vs. millimolar concentrations of these components in solutions of 1.0 mM leu, 0.20 mM PLP + KIC or PM, such that (KIC) = (PM). Over-all dilutions of 1 to 10 were made in EOA or QH reagents, and further 1 to 10 dilutions of these QH solutions were made in 0.01 N HCl after complete reaction.

	<u>Sensitivity, mM/A</u>	<u>To calculate mM</u>
○ A vs. (PLP) by the EOA method	0.257	2.57 A/x
□ A vs. (PLP) by the QH method	0.0572	5.72 A/x - 0.63/x
△ A vs. (KIC) by the QH method	0.0471	4.71 A/x - 0.57/x

(x is aliquot size in ml.)



containing a total of about two micromoles of pyridoxal analog, amino acid imine, and keto acid was added to 5 ml. of 1.0 mM QH in 0.02 N HCl, and was diluted to 10 ml. This QH reagent was reported to be stable for two to three weeks at 4°C, and was prepared by diluting a stock solution 1 to 10. The more concentrated QH stock solution (232 mg/100 ml. or 10.0 mM QH in 0.20 N HCl) was kept frozen at -20°C, where it was reported to be stable for about 18 months (Robins et al., 1956). The final QH concentration of 0.5 mM will be about $2\frac{1}{2}$ times in excess of the amount needed to completely react with model system components.

At room temperature the time for complete reaction may be determined directly by following the increasing absorbancy at 280 mμ of the reaction solution, as suggested by Robins et al. (1956), or by following the increasing absorbancy at 305 mμ of aliquots of the reaction solution diluted 1 to 10 in 0.01 N HCl. The time for complete QH reaction was longer in the presence of buffer. It took 90 to 110 minutes for the absorbancy at 305 mμ to reach a maximum, if 1 M buffer was present in the original model system solution (or about 0.02 M buffer in the QH reaction in HCl). But it took only 50 to 70 minutes for the absorbancy at 305 mμ to reach a maximum, if the original model system solution was unbuffered. After completion of the QH reaction, the maximum absorbancies at 305 and 400 mμ are measured for samples diluted 1 to 10 in 0.01 N HCl, which are stable for at least two hours. Fresh

QH reagent was treated in the same manner as the samples and used as an absorbancy blank. An ultraviolet lamp was found to be convenient and appropriate for absorbancy measurements at both 305 and 400 m μ .

Direct spectral analysis

Model system reactions have also been followed by directly measuring the absorbancies of the reaction solutions at appropriate wavelengths. For solutions with high reactant concentrations calibrated quartz cell spacers (8.0, 9.0, 9.5, 9.9 mm) have been used to decrease the optical path length (to 2.0, 1.0, 0.5, 0.1 mm, respectively) as required. Loss of aldimine has been followed at wavelengths of maximum absorption between 400 and 425 m μ , depending upon both the pH and the pyridoxal analog used. Increases in absorbancy between 320 and 335 m μ have been considered to be due to formation of ketimine and/or pyridoxamine analog after the rate-determining step. Other electronic absorption maxima below 300 m μ changed during a reaction (increased and/or decreased), but it was not understood what molecular changes were responsible for these spectral changes. Furthermore, these changes were not as pronounced as were the other two absorption bands.

Chromatography of reaction solutions

Alpha-ketoisocaproate (KIC) and pyridoxal analog in a portion of reaction solution treated with 2,4-dinitrophenyl-

hydrazine (DNPH) were identified as dinitrophenylhydrazone (DNP) derivatives by one-dimensional, ascending chromatography on paper and on thin-layer silica gel plates. The thin-layer chromatographic techniques were found to be most convenient because of their rapid solvent development (3-4 hours) and clarity of spots. It was found best to spot the reaction solution directly on the chromatogram. When this was followed with the application of DNPH reagent to the spot, yellow DNP-derivatives were produced, both with keto acid formed in the nonenzymic transamination and with the pyridoxal analog remaining in the reaction solution.

After development these same chromatograms were sprayed with a ninhydrin solution to detect the constituents with amino groups: purple or pink spots were identified as amino acids or esters, orange spots as pyridoxamine analogs. When the reaction solutions were not treated with DNPH reagent, but were sprayed with Gibbs' reagent and ninhydrin after development, blue or gray spots for pyridoxal and pyridoxamine analogs, which rapidly faded to brown spots, were detected along with the ninhydrin-sensitive components. Also, both ninhydrin and Gibbs' reagent reacted at the same large spot, which could have been the aldimine of leucine and deoxy-pyridoxal, since a large brown spot with pink or purple edges was found. Gibbs' reagent or N, 2, 6-trichloro-p-benzo-quinoneimine in ammonia solution is specific for para-unsubstituted phenols (See Block et al., 1955, p. 229, for prep-

aration of reagent and chromatographic procedures). When this reagent was applied to spots of the reaction solution before development of the chromatogram, different R_F values were obtained for brown, blue, and gray spots, but these spots were more distinct and the brown background from the Gibbs' reagent spray was avoided.

Solvent systems used were n-butanol (4), acetic acid (1), water (5) and the upper layer of n-butanol (5), ethanol (1), water (4), where parts by volume are in parentheses. Some of these chromatographic techniques have been used previously for qualitative identification of components in nonenzymic transamination reactions (Metzler and Snell, 1952a; Metzler et al., 1954b).

Kinetic Methods

Typical graphs of rate data for the nonenzymic transamination of amino acids with pyridoxal analogs are presented in Results and Discussion. The absorbancies (at wavelengths depending on the particular analytical method) of aliquots of reaction solution were recorded at various times throughout the time of the reaction, and have been previously shown to be proportional to concentrations of a reactant or a product (Figure 8). The rate was determined from these reaction plots by one or more of the following methods.

- 1) Calculation of the initial rate, dP/dt , was made from the slope of the tangent to the curve at time zero,

since the relationship between concentration of the absorbing species changing with time and the measured absorbancy were known. The value dP/dt was converted to a rate constant by means of either the simple 1st or 2nd order rate expressions:

(9) $-dP/dt = k_1 (P)$ or $= k_2 (P)(A)$, where (P) and (A) are concentrations of pyridoxal analog and amino acid, respectively.

This method is not accurate in the determination of rate constants for model transamination reactions because of the uncertainties in the initial or instantaneous concentrations of "effective" reactants, owing to equilibrium conditions (i. e., only a portion of the initial reactants are converted to products at equilibrium).

2) Calculation of the relative rates, in comparing two reactions from the ratio of inverse times required to complete the same percent of reaction in each case, was made from the integrated rate expression: $\log (P_x/P_o) = k_1 t_1 = k_2 t_2$.

$$(10) \quad k_1/k_2 = t_2/t_1$$

This method is able to quickly compare relative rate constants, and if one of the constants is already known from a previous determination, it can be very useful.

3) Calculation of the rate constant was made from a fractional-life period, such as the half-time of reaction from the integrated first-order equation: $t_{\frac{1}{2}} = (\ln 2)/k_1$, where k_1 = first-order rate constant.

$$(11) \quad k_1 = 0.693/t_{\frac{1}{2}}$$

The problem of determining $t_{\frac{1}{2}}$ is complicated because concen-

trations of reactants at equilibrium need to be known, in order to be able to accurately calculate the "effective" concentrations of reactants. But the half-time has been found graphically by following a reaction practically to equilibrium and assuming an equilibrium value.

4) Calculation of rate constants from simple first- and second-order plots:

(12) slope of $\log (X)$ vs. t = first-order rate constant and

(13) slope of $1/(X)$ vs. t = second-order rate constant, where

(X) is some function of the concentration of a reactant or product

The application of simple kinetic relationships has been found to be inadequate in the complex sequence of reaction steps which constitute the transamination reaction, proceeding to an equilibrium position. But kinetic methods have been found to quantitatively characterize such a reaction fairly well. In the reactions of pyridoxal phosphate with leucine, second-order rate constants were calculated by the usual graphical method. These reactions were followed up to 30% completion.

However, reactions having pseudo first-order conditions (excess amino acid) did not follow simple first-order kinetics. (Reactions should be followed for at least 25 to 30% of completion, in order to be able to distinguish first- and second-order.) But when equilibrium values were determined graphically by a method of successive approximations, the

"effective" concentrations of reactants could be calculated from the known initial values and these equilibrium values. A plot of $\log (X_e - X_o / X_e - X_t)$ vs. t , where X is concentration or absorbancy (Figure 9) then gave a straight line, from which the first-order rate constant could be calculated from the relationship:

$$(14) \quad k_{\text{obs}} = -2.303 \text{ slope } (\ln 10 = 2.303)$$

Calculation of pseudo first-order rate constants were made from Guggenheim plots. The Guggenheim method takes equilibrium conditions into account, without requiring measurements of initial or final values (Guggenheim, 1926). It is ideally suited for the determination of pseudo first-order rate constants for these nonenzymic transaminations. The pseudo first-order conditions, in a unimolecular reaction of pyridoxal analog with an excess of amino acid, the following Guggenheim relationship may be derived:

Suppose $A_1, \dots, A, \dots, A_n$ are n readings of absorbancy which are linearly proportional to the concentration of some molecular species, which increases or decreases in time with a rate directly related to the reaction rate of nonenzymic transamination. If these absorbancy readings are taken at times $t_1, \dots, t, \dots, t_n$, without any restriction as to intervals, and n more readings $A'_1, \dots, A', \dots, A'_n$, are taken at times $t_1 + T, \dots, t + T, \dots, t_n + T$, each a constant time interval T after one of the previous set, then, $|A_f - A| = |A_f - A_o| e^{-kt}$ and $|A_f - A'| = |A_f - A_o| e^{-Kt - KT}$, where A_f and A_o are final (equilibrium) and initial values, respectively, and k is the sum of the rate constants of the two opposed processes in this reversible reaction.

Figure 9. First-order plots for the nonenzymic transamination between pyridoxal phosphate and leucine: $\log (A_e - A_o)/(A_e - A_t)$ vs. t , where A_o , A_t , A_e are absorbancy values at time zero, t and equilibrium, respectively, using semi-logarithmic plot, where $(A_e - A_o)/(A_e - A_t)$ ordinate is logarithm scale, but numbers are not logs

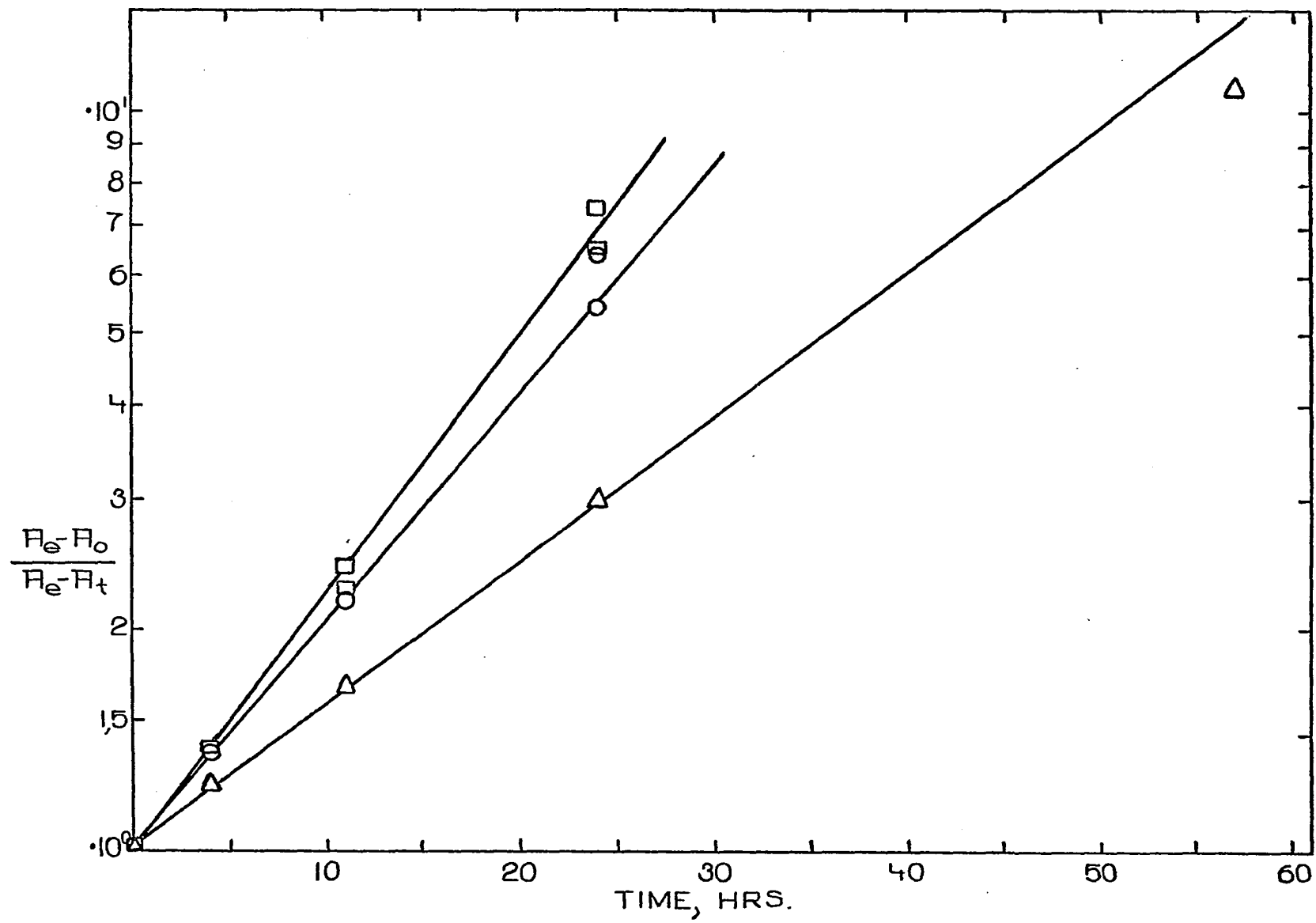
	$k_{obs} \times 10^6 \text{ sec}^{-1}$
○ $A_{350\text{m}\mu}$ vs. (PLP) by EOA method	20.4
□ $A_{400\text{m}\mu}$ vs. (PLP) by QH method	22.2
△ $A_{305\text{m}\mu}$ vs. (KIC) by QH method	12.7

A_e values found graphically by method of successive approximations to give best straight lines in this plot ($T = 30$ hours)

Pseudo first-order conditions: 10 mM PLP

50 mM leu

1.0 M acetate buffer, pH 4.6, 25°C.



Taking natural logarithms of both sides of the former equation and solving for k , one obtains the complete integrated form of the first-order relationship for the reaction which has an equilibrium value for absorbancy or concentration greater than zero: $k = \log_e \frac{A_f - A_0}{A_f - A} \frac{1}{t}$. But combining

both of the above equations by substitution:

$|A' - A| = |A'_f - A_0| (1 - e^{-kt}) e^{-kt}$. Taking logarithms:

$$(15) \quad kt + \log_e |A' - A| = \log_e |A_f - A_0| (1 - e^{-kt})$$

Since the right side of this equation is constant, plotting $\log_{10} |A' - A|$ vs. t gives a straight line with slope of $-k \log_{10} e$; or

$$(16) \quad k = -2.303 \Delta \log |A' - A| / \Delta t$$

There is no extrapolation and every observed reading is used only once. The longer the constant time interval T , the greater the value of $|A' - A|$ for a given t and the more accurate the method. Provided that T is several times as great as the time of half-completion of the reaction, the accuracy will be of the same order as in taking n ordinary readings and further taking n readings of the end-point spread over an interval of time equal to that spent on the ordinary readings; in fact, it is equivalent to obtaining a very accurate end point and using it in the usual manner (Guggenheim, 1926). An analogous method for a second-order reaction with equivalent concentrations has been developed by Roseveare (Frost and Pearson, 1961, p. 150).

Comparisons of Guggenheim plots for two different non-enzymic transamination reactions, using different analytical methods, are presented in Figure 10. Two examples are shown for deviations from linearity or pseudo first-order conditions, in measuring the loss of PLP. These deviations were always concave downward, and two straight lines of different

Figure 10. Guggenheim plots for nonenzymic transaminations of leucine with pyridoxal (PL) and with deoxypyridoxal (DPL), semi-logarithmic scale: $\log \Delta x$ vs. t

mM: change in millimolar concentrations of pyridoxal (PL), as measured by the ethanolimine method, and of ketoisocaproate (KIC), as measured by the dinitrophenylhydrazone method

Points taken from plot of mM vs. t ; $T = 20$ hrs.

Initial conditions:

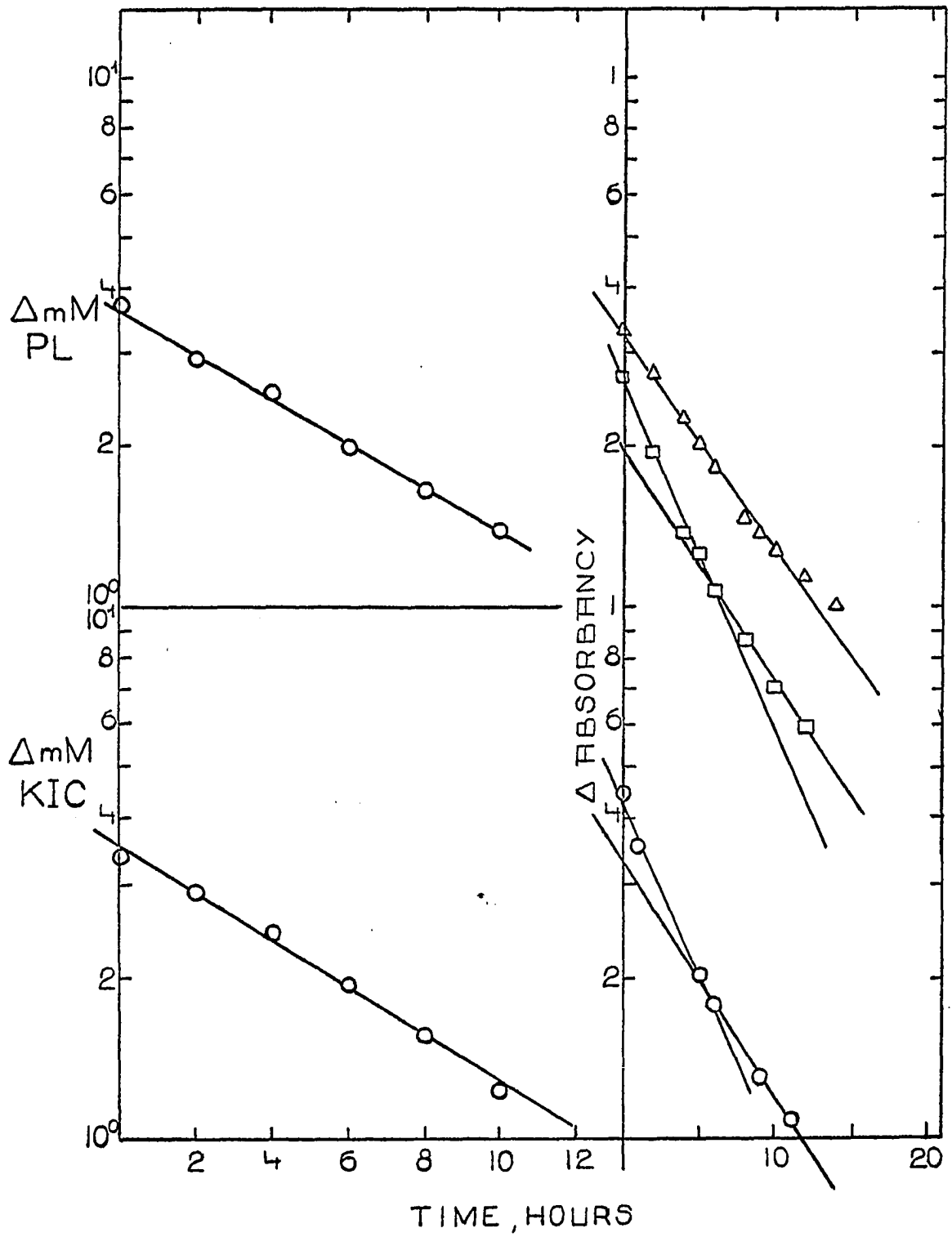
10 mM PL
120 mM leu
2 mM alum
0.1 M acetate buffer, pH 4.2,
25°C

	$k_{\text{obs}} \times 10^5 \text{sec}^{-1}$	dP/dt (2 hrs.) $\times 10^8 \text{M/sec}$	$dP/dt/5 \times 10^{-3} \text{M}$
PL	2.7	15	ca. 3
KIC	2.85	13	ca. 3

$\log \Delta \text{Absorbancy}$ vs. t ; $T = 24$ hrs.

	$k_{\text{obs}} \times 10^5 \text{sec}^{-1}$	
	(initial slope)	(final slope)
Δ 305 m μ vs. (KIC) by QH method	2.80	2.80
\square 400 m μ vs. (DPL) by QH method	4.77	2.76
\circ 413 m μ vs. Decrease in (aldimine) by direct spectral analysis		
	6.4	2.9

Initial conditions: 10 mM DPL
100 mM leu
1.0 M acetate buffer, pH 4.2, 25°C



slope were drawn through the points of the graph. "Initial" and "final" rate constants were determined from these slopes (as usual, by equation (16)), and the initial values were as much as 2 to 3 times higher than the final values.

These deviations were usually only observed for decreasing concentrations of pyridoxal analog, and even then, were observed for probably about half of the reactions studied. Rates based on keto acid formation were generally considered more reliable. For further discussion of kinetic and stoichiometric anomalies, see Results and Discussion. Rate constants obtained by various kinetic methods are compared under the same and different experimental conditions. It was concluded that the simplest and most reliable method was the Guggenheim plot using $\log (A' - A)$ vs. t .

RESULTS AND DISCUSSION

Absorption Spectrum and Ionic Equilibrium of
Pyridoxal-N-methochloride

The absorption of pyridoxal-N-methochloride, in solutions of varying pH in the range of pH 1 to 10, was measured in the region of 220 to 400 m μ (Figure 11). The molar absorptancy indices at the three absorption maxima and at the two isosbestic points were calculated (Table 4). These values generally agreed with those reported by Johnston *et al.*, (1963).

The presence of isosbestic points in the spectra of pyridoxal methochloride solutions of varying pH indicated that only a single equilibrium, involving two ionic forms, was being affected and that it should be possible to calculate the ionization constant directly from the spectrophotometric data. The acid dissociation constant and apparent pK'_a value were calculated from measurements of the absorbancies at 293.5 and 323 m μ at the three pH values nearest 4 (Figure 12). The average of these six calculated pK'_a values was 4.05, and the average deviation from this mean value was ± 0.03 . The corresponding values for the equilibrium or dissociation constant were: $K'_a = 9.1 \pm 0.6 \times 10^{-5}$.

The other data were discarded because the calculated f values were too high (> 0.9). None of the data at 254.5 m μ

Table 4. Comparison of absorption spectra of pyridoxal-N-methochloride and pyridoxal, 25°C

Ionic form ^c	PLM ^a		PL ^{a,b}	
	λ_{max} , m μ	$a_m \times 10^{-3}$	λ_{max} , m μ	$a_m \times 10^{-3}$
Hemiacetal cation (Aa)				
pH 1 ^d	293	7.2	288	9.0
pH 2.3	293.5	7.22		
Dipolar ion (Ab)				
pH 10 ^d	323	7.8	317	8.9
	255	4.8	252	5.8
pH 10.1	323.0	7.78		
	254.5	4.35		
Isosbestic points	267	1.65	263	2.05
	304	4.13	300	4.4

^aStructures - Figure 1

^bSource: Metzler and Snell, 1955

^cStructures - Figure 2

^dSource: Johnston et al., 1963; solutions of 0.5 M in ionic strength with NaCl, compared with experimental values from solutions 0.1 M in ionic strength

(the secondary absorption maximum of the dipolar ion, i. e., $\pi - \pi_2^*$ transition) were used for determination of the pK_a because of interference from carbonate absorption. Thus, carbonate is not a good buffer to use for obtaining the absorb-

Figure 11. Comparison of absorption spectra of pyridoxal-N-methochloride and pyridoxal

———— pyridoxal methochloride

- - - - - pyridoxal

cation forms, pH 1 (HCl solution)

dipolar ion forms, pH 10 (carbonate buffer)

0.5 M ionic strength, 25°C

(Johnston et al., 1963)

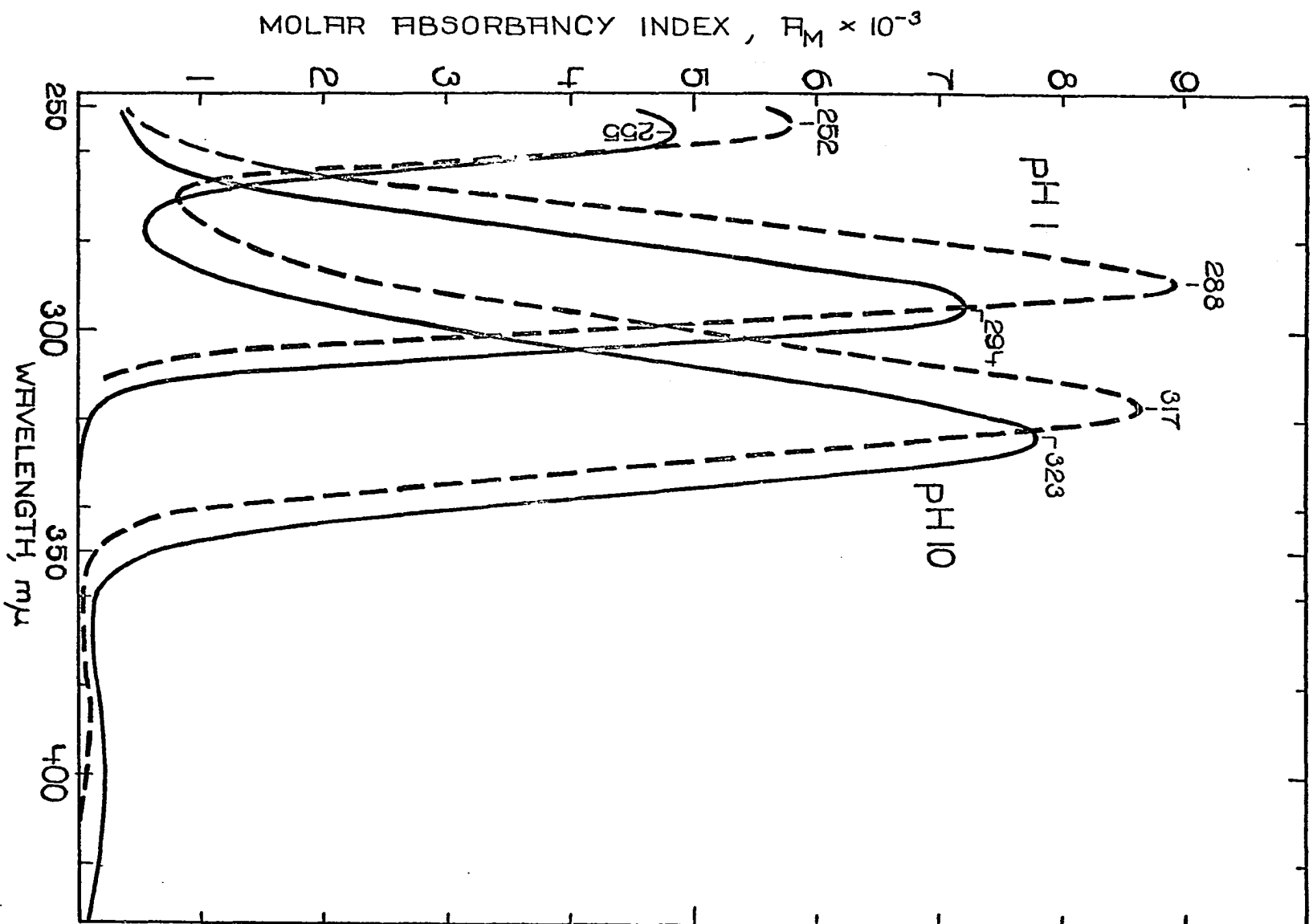


Figure 12. Spectrophotometric titration curves, pH vs. absorbancy of pyridoxal methochloride solutions, constructed from calculated pK' and equations (1) and (2), Experimental ^a

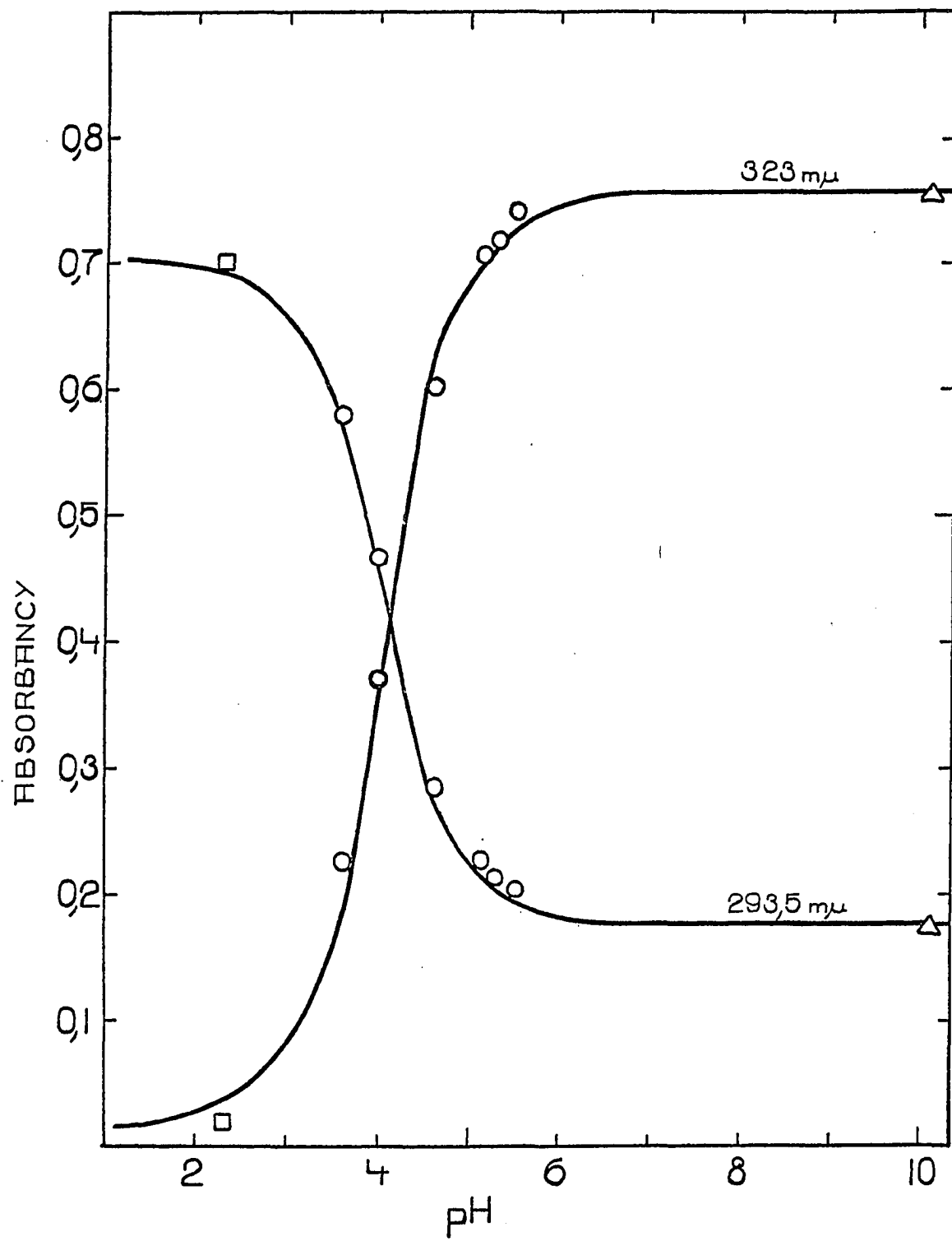
Experimental points for solutions of pyridoxal methochloride with pH controlled by:

□ 0.01 N HCl

○ acetate buffers

△ carbonate buffer

0.10 M ionic strength, 25°C, assumed mainly due to buffer ions



ancy of the dipolar ion ($f = 1$) at 254.5 m μ .

Comparisons with pyridoxal

From this spectral study of the equilibrium between the two ionic forms of pyridoxal methochloride (Aa, Ab - Figure 2, Review of Pertinent Literature), it was concluded that N-methylation of pyridoxal results in slight, but quantitatively distinguishable, changes in its properties. The absorption spectra of pyridoxal and its N-methyl analog were similar, except that N-methylation of pyridoxal resulted in shifts of the absorption maxima to longer wavelengths, by as much as 5-6 m μ for the higher wavelength band (Johnston et al., 1963; Figure 11). This shift is in agreement with earlier observations of 3-hydroxypyridine compounds (Metzler and Snell, 1955). Furthermore, the spectra of both pyridoxal and its N-methyl analog exhibited bathochromic shifts (shifts of absorption maxima to longer wavelengths) with increasing pH (Figure 11). This "red-shift" caused by the ionization of the phenolic hydrogen of pyridoxine analogs has been observed by others (Heyl et al., 1951; Metzler and Snell, 1955; Nakamoto and Martell, 1959a; Williams and Neilands, 1954).

The apparent pK'_a value of 4.05 for pyridoxal methochloride corresponds with the value of 4.20 for dissociation of the phenolic group of pyridoxal (Table 1, Review of Pertinent Literature). This lower pK'_a value for the N-methyl analog may be a reflection on the different relative proportions of

hemiacetal to free aldehyde. A higher fraction of free aldehyde (1.7 times higher) has been estimated for pyridoxal methochloride (Johnston et al., 1963), as compared to pyridoxal under the same conditions. The aldehyde group, an electron-attracting substituent, is expected to enhance the acidity of the phenolic group. Electron-attracting inductive effects of a few other substituent groups on the ionization constants of 3-hydroxypyridine-4-aldehydes, in particular (Nakamoto and Martell, 1959b) and of pyridoxine analogs, in general (Williams and Neillands, 1954), have been discussed.

Significance of ionization constants of pyridoxal analogs

Dissociation constants of pyridoxal analogs have been considered especially useful in any interpretation of pH effects on their reactions with amino acids. These spectrophotometric methods used for the study of ionic equilibria of pyridoxal-N-methochloride should be applicable to other pyridoxal analogs, as they are synthesized for studies on model systems. For example, pK'_a values of 3.96 and 8.4 were spectrophotometrically determined for "alpha⁵-pyridoxalylacetic acid" by F. Scott Furbish (See Table 1 for complete private communication).

Attempts to study mechanisms of reactions of pyridoxal analogs with amino acids spectroscopically in model systems have led to involved but necessary studies of the absorption spectra of hydroxypyridinealdehydes as functions of pH,

because of the complicated equilibria of pyridoxal analogs in solution (Nakamoto and Martell, 1959a; Martell, 1963).

Furthermore, detailed understanding of the biological function and mechanism of action of vitamin B₆ coenzymes requires knowledge of their ionization constants of acidic groups and the relative amounts of various ionic forms present in solution. This information can be used to interpret pH effects on the binding of these coenzymes to apoenzymes and to substrates (Williams and Neillands, 1954; Metzler and Snell, 1955; Snell and Metzler, 1956; Martell, 1963).

Hemiacetal formation

The ratio of hemiacetal dipolar ion to free aldehyde dipolar ion was estimated to be 46 for pyridoxal methochloride, compared to a value of 80 for pyridoxal (Johnston et al., 1963). An explanation for this difference is based upon the relative electromeric or electron-donating, inductive effects of a methyl group and a hydrogen atom bound to the pyridine ring nitrogen atom of pyridoxal. This effect is generally larger for a methyl group than a hydrogen atom and therefore the positive character of the charge on the nitrogen atom is less for pyridoxal methochloride than for pyridoxal, other factors being equal.

This difference in positive character of nitrogen atoms is reflected in a difference in mesomeric or resonance effects of the pyridine ring on the positive character of the formyl

carbon atoms of these two compounds. It follows that this mesomeric effect is less in the case of pyridoxal, leading to a greater positive character on its formyl carbon atom, and thus, a greater electrophilicity for its formyl carbon atom toward the nucleophilic hydroxymethyl group in the adjacent position on the ring. This greater relative amount of free aldehyde in pyridoxal methochloride than in pyridoxal should be considered, along with other effects of N-methylation or protonation, in interpreting their relative reaction rates with amino acids.

Equilibrium Constant for the Formation of
Hydrogen-bonded Aldimine of
Pyridoxal-N-methochloride and Valine

Although the extent of hydrogen-bonded aldimine formation from pyridoxal methochloride and valine was estimated (See Review of Pertinent Literature, Johnston et al., 1963), confirmation by an independent and more quantitative method was made. Relative amounts of imine were determined spectrophotometrically from a series of solutions of varying valine concentration from 0.08 to 0.5 M and of constant pyridoxal methochloride concentration at pH 6.0 (Table 5). The graphical method of Ketelaar et al., (1951, 1952) was used to calculate the molar absorbancy index and the formation constant for the hydrogen-bonded imine dipolar ion (Fc-d, Figure 4). The best experimental data (Table 5) for the equilibrium

under consideration are graphically depicted in Figure 13.

The values for a_{pv} , K_1' and $\log K_1'$ from different methods are compared in Table 6. Application of the method of least squares to the data was considered to yield the most reliable values, and resulted in a calculated $\log K_1'$ value of -0.02 ± 0.1 . It was noted that the relatively small changes in the values for the slope and extrapolated intercept in a plot of $10^3/(a_o - a_p)$ vs. $1/(V)$ led to unexpectedly large changes in the values calculated for a_{pv} , K_1' and $\log K_1'$. These values were calculated from equation (8), Experimental, where $K_1' = \text{intercept/slope}$ and $a_{pv} = 1/\text{intercept} + a_p$, from the plot of the data of Table 5 in Figure 13.

Within experimental error and the error of the graphical extrapolation, the formation constant for the hydrogen-bonded imine dipolar ion of pyridoxal methochloride and valine ($\log K_1' = -0.10$) calculated by an independent method was verified. At pH 6, about 30% of the PLM is converted to the valine imine, under these conditions. The graphical method may be more convenient than the method of successive approximations (Johnston et al., 1963), but it is not more accurate. The dissociation of the hydrogen-bonded proton from this dipolar ionic form is governed by $pK_{2pv} = 8.0$, calculated by a successive approximation method (Johnston et al., 1963). All equilibrium constants are apparent constants expressed in terms of molar concentrations, except that apparent hydrogen ion activities (from pH - meter readings) were used instead of

Table 5. Data for the determination of the formation constant for the hydrogen-bonded imine of pyridoxal methochloride and valine^{a,b}

Graph points ^c	(V), M	1/(V), M ⁻¹	A _{419 mμ} vs. blank	a _o X 10 ⁻³	(a _o -a _p) X 10 ⁻³	$\frac{10^3}{(a_o - a_p)}$
1	0.5000	2.000	0.885	2.375	2.276	0.4396
2	0.4000	2.500	0.745	2.000	1.901	0.5260
3	0.3330	3.000	0.648	1.739	1.640	0.6098
4	0.2500	4.000	0.515	1.383	1.284	0.7903
5	0.2000	5.000	0.431	1.157	1.058	0.9450
6	0.1670	6.000	0.377	1.012	0.9132	1.095
7	0.1428	7.000	0.330	0.8860	0.7872	1.271
8	0.1250	8.000	0.292	0.7839	0.6851	1.460
9	0.1000	10.000	0.245	0.6579	0.5591	1.789
10	0.0833	12.000	0.213	0.5719	0.4731	2.113

^aExperimental conditions were 0.1 M phosphate, pH 6.00 ± 0.01, 0.500 M in ionic strength, 25°C

^bSymbols used in the headings for this table are: (V), valine concentration; A, absorbancy; a_o, molar absorbancy index for solutions of hydrogen-bonded pyridoxal methochloride-valine imine, PV⁺, and a_o = A₄₁₀/3.726 X 10⁻⁴ M (initial concentration of pyridoxal methochloride; a_p, molar absorbancy index for pyridoxal methochloride dipolar ion, P⁺, at 419 mμ

^cIn Figure 13

Figure 13. Formation of hydrogen-bonded imine of pyridoxal methochloride and valine as a function of reciprocal valine concentration, 30 min. after mixing in the dark, pH 6 phosphate buffer, 0.5 M ionic strength, 25°C (Plot of data of Table 5:

$$10^3/(a_o - a_p) \text{ vs. } 1/(V)$$

Possible slopes and intercepts (See Table 6)

Maximum slope: straight line through first 4 points

Minimum slope: straight line through points 4,5,6

Average of minimum and maximum slopes

Least-squares slope: straight line through all ten points calculated by method of least squares

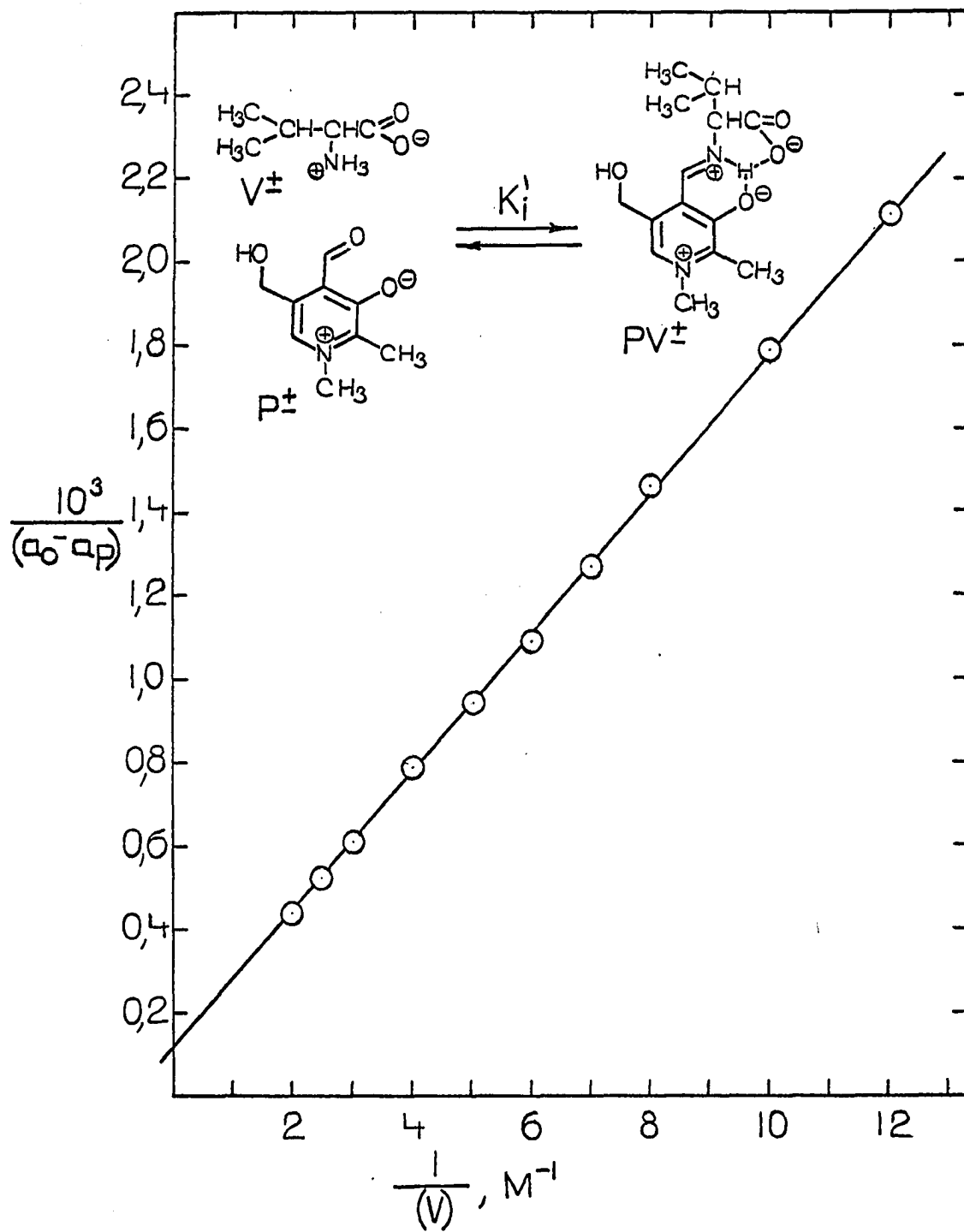


Table 6. Comparison of results from spectrophotometric measurements of the formation of hydrogen-bonded aldimine of pyridoxal methochloride and valine, calculated by different methods

Method	Slope $\times 10^3$	Intercept $\times 10^3$	a_{pv} $\times 10^3$	K_1'	$\log K_1'$
Straight line through 1st 4 points ^a	0.1753	0.087			
Straight line through points 4,5,6 ^a	0.1526	0.179			
Ave. of graphical extremes ^a	0.1639 ± 0.0113	0.133 ± 0.046	7.62	0.812 ± 0.36	-0.09 ± 0.26
Least squares method applied to data ^b	0.1612	0.1541	6.6 ± 8	0.954 ± 0.3	-0.0205 ± 0.1
Indirect estimate ^c	---	---	7.5	0.788	-0.10
Best line fitted to points by eye ^d	0.168	0.105	9.6	0.624	-0.205

^aFigure 13

^bTable 5

^cSource: Johnston et al., 1963

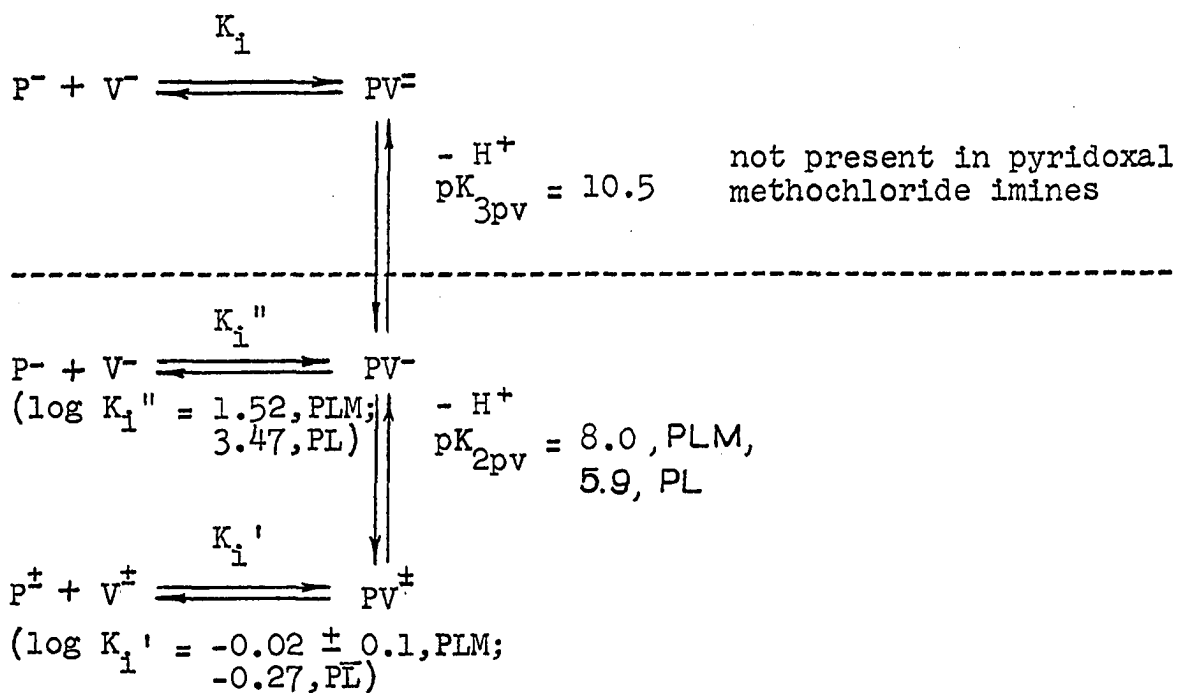
^dReported as note in Johnston et al., 1963; a values of imine solutions were determined at 419 m μ , n8t at 319 m μ (typographical error in publication)

hydrogen ion concentrations.

Comparisons with imine formation constants for pyridoxal and valine

Furthermore, it can be concluded that pyridoxal methochloride and pyridoxal are quite similar in the extent of imine formation with valine, except for the lack of an equilibrium for the imine dianion, K_1 , in the case of the former (Figure 14). The relative $\log K_1'$ values indicate that the PLM-valine imine ($\log K_1' = -0.02 \pm 0.1$) is just slightly more stable than the corresponding pyridoxal imine ($\log K_1' = -0.27$). Also, from calculated values of $\log K_1''$ (Figure 14), the hydrogen-bonded anionic imine from pyridoxal is approximately 100 times more stable than the non-hydrogen bonded form from pyridoxal methochloride. Hydrogen-bonding lends stability to the imine, because the equilibrium constant for transfer of the proton from the ring nitrogen to the hydrogen-bonded position was estimated to be 125 (Johnston et al., 1963). Yet in pyridoxal itself the tendency is for the proton to stay on the ring nitrogen, with $K = 0.14$ for transfer of the proton to the phenolate anion position. (For comparison with enzyme spectra and ionic equilibria see Review of Pertinent Literature.)

Figure 14. Equilibria in pyridoxal - valine or pyridoxal-
N-methochloride - valine solutions (Johnston
et al., 1963)



$$K_1' = K_1 \cdot \frac{K_{2p} K_{2v}}{K_{2pv} K_{3pv}} = K_1'' \cdot \frac{K_{2v}}{K_{2pv}}$$

$$K_1'' = K_1 \cdot \frac{K_{2p}}{K_{3pv}}$$

Equilibrium Constants for the Formation of Aldimines
of Leucine and Deoxypyridoxal, Leucine and Pyridoxal
Phosphate as a Function of pH

(Most of the experimental work, calculations and curve-fitting was done by Erika Rommel, Louise Hodgin, and David E. Metzler, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Private communication. 1964)

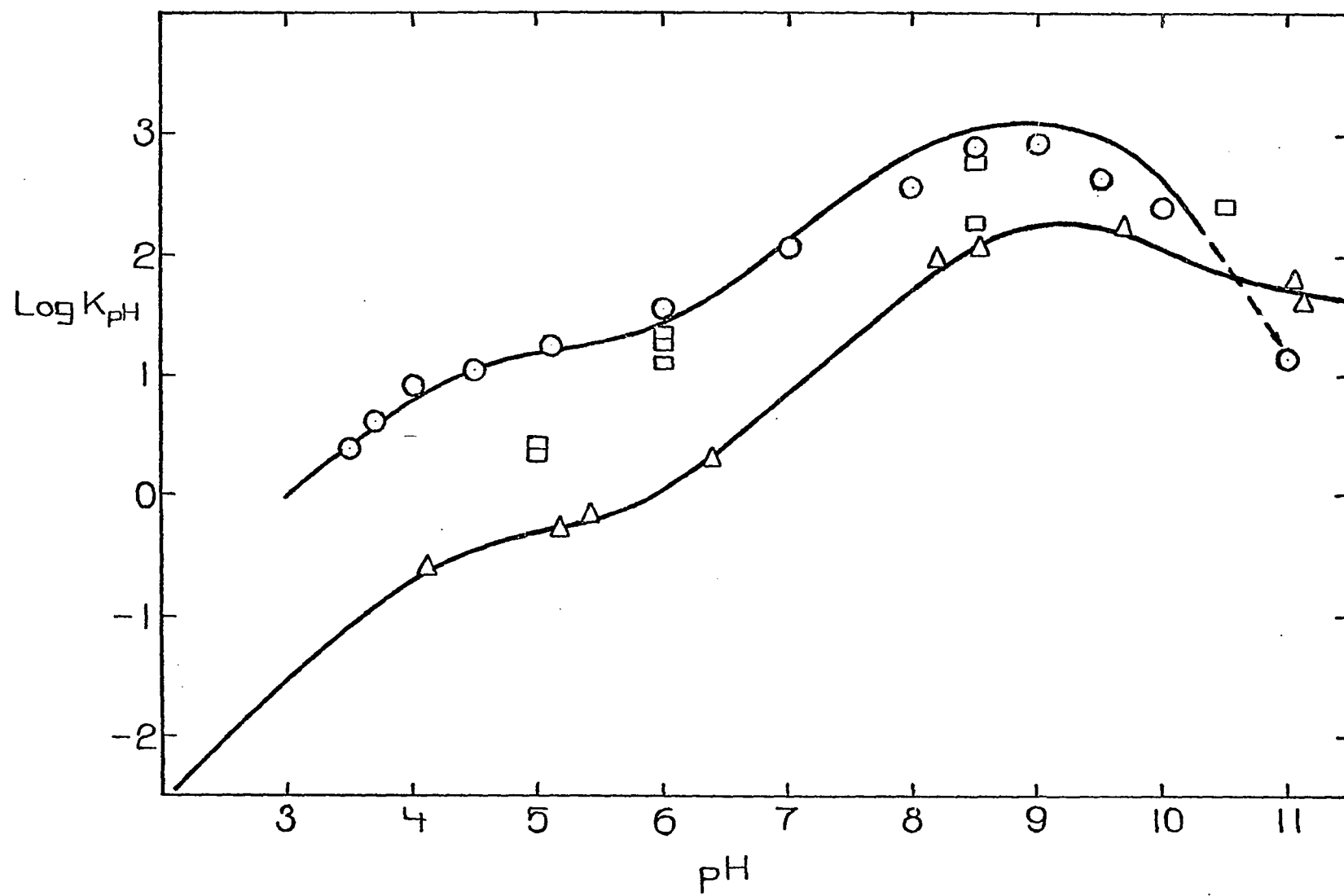
The appearance of absorption bands in the region 400 to 420 m μ indicated the formation of imines in solutions of pyridoxal phosphate and leucine and of deoxypyridoxal and leucine. The extent of formation of these imines is dependent upon the amino acid concentration (or the ratio of concentrations of amino acid and pyridoxal analog, since imine formation is an equilibrium) and on the hydrogen ion concentration of the solution. By measuring the relative extent of imine formation spectrophotometrically in solutions of varying amino acid concentration, at constant deoxypyridoxal (or pyridoxal phosphate) concentration and constant pH, an imine formation constant was calculated by the graphical method previously described for imine formation from pyridoxal methochloride and valine (Experimental).

The equilibrium constants for the formation of these imines were then determined as a function of pH, and the logarithm of K_o values (observed imine formation constants) were plotted against pH (Figure 15). The shape of the curve for imine formation from deoxypyridoxal and leucine was similar

Figure 15. Variation of the logarithm of the equilibrium constants for imine formation with pH

Imines formed from:

- Deoxypyridoxal + leucine (K_o)
- Pyridoxal phosphate + leucine (K_o)
- △ Pyridoxal anion + valine anion (K_1)



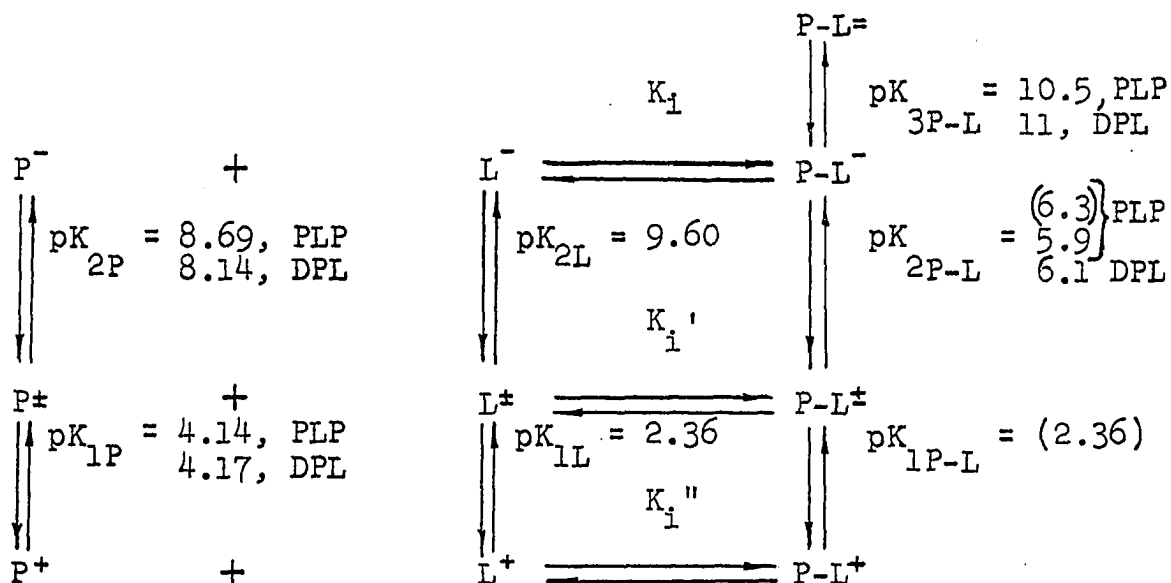
to that from pyridoxal and valine, which was reported by Metzler (1957). Experimental points were in agreement with the theoretical curve calculated by a method previously described (Metzler, 1957) and outlined in Figure 16, using the pK_a values of the species involved and the K_1' value for the hydrogen-bonded imine.

From the calculated formation constants the molar fractions of total imine formed from 0.01 M deoxypyridoxal, 0.05 M deoxypyridoxal, and 0.05 M leucine were calculated by a method of successive approximations (instead of solving quadratic equations), and plotted against pH (Figure 22). The molar fraction of protonated imine (protonated at the pyridine ring nitrogen atom) was calculated from equation (17) relating the fraction of total imine at each pH value, assuming $pK_{2P-L} = 6.10$ (Figure 16). Evidence for this pK was obtained from the intersection of curves for a plot of the molar absorbandy indices of the imine at 414 and 294 $m\mu$ (which in turn were obtained from intercepts found by extrapolation in the plots used to determine the imine formation constants) as a function of pH.

$$(17) \quad (\text{Fraction total imine}) (1 - f_{P-L}) = (\text{Fraction imine } H^+)$$

A few experimental points for the observed equilibrium constants for the formation of imine from pyridoxal phosphate and leucine were included in Figure 15. The shape of the curve (for $\log K_0$ vs. pH) was assumed to be similar to those for imine formation from deoxypyridoxal and leucine and from

Figure 16. Equilibria of imine formation from pyridoxal phosphate (PLP) and leucine, deoxypyridoxal (DPL) and leucine, and equations for calculation of formation constants



$$\begin{aligned}
 K_o &= \frac{(P-L)}{(P)(L)} = \frac{(P-L^+) + (P-L^\pm) + (P-L^-) + (P-L=)}{(P^+ + P^\pm + P^-)(L^+ + L^\pm + L^-)} \\
 &= \frac{(P-L^\pm)}{(P^\pm)} \left[\frac{1 + \frac{(H^+)}{K_{1P-L}} + \frac{K_{2P-L}}{(H^+)} + \frac{K_{2P-L}K_{3P-L}}{(H^+)^2}}{\left(1 + \frac{(H^+)}{K_{1P}} + \frac{K_{2P}}{(H^+)}\right)(L^\pm) \left(1 + \frac{(H^+)}{K_{1L}} + \frac{K_{2L}}{(H^+)}\right)} \right] \\
 &= K_i' \left[\frac{1 + \frac{H^+}{K_{1P-L}} + \dots}{\left(1 + \frac{H^+}{K_{1P}} + \dots\right) \left(1 + \frac{H^+}{K_{1L}} + \dots\right)} \right] \\
 \log K_o - \log K_i' &= \log \left[1 + \frac{(H^+)}{K_{1P-L}} + \frac{K_{2P-L}}{(H^+)} + \frac{K_{2P-L}K_{3P-L}}{(H^+)^2} \right] \\
 &\quad - \log \left[1 + \frac{(H^+)}{K_{1P}} + \frac{K_{2P}}{(H^+)} \right] - \log \left[1 + \frac{(H^+)}{K_{1L}} + \frac{K_{2L}}{(H^+)} \right]
 \end{aligned}$$

pyridoxal and valine. Since the curves are parallel, the relative extent of imine formation from these pyridoxal analogs is also dependent on pH.

Imine concentrations were calculated from direct spectral data and compared to values predicted for the corresponding conditions (Table 7):

Table 7. Comparison of imine concentrations from direct spectral data of reaction solutions at time zero with predicted from the theoretical curve (Figure 15)

0.10 M leucine + M DPL,	pH	(DPL-leu imine), spectral	M X 10 ⁴ predicted
0.00113	4.6	6.3	6.02
0.01	4.2	29 ± 1	43.7
0.01	7.1	48	67.5

The graphical methods of Harbury and Foley, (1958) and of Isenberg and Szent-Györgi (1958) also could have been used for the evaluation of imine formation constants from the data obtained. These methods may have some advantage over that of Ketelaar et al., (1951, 1952), in that the apparent molar absorbancy indices for solutions of imine do not have to be calculated, since absorbancies are used directly, and the molar absorbancy index and concentration of pyridoxal analog would not have to be determined. (Compare equations given in Review

of Pertinent Literature.)

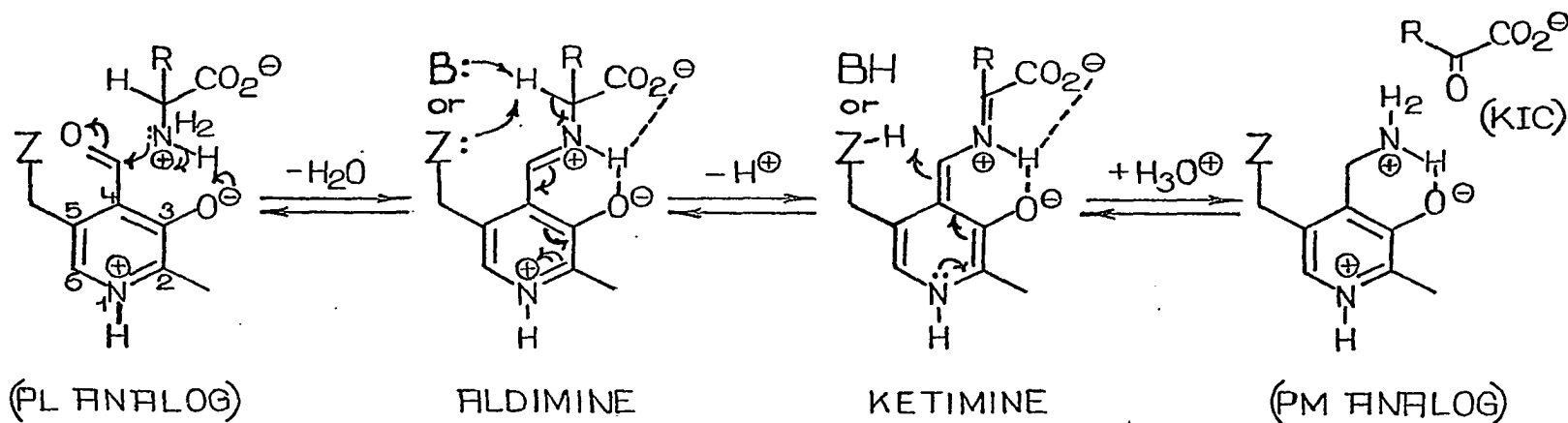
Nonenzymic Transamination of Leucine with Deoxypyridoxal

The roles of the nitrogen atom of the pyridine ring, the 3-phenolic, 4-formyl and 5-hydroxymethyl groups of pyridoxal (vitamin B₆ aldehyde), the most thoroughly studied amino group acceptor in nonenzymic transamination of amino acids, have been discussed (Review of Pertinent Literature). However, the effects of these groups and of other substituents at the 5-position of pyridoxal (Figure 17) have not been studied quantitatively in such model systems. In order to quantitatively measure the effects of these groups in a model transamination system, 5-deoxypyridoxal, which has a methyl group in place of the hydroxymethyl group of pyridoxal, was studied as a reference amino group acceptor.

Leucine was chosen as the amino group donor because of its relatively high imine formation constant (Metzler, 1957) and transamination rate, (Metzler and Snell, 1952b) compared to that of other naturally-occurring amino acids, and its expected lack of side reactions encountered with amino acids (such as serine, threonine, glutamate, cysteine, lysine), having extra functional groups. Furthermore, its transamination product, alpha-ketoisocaproate was expected to be more stable than keto acids with smaller side chains, such as pyruvate (from alanine).

The temperature of 25°C was chosen for this kinetic model

Figure 17. Model systems of nonenzymic transamination between pyridoxal analogs and amino acids (See Figure 1 for nomenclature of analogs)



5-substituted PL analogs (intramolecular catalysts)

Z = -OH	PL	Pyridoxal; PM Pyridoxamine Analogs
= -OPO ₃ H ⁻	PLP	Pyridoxal-5-phosphate
= -H	DPL	5-Deoxypyridoxal
= -O ₂ ⁻	CPL	"5-Carboxypyridoxal" or "5-Pyridoxalylate"
= -CO ₂ ⁻	FPL or PLF	"alpha ⁵ -Pyridoxalylformate"
= -CH ₂ CO ₂ ⁻	APL or PLA	"alpha ⁵ -Pyridoxalylacetate"

R = -CH₂CH(CH₃)₂,
 for leucine
 KIC = alpha-keto-
 isocaproate
 B = general base
 catalyst (inter-
 molecular),
e.g., buffer

system, because formation and acid dissociation constants of imines of pyridoxal and amino acids are known, or can readily be determined, and pH measurements are more reliable at room temperature than at 100°C. This information is necessary for the calculation of precise rate constants, because the true reaction rate is a measure, of not the amount of pyridine aldehyde lost or of keto acid formed, but of the amount of transamination intermediate converted to products in a unit of time.

This nonenzymic transamination does proceed at 25°C, and may be followed both qualitatively and quantitatively by several techniques. Identification of products has been made by paper and thin layer chromatography. The rates of change in pyridoxal analog and in keto acid have been followed by several chemical- spectrophotometric methods. Spectral changes in a reaction solution (attributed to changes in concentrations of reactants, intermediates and products) are in quantitative agreement with rates obtained by the chemical methods.

Chromatography of reaction solutions

Ketoisocaproate (KIC) was identified as its 2,4-dinitro-phenylhydrazine (DNP), most conveniently, by separating this yellow DNP-derivative from that of deoxypyridoxal on a thin-layer silica gel plate. This was qualitative evidence for the production of KIC from the nonenzymic transamination of leucine with deoxypyridoxal. Usually two yellow spots were detected

for KIC, corresponding to the syn- and anti-forms of DNP-KIC (Metzler and Snell, 1952a). A yellow spot for the DNP-pyridoxal analog had a slightly lower R_F value than those of DNP-KIC. The DNPH reagent itself lagged a little behind the solvent front and appeared as a pair of yellow spots or as a streak. (See Table 8 for comparison of R_F values).

Constituents with amino groups were identified with a ninhydrin spray reagent. Leucine, having a low R_F value of 0.3, was easily identified as a purple or pink spot. Deoxypyridoxamine (if present in high enough concentration) gave an orange spot which migrated very little from the origin, as did a pyridoxamine standard (Figure 18).

No para-unsubstituted phenols other than deoxypyridoxal and deoxypyridoxamine, and perhaps the leucine aldimine of deoxypyridoxal were detected by Gibbs' reagent. Thus, no intermediates or products other than those expected from the nonenzymic transamination of leucine with deoxypyridoxal to yield ketoisocaproate and deoxypyridoxamine were detected by these chromatographic techniques. These results are further evidence that the changes observed by quantitative analytical techniques, especially spectral, are indeed related to an actual nonenzymic transamination process.

Stoichiometric and kinetic anomalies

By comparing the changes in concentrations of ketoisocaproate and deoxypyridoxal in the reactions studied at dif-

Table 8. Chromatography of a solution after about 200 hours reaction, 25°C

Initial conditions: 50 mM leu, 10 mM DPL, pH 6.6, unbuffered
(dinitrophenylhydrazine (DNPH) solutions spotted; ninhydrin (Nin) solution sprayed after development)

R_F values on paper - developed with upper layer of n-BuOH (5), H_2O (4), EtOH (1); BEW, 514

DNP - KIC	0.86	yellow	lit. values 0.79 - 0.86 ^a
Nin - leu	0.59	purple	
DNPH	0.95	yellow	

R_F values on thin-layer silica gel plates - developed with upper layer of n-BuOH (5), H_2O (4), EtOH (1)

			<u>Standards</u>
DNPH	(2) 0.67 - 0.76	yellow	
DNP - KIC	0.59	yellow	0.54 - 0.62
	0.50 - 0.53	yellow	0.45 - 0.51
DNP - DPL	0.46 - 0.49	yellow	0.62 - 0.68
Nin - leu	0.35	purple	0.31 - 0.4
Nin - DPM	0.05 - 0.09	orange	0.05 - 0.09 (Nin - PM)

Developed with n-BuOH (4), HOAc (1), H_2O (5)

DNP - KIC	0.7	yellow
Nin - leu	0.3	purple
Nin - DPM	0.06	orange - red

R_F values of spots from Gibbs' Reagent sprayed on thin-layer chromatograms not treated with DNPH; developed with upper layer on n-BuOH (5), H_2O (4), EtOH (1)

DPL	0.65
DPL - leu aldimine	0.31

^aSource: Meister and Abendschein, 1956

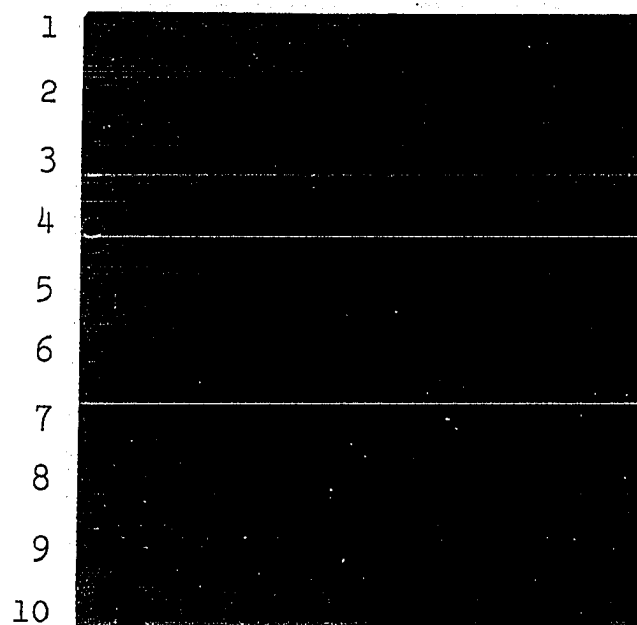
Figure 18. Identification of amino group acceptors and donors in nonenzymic transamination by thin-layer chromatography

Standards and samples			Reagents		
	conc, M	no. spots	spot	no.	Nin-hydrin spray after development
1 PM	0.01	15	_____		
2 Leu	0.16	5	_____		
3 DPL	0.001	25	DNPH	8	
4 DPL + Leu (8×10^{-4} + 0.03, pH 6.2)		50	DNPH	12	
5 4 at t_e		50	DNPH	12	
6 KIC	0.05	20	DNPH	12	
7 as 4		50	Gibbs'	20	
8 as 5		50	Gibbs'	20; DNPH, 4	
9 DPL	0.001	40	Gibbs'	20	
10 PM	0.01	15	Gibbs'	20	

Solvent system: upper layer of BEW, 514

Results: Color (identity) Photo taken 1 day after color development

- 1 Orange (PM - Nin)
- 2 Purple (Leu - Nin), (color fades almost completely in one day)
- 3 Yellow (DPL - DNP) DNPH (2)
- 4 Purple (Leu - Nin); Yellow (DPL - DNP)
- 5 Orange (PM - Nin); Purple (Leu - Nin); Yellow (DPL - DNP); (KIC - DNP)
- 6 (2) Yellow (KIC - DNP) (2)
- 7 Purple (Leu - Nin); Brown, Gray (DPL - Gibbs')
- 8 Orange (PM - Nin); Purple (Leu - Nin); Yellow (KIC - DNP) (2); Brown
- 9 Brown (DPL - Gibbs') (DPL - Gibbs')
- 10 Orange (PM - Nin); Brown (DPL - Gibbs')



origin

solvent front

ferent pH's (Table 9), it can be noted that initially, i. e., after 48 hours reaction, the changes in concentration of deoxypyridoxal are at least equal to, but usually greater than (depending upon pH) the corresponding changes in concentration of keto acid. These concentrations were calculated from standard calibration curves based on known amounts of keto acid and pyridoxal analog (See Experimental, Figure 8). However, at a much later time in each of these reactions (approaching equilibrium), the changes in concentration of deoxypyridoxal are less than the corresponding changes in concentration of keto acid, except at the pH 4 optimum (Figure 19). This greater initial loss and lesser later loss of deoxypyridoxal than would be expected from the corresponding keto acid formation can not be explained solely on the basis of a side reaction involving transamination products.

It has generally been observed that the rate of decrease in pyridoxal analog, followed by decrease in absorbancy at 400 m μ by the quinolyldiazotization (QH) method, is measurably greater than (up to 3 times greater under certain conditions of pH and concentration, especially at pH 11.6) the corresponding rate of increase in ketoisocaproate, followed by increase in absorbancy at 305 m μ by the QH method (Table 9). If it is assumed that the quinolyldiazotization method is able to detect aldimine and ketimine intermediates, as well as pyridine aldehyde and alpha-keto acid, then overall rates of the transamination reaction would be the same, regardless if they were based on

Table 9. Stoichiometric and kinetic results of transamination of leucine with deoxypyridoxal^a

pH after 48 hrs.	after t hrs.	Stoichiometric (changes in millimolar concentrations)				Kinetic ^b k _{obs} x 10 ⁷ sec. ⁻¹	
		KIC	DPL	KIC	DPL	KIC	DPL
1.2		0	0			1 ^c	0
1.7		0	0			1 ^c	0
4.1	504	0.9	2.1	4.5	8.4	11	17.5
5.75	648	1.0	1.35	6.9	6.15	± 2 7 (16)	± 1.5 3.5
<u>5.2</u>						± 1	± 1
7.2	768	0.65	0.9	8.0	5.6	4 (8)	4.5
<u>7.8</u>						± 2	± 2.5
9.2	648	1.0	1.0	7.7	6.4	6.1 (11)	6.4
<u>8.8</u>							
9.35	648	1.3	1.7	7.7	6.4	6.5 (24)	15
						± 2.5	± 5
11.65	456	1.8	3.4	7.1	5.9	15	37
						± 2	± 9
12.55	456	1.5	1.75	7.3	6.8	13	17
						± 3	± 10

^aInitial conditions: 50 mM leu, 10 mM DPL, unbuffered, 25°C

^bCalculated from adjacent Guggenheim points and/or determined from Guggenheim plots; pH profile - Figure 21 (Initial rate -- deviation from first order plot during at least first 24 hrs.)

^c(Estimated from approximate $t_{\frac{1}{2}}$ as compared to reactions at higher pH, during first $\frac{1}{2}$ week of reaction, but little further reaction detected after two months)

Figure 19. Rate curves for reaction between leucine and deoxypyridoxal: Absorbancy vs. time (hours), indicating (mM DPL or KIC at 0, 48, 504 hours).

Initial conditions: 0.05 M leu, 0.01 M DPL, unbuffered, 25°

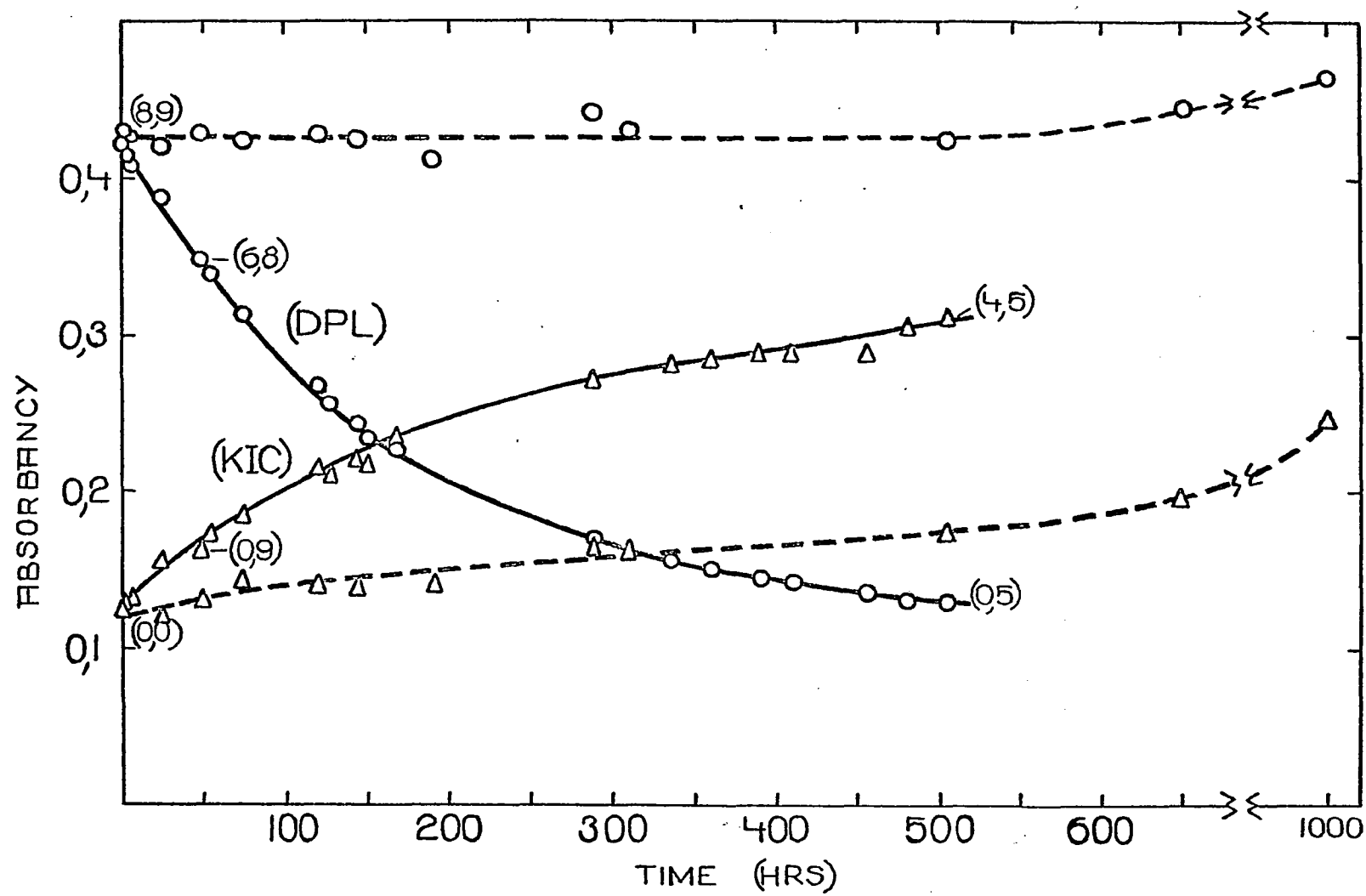
Aliquots analyzed by QH method

○ $A_{400} \text{ m}\mu = f \text{ (DPL)}$

△ $A_{305} \text{ m}\mu = f \text{ (KIC)}$

— pH 4.1

---- pH 1.7



absorbancy at 305 or at 400 mμ. This assumption seems to be valid because rates based on other analytical methods, including the ethanol imine method and direct spectral measurements used in other experiments, are in agreement with those obtained from the QH method.

Side reactions involving the pyridoxal analog are suggested to account for this anomaly. Such reactions may include air oxidation of the pyridoxal analog or formation of the imine from the pyridoxal analog and its corresponding pyridoxamine analog, as suggested by Banks et al. (1961) and by Bruce and Topping (1963a). In the related study by Banks et al., (1961) the straight line curves obtained from the reciprocal plot of the data fitted the equation derived for spectrophotometric determination of the imine formation constant (See Review of Pertinent Literature). This indicated the formation of ketimine from pyridoxamine and pyruvate at pH above 8 involved equimolar amounts of reactants.

At pH 10, however, the forward reaction between pyridoxal and alanine did not yield equimolar amounts of products, but a 2 to 1 ratio of pyridoxamine to pyruvate. This stoichiometric anomaly was explained by possible side reactions such as air oxidation of pyridoxal or decarboxylation of pyruvate. Furthermore, a reaction between pyridoxal and pyridoxamine at pH 11 was found to yield twice the initial molar amount of pyridoxal. This reaction was explained on the basis of air oxidation of an imine intermediate (Banks, et al., 1961).

In another kinetic study of nonenzymic transamination, a greater rate of cleavage of the ketimine of phenylglycine and pyridoxal in alcohol, as compared to aqueous solution, resulted in stoichiometric amounts of products and indicated negligible side reactions (Bruce and Topping, 1963a). However, this imidazole-catalyzed reaction in aqueous solution (pH 8.6) had side reactions resulting from the slow breakdown of ketimine. Imidazole catalysis had little effect on ketimine cleavage, although it facilitated the rate-determining aldimine to ketimine transformation. At less than 0.3 M imidazole the formation of imine from pyridoxal and pyridoxamine was significant and rate constants could be determined (Bruce and Topping, 1963d). In these experiments air oxidation of pyridoxal was minimized by several precautions.

Side reactions of an uncharacterized nature occurred also in alkaline medium (pH 9.6) in the oxidative deamination of amino acids by pyridoxal (Ikawa and Snell, 1954b). There was a greater loss of pyridoxal than expected, since pyridoxal decreased in smaller amounts than the ammonia formed. In some reaction mixtures 4-pyridoxic acid, the oxidation product of pyridoxal with the 4-formyl group converted to a carboxylic acid, was found.

The rate of racemization of alanine in the reaction with pyridoxal, observed by changes in optical rotation, did not equal the rate associated with the third relaxation time in spectral measurements (Fleck and Alberty, 1962). Both meas-

urements were expected to agree since both depend on the aldimine-ketimine tautomerization (in which the optically-active center at the alpha-carbon is lost after removal of the proton in the rate-limiting step in transamination).

Some of these stoichiometric and kinetic anomalies may be due to the instability of aqueous solutions of pyridoxal observed by Christensen (1958), Davis et al., (1961), Fleck and Alberty (1962). Spectral changes were observed even in sterilized solutions of pyridoxal at pH 8 kept in total darkness over periods of days at room temperature (Fleck and Alberty, 1962). The total magnitude of these absorbancy changes per mole of pyridoxal was about 5% of the changes observed when alanine was also present at concentrations of about 0.2 M, but the magnitudes of these changes were not reproducible. However, deoxypyridoxal, 0.01 M in an unbuffered solution of 0.05 M leucine at pH less than 2, was stable for at least 1000 hours, since there was no significant change in absorbancy of aliquots analyzed by the quinolyldrazone method at 400 mμ (Figure 19).

Direct spectral measurements were obtained for two reactions in unbuffered solution near neutral pH. For the reaction with an initial pH of 6.6, decreases in absorbancies at 415 and 234 mμ, maxima for aldimine absorption followed pseudo first-order kinetics. The rate constants were 7.1 and 10×10^{-7} /sec, respectively, as compared to those obtained from data by the QH method: 6.9 ± 0.8 and $5.9 \pm 0.5 \times 10^{-7}$ /sec,

for increase in absorbancy at 305 m μ and decrease in absorbancy at 400 m μ , respectively.

An example of such spectral changes may be seen in Figure 20. In this reaction of deoxypyridoxal with leucine the maximum absorbancy at 288 m μ increased and was also followed as a function of time. For the reaction with an initial pH of 7.1, decrease in aldimine absorbancy at 420 m μ and increase in absorbancies at 325 and 285 m μ (probably due to ketimine and/or deoxypyridoxamine products) also yielded pseudo first-order rate constants, but that obtained from the data at 325 m μ was about twice the rates obtained from data at 420 and 285 m μ . All of these rates, however, were considerably higher than the corresponding constants from QH data (Table 10).

Table 10. Comparison of rate constants from the quinolyldihydrazone analyses with those from direct spectral analyses of the reaction of leucine (leu) with deoxypyridoxal (DPL)^a

Analytical method:	QH		Direct spectra		
	+A ₃₀₅	-A ₄₀₀	-A ₄₂₀	+A ₃₂₅	+A ₂₈₅
k _{obs} X 10 ⁷ sec ⁻¹ :	7.2	11.9	15.6	34.7	19.6

^aInitial conditions: 0.01 M Leu, 0.01 M DPL, pH 7.1, unbuffered, 25°

Spectral changes noted in the deoxypyridoxal control solution may be due to photodecomposition or air oxidation (Figure 20). Furthermore, spectra and ethanolimine analyses of deoxypyridoxal (DPL) solutions showed no significant changes during the first day after mixing. However, gradual pH and spectral changes occurred especially in unbuffered solutions of DPL after one day, and indicated loss of the free aldehyde form at about 380 m μ and increase in the 325 m μ absorption band (See Figure 20). Air oxidation may be at least partly responsible since these changes were inhibited by occasionally bubbling nitrogen gas into the solutions (Robert Johnson, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Non-enzymatic transamination reactions of amino acid esters with pyridoxal analogs. Private communication. 1964.)

Variation of pH in unbuffered reaction solutions was considered as a possible cause of the kinetic anomalies. The pH values used in Figure 21, the pH profile for the reaction rate of leucine and deoxypyridoxal, were obtained during the first day of reaction. These reactions were followed over periods of from three to eight weeks, and pH changes were noticed in these unbuffered solutions. These changes varied both in magnitude and in direction from the initially determined pH values. Changes of 0.5 to 1 pH unit were recorded after about four weeks reaction. These changes are indicated

Figure 20. Spectral-time studies at 25°C of

a) an aqueous solution of DPL (unbuffered)

spectrum no. time after dilution pH

1		55'	
3 ^a	6 hr.	25'	5.00
4 ^a	21 hr.	25'	
5	48 hr.		6.8

b) an aqueous solution of DPL in 0.10 M
Leu (unbuffered)

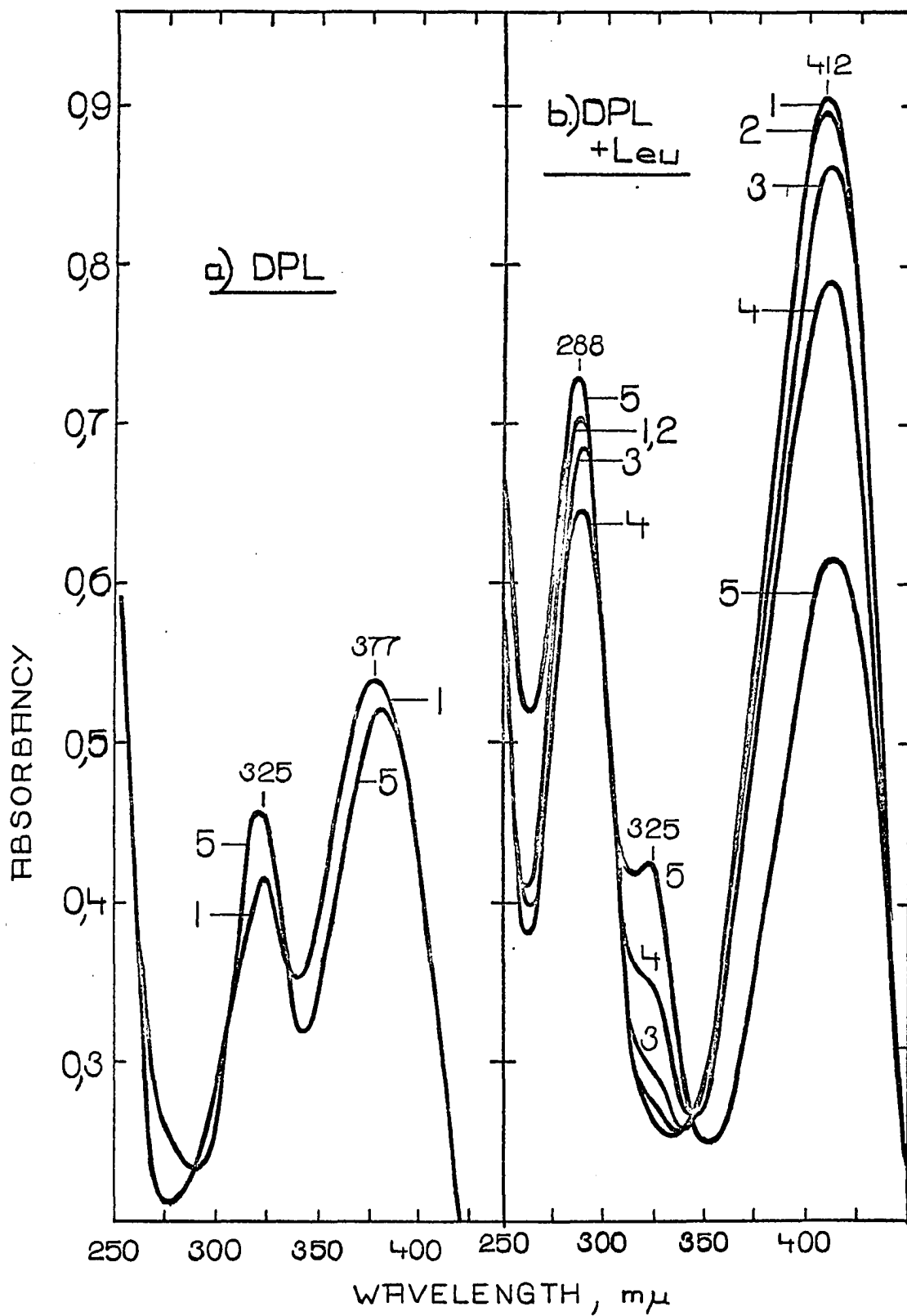
1		1'	
2		35'	4.64
3	5 hr.	10'	
4	21 hr.	5'	
5	47 hr.	50'	6.6

Spectral analysis of DPL in 0.1N HCl:

1.13×10^{-3} M DPL in a) and b, 9.0 mm
 ± 0.01

cell spacers required

^aSpectra not shown



by the sizes of figures (width of triangles or rectangles) surrounding the experimental values of Figure 21. (Also, see Figure 20 for pH changes observed in unbuffered solutions of deoxypyridoxal).

It was thought that the pH of an unbuffered solution of leucine and deoxypyridoxal would remain fairly constant throughout the reaction, since there is no net gain or loss of protons in the nonenzymic transamination reaction (Figure 17). Also, the pH values of unbuffered solutions of pyridoxal phosphate and leucine remained fairly constant over periods of several days. Although boiled, redistilled water was used in the preparation of reaction solutions, pH changes observed in these solutions might be due to reabsorption of carbon dioxide and oxygen from the atmosphere when the tubes containing the solutions were opened to withdraw aliquots for analysis.

In some cases the Guggenheim plots used to calculate the pseudo first-order rate constants, based on the deoxypyridoxal data were concave downward and initial and final rates could be calculated from two linear sets of points (Figure 10). Fleck and Alberty (1962) also noted a curvature concave downward in some of their Guggenheim plots during the first 10% of the time period. These deviations seemed to occur predominantly at low pH values or at high amino acid concentrations. Perhaps, intermolecular acid-base catalysis of two molecules of imine or by amino acid anion with one molecule of imine would explain this departure from pseudo first-order kinetics.

Another possible side reaction to account for greater loss of deoxypyridoxal is the condensation of the alpha-carbon of an imine with the aldehyde group of deoxypyridoxal. This condensation was shown to take place in the reaction of pyridoxal phosphate with cycloserine (Roze, 1964).

In considering these kinetic and stoichiometric anomalies, it seems that the most reliable rate data are probably those based on keto acid formation, or on loss of aldimine followed spectrophotometrically, until further investigation into the nature of the possible side reactions of pyridoxal analogs yields an explanation which quantitatively accounts for these anomalies.

Rate of reaction as a function of pH

Pseudo first-order rate constants for transamination of deoxypyridoxal by a 5 to 1 excess of leucine, in unbuffered aqueous solutions, were determined and plotted against the initial pH of the reaction solutions in Figure 21. Two rate maxima exist - one at about pH 4 and a higher one above pH 11, with a minimum between them at neutral pH values. It was observed that reaction solutions of pH less than 3 had only a slightly yellowish tinge compared to brighter and deeper yellow solutions at higher pH, indicating relative extent of imine formation as a function of pH in these solutions. The bright yellow solutions also faded relatively rapidly corresponding to their rates of conversion of imine to products.

In order to interpret this pH profile for the rate of non-enzymic transamination quantitatively, the pH profile for the corresponding imine formation constants must be used (Figure 15). Consider the reaction, $P + L \xrightleftharpoons[\text{fast}]{K_o} P-L \xrightarrow[\text{slow}]{k_1} K + PM$, where P = pyridoxal analog, PM = pyridoxamine analog, L = leucine, P-L = imine intermediate, K = keto acid (from leu), and equilibrium constant for imine formation (observed at any pH), $K_o = \frac{(P-L)}{(P)(L)}$, and k_1 is the first-order rate constant for conversion of P-L to products. First-order rate constants for formation of keto acid in unbuffered solutions may be expressed by equation (18).

$$(18) \quad k_1 = \frac{-d(P-L)/dt_o}{(P-L)} = \frac{+d(K)/dt_o}{(P-L)}$$

The imines, P-L, are formed rapidly and the rate measured for loss of pyridoxal analog, formation of keto acid, or loss of aldimine, by several methods, must be proportional to that of the subsequent conversion of the imine to products.

The optimum at pH 4 may be consistent with the reactive intermediate being the hydrogen-bonded aldimine with a protonated nitrogen atom in the pyridine ring. This protonated imine species becomes predominant below pH 6 (pK 6.1). Below pH 4 the observed rate constant falls off sharply, probably due to the decrease in the extent of imine formation. In this low pH range the cation forms of deoxypyridoxal and an amino acid would not be expected to form a very stable imine (a dication). Furthermore, dehydration of the carbinolamine

Figure 21. Pseudo first-order rate constants for trans-amination of leucine with deoxypyridoxal as a function of pH

Initial conditions: 0.05 M Leu, 0.01 M DPL, unbuffered, 25°

QH analytical method

Δk_{obs} for KIC formation from $\Delta A_{305 \text{ mu}}$

$\square k_{\text{obs}}$ for DPL decrease $\Delta A_{400 \text{ mu}}$

\otimes estimated values (see footnote a in Table 9)

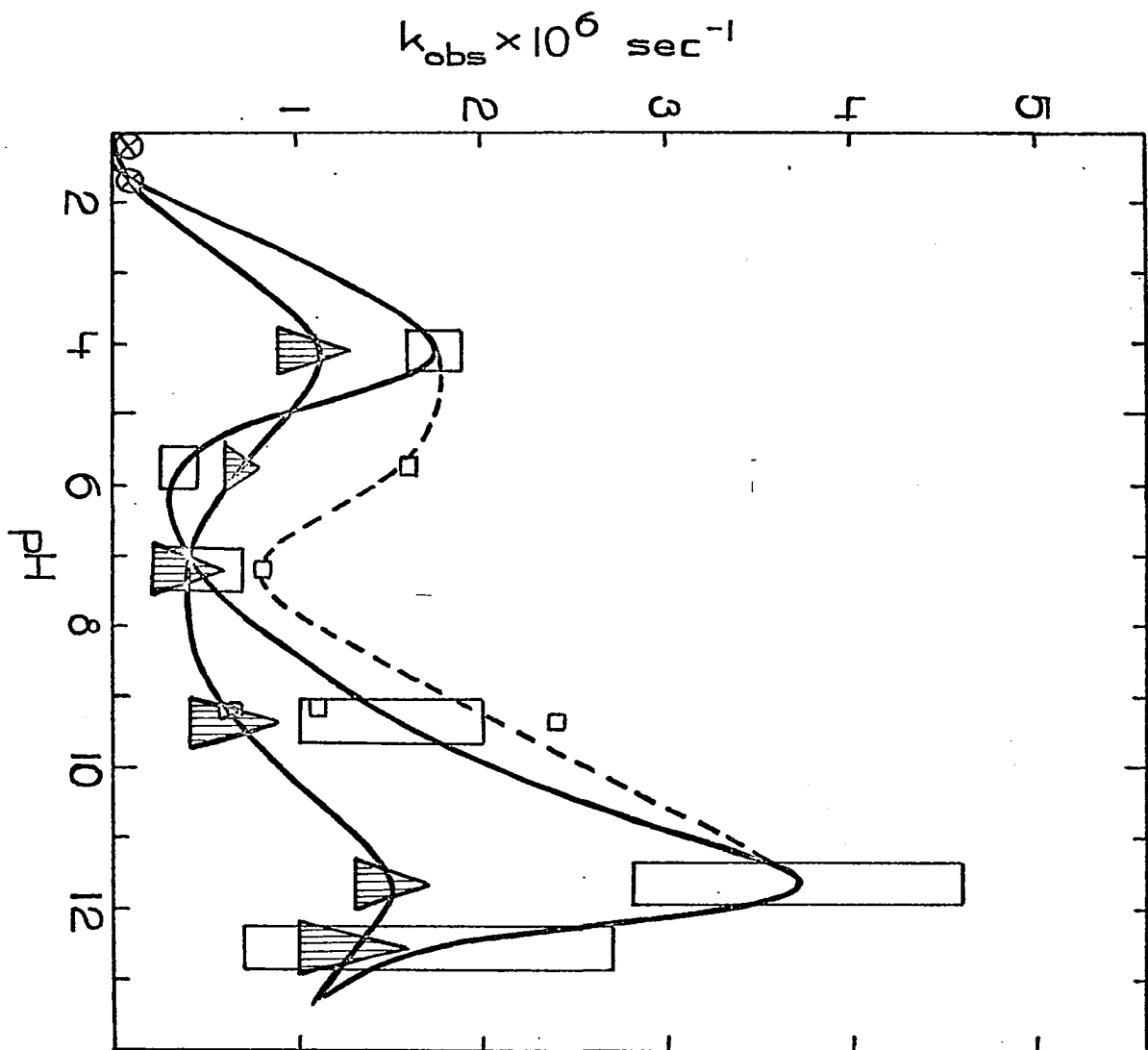
Guggenheim kinetic method used where $k_{\text{obs}} = -2.303$

$\log \Delta A / \Delta t$, equation (16), calculated from pairs of adjacent points to obtain average; sizes of symbols indicate relative certainty in k_{obs}

(average error) and in pH of solutions over time of measurements; Table 9 data

----- initial
 final pseudo first-order rates of re-

actions having deviations from linear Guggenheim plot: initial rates from measurements during at least first 24 hours and 24 + T hours of reaction, where T is $> t_{\frac{1}{2}}$ (See Experimental for sample plots obtained: Figure 10)



intermediate in semicarbazone formation from pyridoxal has been found to be rate-limiting below pH 4 (Cordes and Jencks, 1962a, b). However, at about pH 4 the dipolar ions of deoxypyridoxal and amino acid form a more stable imine, which also readily breaks down to transamination products, because the positively-charged pyridine ring nitrogen atom aids in withdrawal of electrons from the alpha-carbon atom of the amino acid residue.

As the pH increases toward neutrality the reaction rate decreases, probably as a result of dissociation of the pyridinium group ($pK_a 6$), and thus decreasing concentration of the reactive protonated imine, and formation of increasing concentration of the relatively unreactive imine having no positive charge on its pyridine ring nitrogen atom. The fractions of total, $(I)/(P + I)$, and protonated, $(IH^+)/(P + I)$, aldimine from leucine and deoxypyridoxal were calculated from the imine formation constants as functions of pH and from pK_{2P-L} of 6.1, where I is P-L. These values are presented in Figure 22 along with rate constants obtained for the formation of ketoisocaproate in the nonenzymic transamination, pH 4 to 10.

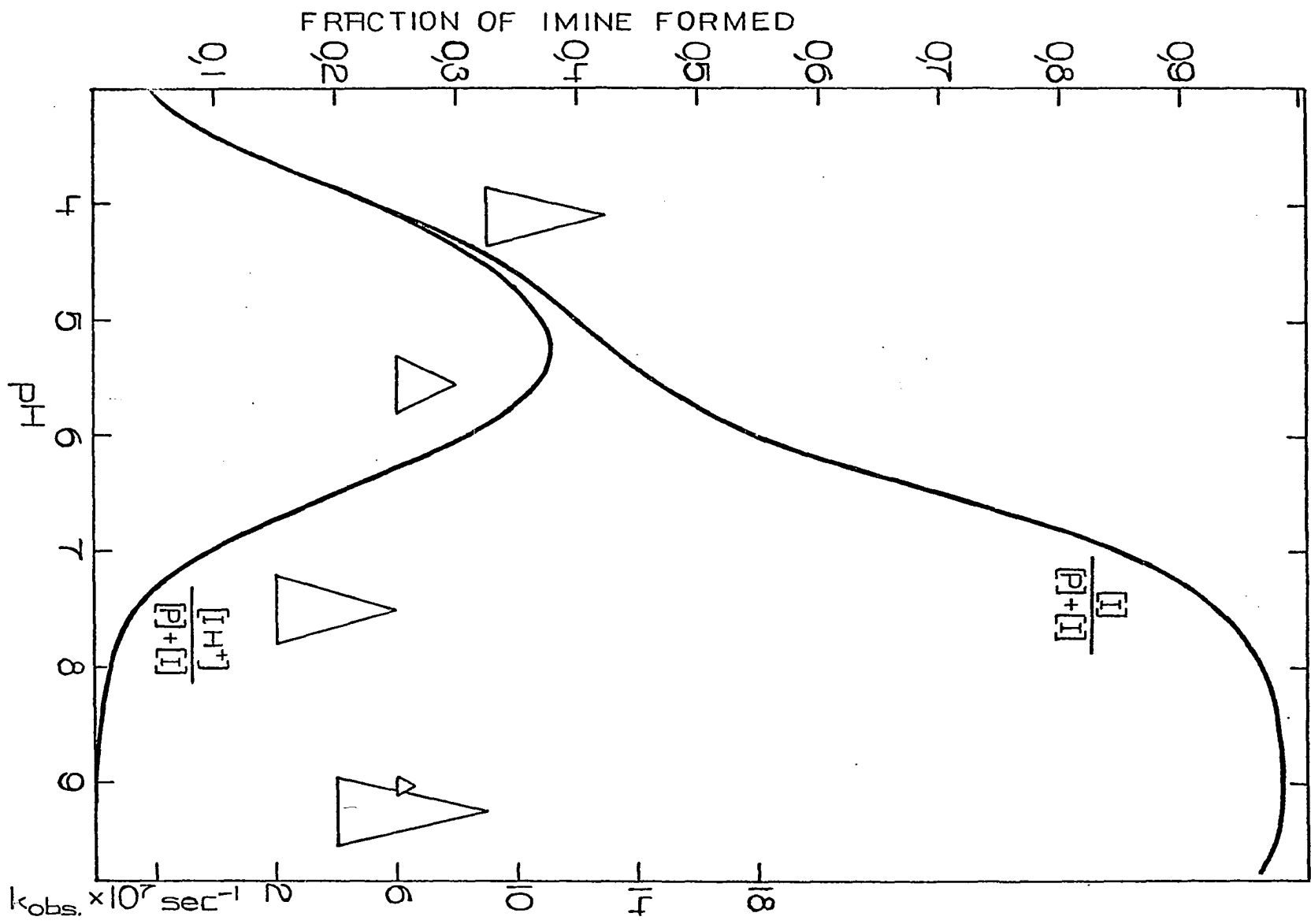
Keto acid may be formed primarily from the protonated aldimine below pH 7 and the rate optimum in the acid region may be explained on the basis of a direct proportionality relationship of the rate to the fraction of concentration of protonated aldimine: $I + H^+ \xrightleftharpoons{pK\ 6.1} IH^+$; observed rate = $k(IH^+)$. An alternative explanation for the rate of the acid pH region is that it may be proportional to the product of total imine

Figure 22. Fraction of deoxypyridoxal-leucine aldimine as a function of pH

Upper curve: Fraction of deoxypyridoxal as aldimine calculated from imine formation constants, by a method described earlier (Metzler, 1957), outlined in Figure 16, and from successively approximating the imine concentration in solutions of 0.01 M DPL and 0.05 M leu, at each pH

Lower curve: Fraction of protonated imine calculated from $pK_{2P-L} = 6.1$ (verified by a plot of a_{P-L} vs. pH)

△ Transamination rates: (Figure 21, Table 9)



concentration and hydrogen ion concentration at each pH: rate = $k(H^+)(I)$.

Above pH 7 the rate begins to increase, despite the decrease in the fraction of protonated imine below 10%. Although the fraction of total or unprotonated imine is greater than 90% from about pH 7.5 to above 10, the increase in rate in the alkaline pH range may be partly due to general base catalysis of the conversion of aldimine to ketimine by the excess amino acid anion, by another molecule of unprotonated aldimine acting intermolecularly or by hydroxide ion, in the extraction of the hydrogen from the alpha-carbon of the amino acid residue in the unprotonated imine. However, at very high pH, imine formation decreases probably as a result of dissociation of the imine hydrogen-bonded proton (pK_a 11). Hydrogen-bonding probably stabilizes the imine and loss of this stabilizing effect on imine formation probably begins to show up in the over-all transamination rate at least at pH 12.

The pH profile for transamination of leucine by deoxy-pyridoxal, may be compared with that obtained for transamination of alanine by pyridoxal (Blake et al., 1963). At 100°C, in the absence of added buffer the latter had a broad maximum near pH 5 and a much higher, sharper peak at pH 9.5 (pH's measured at 25°). Other pyridoxal model transamination reactions studied indicated that the rates increased from pH 7 to 10 in buffered systems with phenylglycine (Bruice and Topping, 1963b) and with alanine (Banks et al., 1961; Fleck and Alberty,

1962). Faster nonenzymic transamination rates were observed at acid pH values than in neutral or slightly alkaline solutions (Banks et al., 1961). Similar results were obtained from reactions of pyridoxal phosphate and amino acids catalyzed by metal ions (Cattaneo et al., 1960; Review of Pertinent Literature).

The optimum pH for metal ion-catalyzed transamination of glutamate with pyridoxal depended somewhat on the metal ion, according to Longenecker and Snell (1957). For the best metal ion catalysts, Cu (II), Al (III), Fe (II, III), the optimum pH was about 4.8, but for Zn (II) and others, it was near pH 7. An optimum of about pH 5 was found for unbuffered solutions. In general, metal ion-catalyzed transamination of amino acids with pyridoxal had a maximum rate near pH 4.5 in the range of pH 2 to 9 at 100°C and at about pH 5.2 at 25°C (Metzler and Snell, 1952b). Little or no transamination was found to occur at pH 9.6, where certain amino acids are oxidized optimally.

The question arose as to whether the pH 4 optimum was due to traces of metal ion in these unbuffered systems. Reaction solutions containing 1 mM EDTA (ethylenediaminetetraacetate), a metal ion-chelating agent, were compared with those lacking EDTA under the same conditions. No effect of inhibition of catalysis by metal ions was observed, within experimental error in determination of the rate constants (Table 11).

However, acetate buffer at a concentration of 1 M, may have complexed with any contaminating metal ions and thus no effect of EDTA could be observed. Indeed, in reaction solu-

Table 11. Observed pseudo first-order rate constants ($\times 10^5/\text{sec.}$) for nonenzymic transamination^a

	QH method		Direct spectra
	$A_{305} \text{ m}\mu$ (KIC)	$-A_{400} \text{ m}\mu$ (DPL)	$-A_{413} \text{ m}\mu$ (aldimine)
No EDTA	2.8	2.8	3.0
+1.0 mM EDTA	2.60 ± 0.3	3.1	3.3

^aInitial conditions: 0.10 M leu, 0.01 M DPL, 1.0 M acetate, pH 4.2, 25° (Although Guggenheim plots are not linear over the time period studied the slopes were taken as the best straight lines through most of the points).

tions which had the acetate buffer concentration decreased by a factor of 100, a small effect of EDTA was noted. Although quantitative rate constants were not obtained from these QH data, the relative rates found by taking the inverse ratio of times to attain the same amount of reaction, as measured by changes in absorbancies at 305 and 400 $\text{m}\mu$ in QH-treated aliquots, indicated that the reaction in the absence of EDTA was at least about 1.5 times faster than the reaction in its presence (but still only half as large as the effects observed by Metzler and Snell, 1952b). Also, the yellow color (due to aldimine) in the reaction solution containing no EDTA faded faster than that containing EDTA. However, it is not expected that this

small rate enhancement by contaminating metal ions in unbuffered systems would significantly shift or eliminate this acid pH optimum, especially since Cennamo (1964) found that the addition of metal ions does not enhance the transamination of amino acid esters by pyridoxal at 100° - a reaction which has an optimum at pH 5.3, measured at 25°.

Effect of leucine concentration

The rate of KIC formation at pH 7.1 to 7.2 increased by a factor of more than two (Table 12) when the leucine concentration was doubled (from 5/1 to a 10/1 excess over that of deoxypyridoxal). This may be evidence of general acid catalysis by leucine. The observed overall rate constants were changed to k'_o values by dividing by the appropriate leucine concentration, equation (19). For pseudo first-order conditions (excess leucine). $-d(P)/dt = k_{obs}(P) = k'_o(L)(P)$, where k_{obs} is the observed rate constant, and k_o is the pseudo first-order rate constant in an unbuffered system.

$$(19) \quad k'_o = k_{obs}/(L)$$

In 0.50 M acetate buffer solutions near the pH optimum, leucine concentration was varied between 0.01 and 0.1 M with constant deoxypyridoxal concentration at 1.0 mM. Pseudo first-order rate constants were determined from Guggenheim plots of logarithms of absorbancy changes (increasing at 325 mμ and decreasing at 415 and 400 mμ) vs. time. Ketoisocaproate and deoxypyridoxamine were identified as reaction products by the

Table 12. Effect of leucine concentration on pseudo first-order rate constants^a

QH method: + A ₃₀₅ mμ (KIC) -A ₄₀₀ mμ (DPL)			
k _{obs} X 10 ⁷ /sec:	2.65	7.9	("initial" rate in Guggenheim plot)
	7.2 ^b	2.35	("final" rate in Guggenheim plot)
k' _o X 10 ⁷ /sec:	53 ^b 72 ^b		

^aInitial conditions: 0.05 M Leu, 0.01 M DPL, pH 7.2, 25°

^bSame conditions except 0.10 M Leu

thin-layer chromatographic techniques. (These experiments were carried out by Louise Hodgins in this laboratory.) The linear relationship of the observed rate constants with increasing leucine concentration (Figure 23) may be partly due to increasing concentrations of reactive intermediate at this pH, where aldimine formation constants are not optimal (Figure 15).

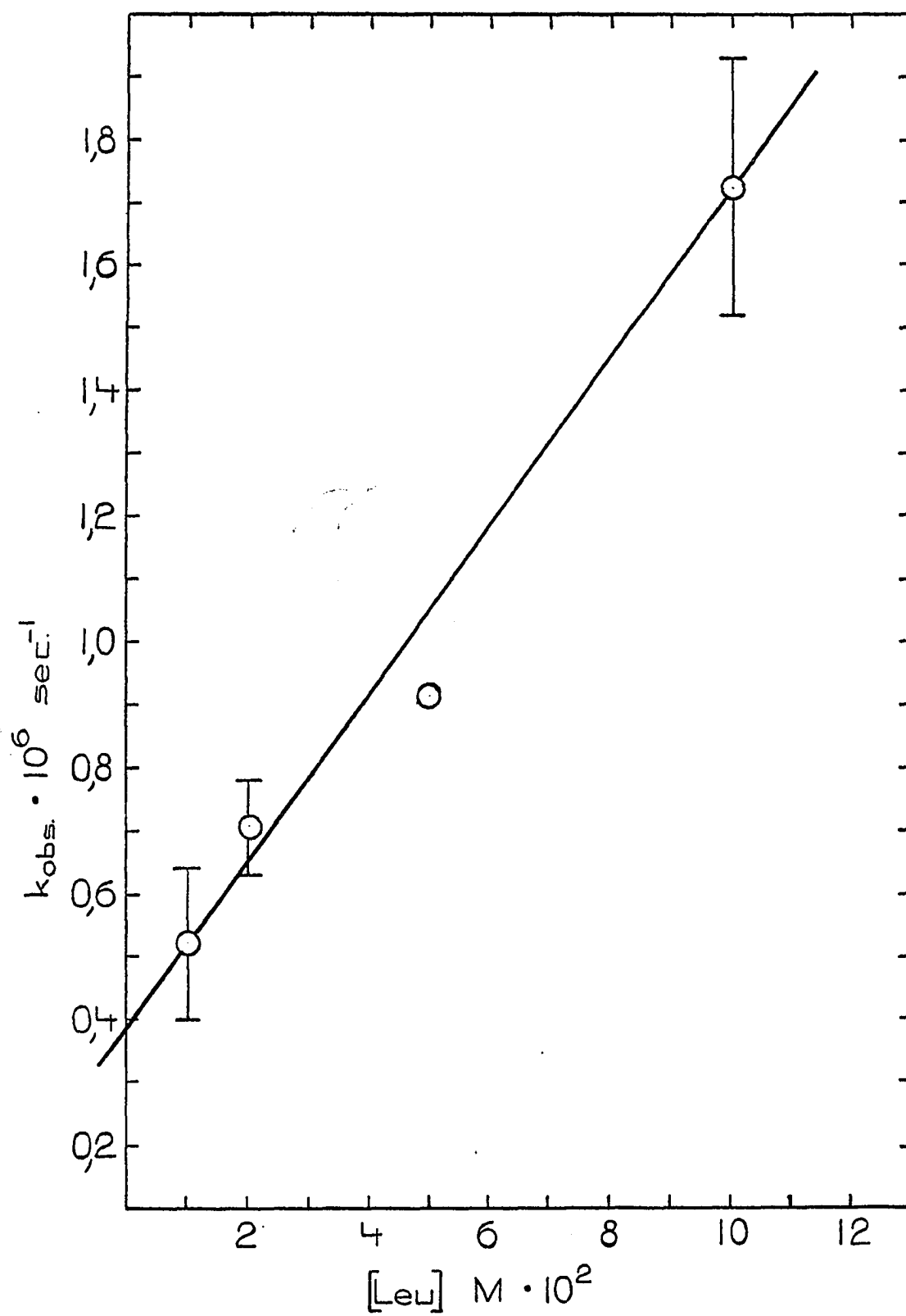
The fraction of deoxypyridoxal calculated as imine present under these conditions increases non-linearly from 0.44 at 0.01 M leucine to 0.88 at 0.10 M leucine. Such a curve would intersect, of course, with the origin (no leucine), and coincide with the first three points of Figure 23. Thus, this

Figure 23. Effect of leucine concentration on the pseudo first-order rate constant for the nonenzymic transamination of leucine with deoxypyridoxal^a

$k_{\text{obs}} \times 10^6 / \text{sec.}$ plotted vs. $(\text{leu}), \text{M} \times 10^2$, where k_{obs} values are averages of three determinations, with lines extending from points indicating average deviations, from Guggenheim plots of logarithms of absorbancy changes ($-A_{415}$, $-A_{400}$, $+A_{325} \text{ m}\mu$) vs. time.

^aInitial conditions: 1.0 mM DPL, 0.50 M acetate buffer

<u>(leu), M</u>	<u>pH</u>
0.01	4.3-4.4
0.02	4.2
0.05	4.1
0.10	4.05



part of the rate-leucine dependence curve (Figure 23) can be explained on the basis of increasing formation of imine, but at 0.1 M leucine the greater rate observed than would be expected from this relationship could have been acid-base catalysis by leucine dipolar ion.

From Figure 23 the parameters in equation (19a) may be determined.

$$(19a) \quad k_{obs}^{\prime} = k_o^{\prime} (L) + c$$

The intercept, c , in the plot of k_{obs}^{\prime} vs. (L) was about 0.4×10^{-6} /sec. when the linear plot was extropolated to $(L) = 0$. Thus, the observed rate constant is not directly proportional to very low leucine concentrations, as equation (19) would predict.

In comparing these results to those obtained by others, the following observations are pertinent. The rate of aldimine formation from the carbinolamine of pyridoxal and alanine was dependent on alanine concentration at each pH and ionic strength studied (Fleck and Alberty, 1962). An equation entirely analogous to (19a) fitted the observed rate constants. However, no proportionality between the reaction rate and alanine concentration was found for the rate-determining step in the transamination at pH 8 (ionic strength of 0.05 M in sodium acetate, 25°).

The same reaction was studied at 100°, and the observed rate constants were directly proportional to alanine concentration between 0.2 and 0.4 M in a 1 to 1 acetic acid-acetate

buffer (Blake et al., 1963). The linear plots all extrapolated through the origin (ala conc. = 0). Kinetics of the non-enzymic transamination reaction, in general, has been found to be first-order with respect to both pyridoxal and amino acid concentrations (Bruce and Topping, 1963d; Blake et al., 1963; Banks et al., 1961).

The dependence of the rate constant on alanine concentration was not an effect of changing activity coefficient (Fleck and Alberty, 1962). None of the parameters of the kinetic expression was dependent on ionic strength adjusted with sodium acetate, so that the total carboxylate concentration was constant. Therefore, the dependence of the rate constant on alanine concentration was due neither to changes in ionic environment of the reactants nor to a specific dependence on carboxylate ion or sodium ion concentrations. This thorough study of the effect of ionic strength on the rate of nonenzymic transamination by Fleck and Alberty (1962) indicated that rate constants of these reactions do not depend on ionic strength. This conclusion was independently reached by Banks et al., (1961). For these reasons and from the observations in preliminary experiments that simple inorganic salts have no effect on the rates, ionic strength or its adjustment to a constant level was considered irrelevant in the model transamination systems studied here.

Nonenzymic Transamination of Leucine with Pyridoxal Phosphate

Stoichiometry and kinetics

Loss in pyridoxal phosphate (PLP) was followed up to 24% decrease in PLP at the optimum pH by the ethanolicimine method and checked by the quinolyldiazotization (QH) method (although the keto acid data were inconsistent.) Although these reactions followed simple second order kinetics initially (to about 25% reaction), equilibrium conditions (correction for equilibrium concentration of PLP) would have to be taken into account if the reactions were followed for a longer period of time.

Since the reaction rate can be increased by increasing one of the reactants, the best kinetic studies were made under pseudo first-order conditions with respect to the pyridoxal analog. The limiting reactant concentrations for this condition were the solubility of leucine (0.16 M), and the minimum concentration of pyridoxal analog to effect a reaction of easily measurable rate (0.01 M for PLP). The reactant concentrations used (leucine to pyridoxal phosphate) were 5, 7.5 and 10 to 1. The rate of keto acid formation, as measured by the QH method and (checked once by the DNPH method at relatively high keto acid concentration), was close to first-order kinetics by the Guggenheim method, even for approaching equilibrium conditions.

The rate of decrease in PLP, on the other hand, was only close to first-order kinetics for probably up to 60% of the

PLP decrease. After that, as equilibrium was approached, side reactions involving the pyridoxal analog may become significant as previously suggested for deoxypyridoxal. Although the rate of PLP decrease was about twice as great as the rate of ketoisocaproate (KIC) formation at pH 4.1 these rates became practically identical at pH 6.8 indicating that any side reactions of PLP predominate at lower pH's in the acid range, at least.

Although the total concentrations of ketoisocaproate and pyridoxal phosphate analyzed decreased gradually in reaction solutions, a control solution containing only these two carbonyl components, was quite stable during a five-day period at room temperature. Very little loss of PLP or KIC occurred, as analyzed by the quinolyldiazotization method (Table 13). This method also gave a linear correspondence between $A_{400 \text{ m}\mu}$ (pyridoxal phosphate) and $A_{305 \text{ m}\mu}$ (keto acid) in a reaction solution from start of the reaction ($t = 0$) to equilibrium ($t = e$) (Figure 24).

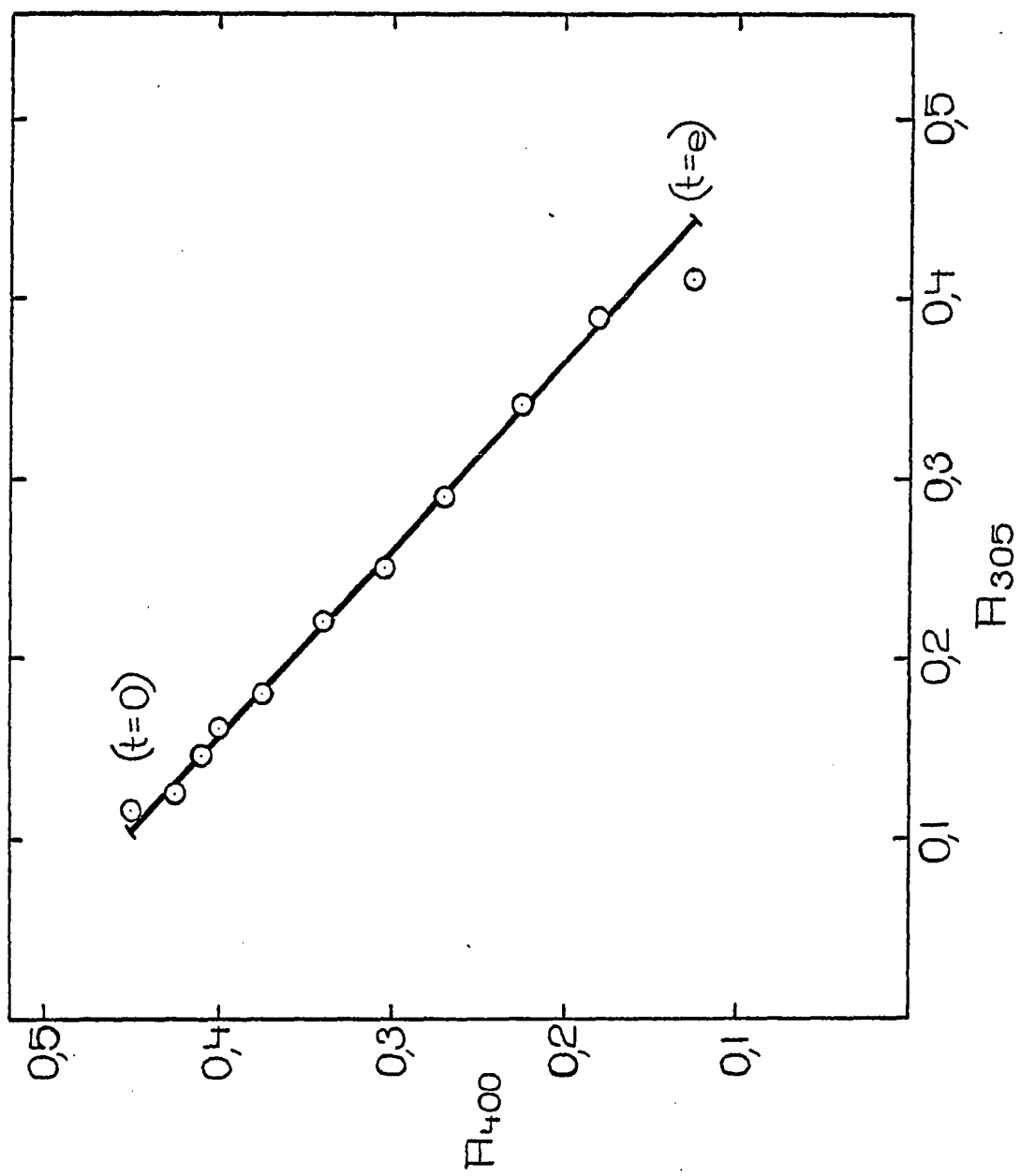
Table 13. Stability of pyridoxal phosphate (PLP) and ketoisocaproate (KIC) in a control solution at room temperature

Millimolar concentrations	At time 0	After 5 days
PLP	15.3	15.2
KIC	3.77	3.72

Figure 24. Linearity of absorbancy at 305 m μ (QH - KIC) and absorbancy at 400 m μ (QH - PL analog) during nonenzymic transamination of leucine with pyridoxal analog, as measured by the modified QH method, from start of the reaction ($t = 0$), buffered or unbuffered, to equilibrium ($t = e$)

Initial conditions:

75 mM leu
10 mM PLP
0 to 1.0 M acetate buffer, for several experiments
pH 4.6-5.1
25°



Rate of reaction as a function of pH

Apparent second-order rate constants were determined for unbuffered reaction solutions of leucine and pyridoxal phosphate (PLP) and plotted as a function of pH (Figure 25). The optimum reaction is at about pH 4, with the rate falling off much more sharply on the acid side than on the alkaline side. At pH 8.5 little reaction could be detected after seven days, although the analytical data were somewhat inconsistent.

Pseudo first-order rate constants for the transamination of leucine with PLP were found to decrease with increasing pH in the pH range of 3.9 to 6.8 in unbuffered solutions (Table 14). By trial and error the equilibrium constant was calculated to be 4.1 for the system at the lower pH values, and this indirectly-determined equilibrium position was taken into account in calculating the corresponding rate constants. The Guggenheim method was used for calculating the rate at pH 6.8, but cruder methods were used to estimate the rates at in-between pH values, as indicated in Table 14.

Intramolecular (Acid-Base) Catalysis of Nonenzymic Transamination of Leucine with Pyridoxal Phosphate

Pseudo first-order rate constants for the formation of ketoisocaproate in the nonenzymic transamination of leucine with pyridoxal phosphate (PLP), were compared with those for the corresponding reaction with deoxypyridoxal (DPL) in unbuffered solutions in the pH range of 4.1 to 6.8. The rate constants of reactions with PLP were 3 to 4 times greater than those with DPL. (Compare k_0 values, Figure 26).

Figure 25. Apparent second-order rate constants ($k_2 \times 10^5/\text{M}/\text{sec.}$) for non-enzymic transamination of leucine with α -pyridoxal phosphate as a function of pH

EOA analytical method for PLP decrease from initial concentrations, 0.02 M = (PLP) = (leu), unbuffered, 25°C

(Size of rectangles around some points indicate magnitude of average error; also value of k_2 estimated at pH 8.5)

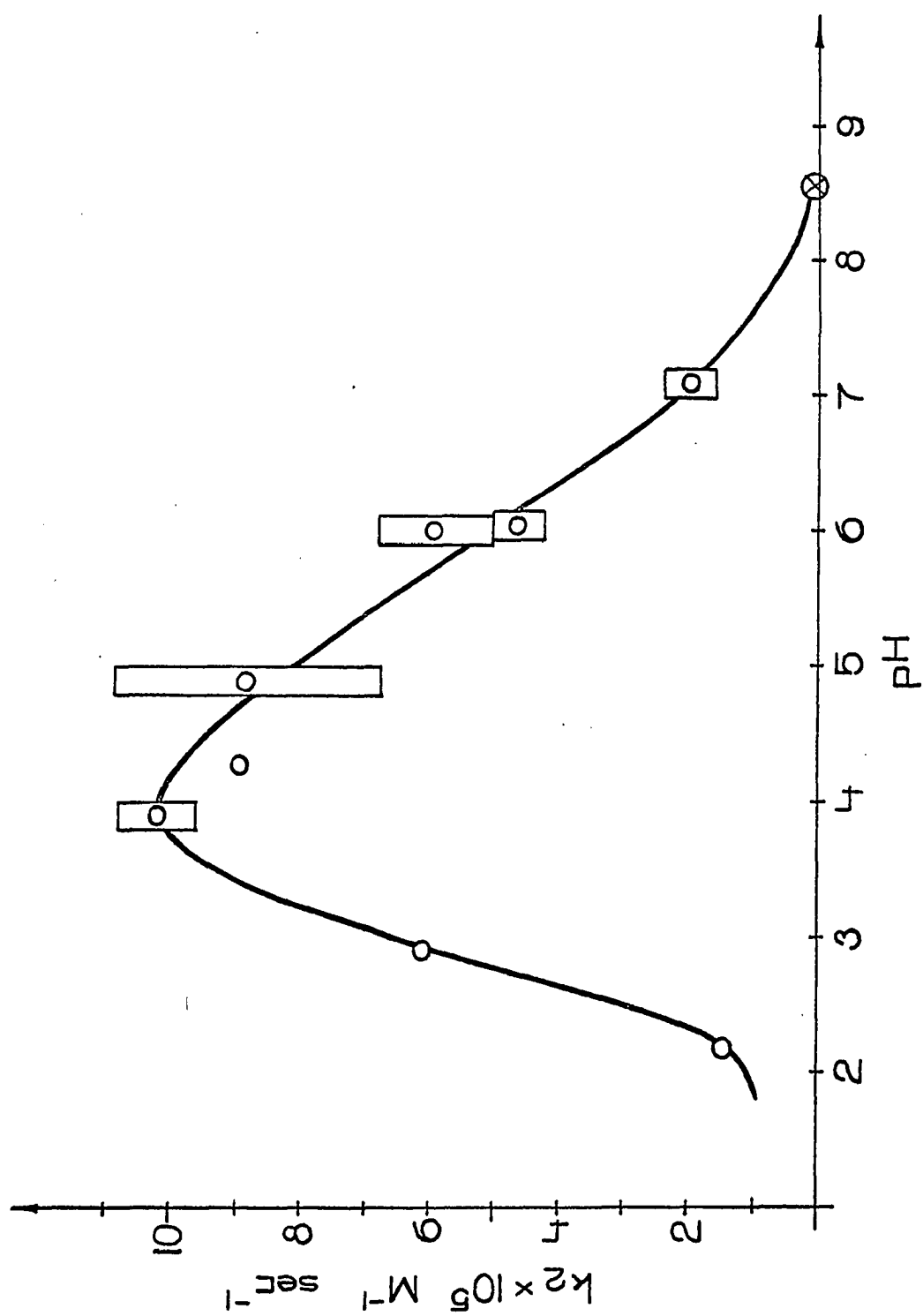


Table 14. Pseudo first-order rate constants for the non-enzymic transamination of leucine with pyridoxal phosphate in unbuffered solutions at 25°C

pH	Initial mM		Analytical method	Kinetic method	$k_{\text{obs}} \times 10^6/\text{sec.}$		
	(PLP)	(leu)			X:	KIC	-PLP
3.9	20	100	EOA QH	$\log(X_e/X_e - X_t)^a$	4.5	9.49 ± 0.27 -----	
4.1	10	75	EOA QH	" "	----- 3.40 3.94 5.47	6.30 6.98	
4.4	20	100	EOA	"	-----	5.8 ± 0.3 7.73 ± 0.24	
5.1	10	50	QH	$\log \frac{(A_e - A_0)}{(A_e - A_t)}$	2.92	3.94	
			EOA QH	" $(t_y/t_x)k_y$	----- 2.0	3.48 3.3	
				$dA/dt \times \frac{23.5 \text{ mM KIC}}{(A-A_0) 1.7}$			
6.2	10	50	QH	$(t_y/t_x)k_y$	1.25	2.45	
6.4	10	50	QH	" $\log X$	4.0 2.3-3.1	5.5 1.6- 2.8	
				$\log A_{405 \text{ m}\mu}$ $\log A_{330 \text{ m}\mu}$	----- 2.0	2.0 -----	
6.8	10	50	QH	$\log (A' - A)$	1.25	1.13	

^aUsing $K_e = 4.1 = (\text{PMP})(\text{KIC})/(\text{PLP})(\text{leu})$

Figure 26. Comparison of pseudo first-order rate constants^a for transamination of leucine^b (25°) as a function of pH by:

A. deoxypyridoxal, 0.01 M

○ k_o (unbuffered)

□ k_{obs} (estimated for 0.01 M phosphate buffer)

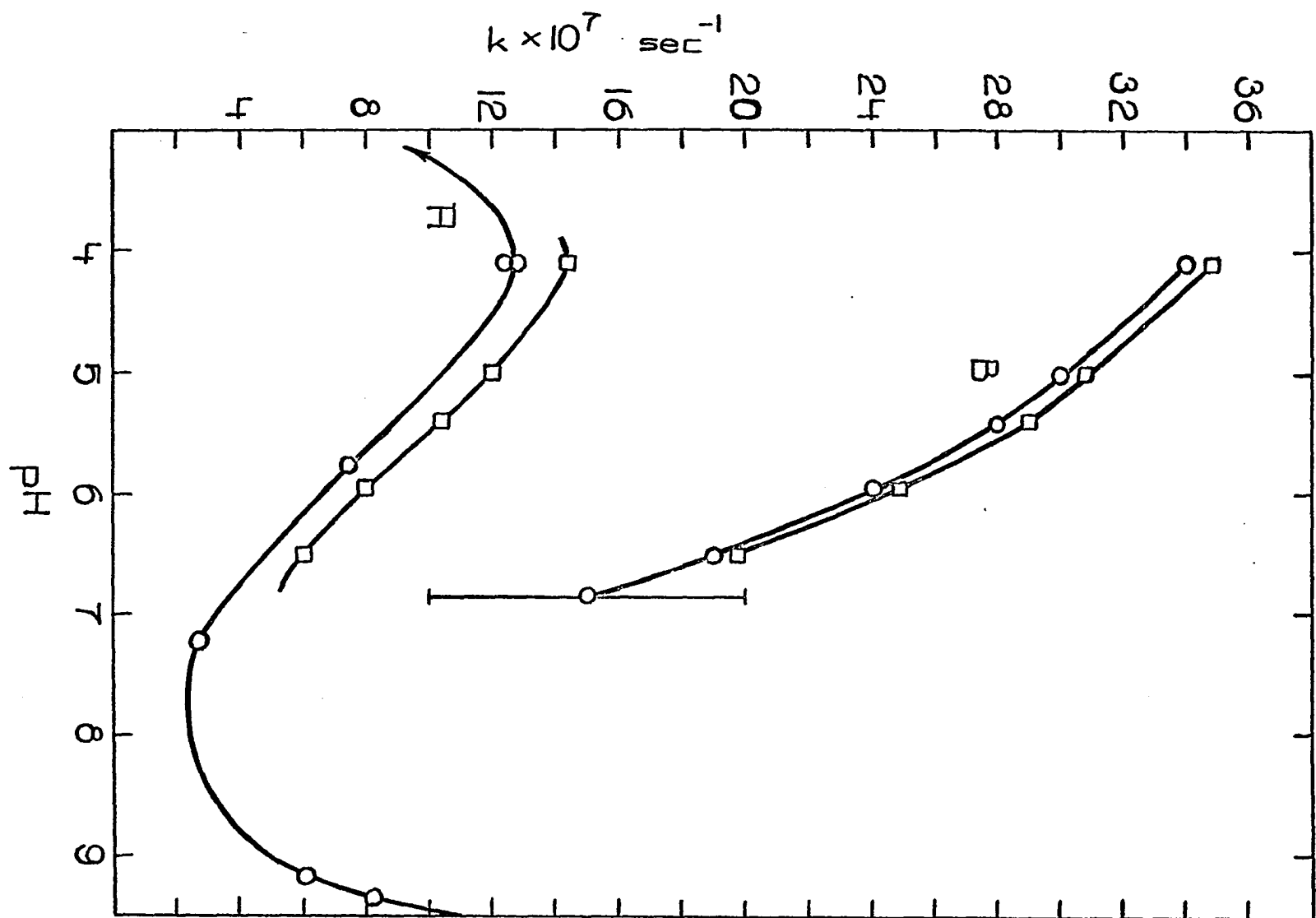
B. pyridoxal phosphate, 0.01 M

○ k_o (unbuffered)

□ k_{obs} (calculated for 0.01 M phosphate buffer from k_B values)

^a Based on KIC formation by QH method

^b 0.05 to 0.10 M leu; assumed k_{obs} and k_o independent of concentrations



However, since the imine formation constants, $\log K_{\text{pH}}$, for leucine and deoxypyridoxal were found to be greater than those for leucine and pyridoxal phosphate (Figure 15), the relative concentrations of imine, at each pH in the range studied, were taken into account in comparing these relative rates of nonenzymic transamination, using equation (20). From equation (18) and the preceding relationships, it can be shown that $-(d(P-L)/dt)_0 = k_i K_o (P)(L) = + (d(K)/dt)_0 = k_{\text{obs}} (P)(L)$, or $k_i K_o = k_{\text{obs}}$, or

$$(20) \quad k_i = k_{\text{obs}} / K_o.$$

Furthermore, in order to compare rigorously and quantitatively the inter- and intramolecular catalysis of phosphate, catalysis of nonenzymic transamination of leucine by pyridoxal phosphate in unbuffered solutions should be compared with that of deoxypyridoxal in the presence of an equimolar concentration of phosphate buffer. (In such a comparison, the concentrations of pyridoxal analog and total phosphate should be equal, as well as that of hydrogen ion, in both systems.) Such a comparison was made with the available data in the following manner. Assuming that the phosphate buffer catalytic constant for transamination of deoxypyridoxal with excess leucine is about twice that for the buffer catalytic constant for the pyridoxal phosphate reaction (on the basis of the comparison of the acetate buffer catalytic constants for the two reactions at pH 4.2, Table 20), and assuming that this ratio does not change significantly from pH 4 to 6.5, then

using equation (21) and points for curve A, Figure 26, the rates for the deoxypyridoxal reaction catalyzed by 0.01 M phosphate may be estimated for this pH range.

(21) $k_{\text{obs}} = k_o + k_B(B)$, where (B) = total buffer (phosphate) concentration, k_B is the buffer catalytic constant, k_o is the rate constant for unbuffered solutions.

The ratio of the observed rate constants, (k_o , PLP/ k_{obs} , DPL), for the unbuffered reaction catalyzed by 0.01 M pyridoxal phosphate to that for the reaction catalyzed by 0.01 M phosphate buffer and 0.01 M deoxypyridoxal (estimated as described above) was about $2\frac{1}{2}$ to $4\frac{1}{2}$, between pH 4.1 and 6.8 (Table 15). The gradual increase of this ratio with pH, except for a peak at pH 4.2, may have been a result of a change in the ratio of buffer catalytic constants for the two reactions with pH, i.e., the second assumption, above, probably was not good enough for an explanation of this pH effect with the available data.

From the ratios of calculated k_i values for PLP and DPL as a function of pH (Table 15) it can be seen that the leucine imine of PLP yields keto acid at rates between 5 to 11 times faster than that of DPL in the pH range 4 to 7. This ratio also increased with pH, except for an optimum again at pH 4.2. The phosphate group at the 5-position of pyridoxal indeed catalyzed the conversion of the leucine imine to ketoisocaproate and pyridoxamine phosphate. The explanation of the pH effect was sought in terms of the catalytic activity of the

Table 15. Comparison of inter- and intramolecular catalysis of nonenzymic transamination by phosphate^a

pH	4.1	4.2	5.0	5.4	6.0	6.5	6.8
$k_{obs} \times 10^7/\text{sec.}, \text{DPL}$	14.4	12.5	12	10.4	8.0	6.0	2.7
$k_o \times 10^7/\text{sec.}, \text{PLP}$	34	42	30	28	24	19	12
$k_o, \text{PLP}/k_{obs} \text{DPL}$	2.4	3.4	2.5	2.7	3.0	3.2	4.4
$K_o/M, \text{DPL}$	7.24	8.12	14.4	17.8	27.6	52.5	91.2
$K_o/M, \text{PLP}$	2.3	2.5	7.25	9.12	13.2	20.9	31.7
$K_o, \text{DPL}/K_o, \text{PLP}$	3.15	3.25	1.99	1.95	2.09	2.52	2.88
$k_i \times 10^7/\text{sec.}, \text{DPL}$	1.99	1.54	0.83	0.53	0.29	0.11	0.03
$k_i \times 10^7/\text{sec.}, \text{PLP}$	14.8	16.8	4.13	3.07	1.82	0.91	0.28
$k_i, \text{PLP}/k_i, \text{DPL}$	7.4	10.9	5.0	5.3	6.3	8.3	9.3

^a Nonenzymic transamination of leucine to ketoisocaproate, comparing deoxypyridoxal (DPL) and pyridoxal phosphate (PLP) as amino group acceptors in pseudo first-order conditions (excess leucine and 0.01 M PLP vs. excess leucine and 0.01 M DPL, catalyzed by 0.01 M phosphate; calculated rate constants from k_o values for DPL and k_B for phosphate from phosphate catalysis of reaction of PLP and leucine using equation (21a); assumed K_o and k_{obs} independent of concentrations

ionic forms of the phosphate group of PLP.

Calculation of specific phosphate catalytic constants for the phosphate groups of pyridoxal phosphate was made using

equation (21a), where k_{obs} is k_o for the formation of keto-isocaproate from leucine in nonenzymic transaminations with pyridoxal phosphate, k_o is that for the same reaction in the absence of the intramolecular phosphate catalysis, using deoxypyridoxal in unbuffered solutions as the amino group acceptor, (B) is the pyridoxal phosphate concentration of 0.01 M, and k_B' is the specific catalytic constant for the 5-phosphate ester group of pyridoxal phosphate.

$$(21a) \quad k_{\text{obs}} = k_o + k_B'(B)$$

The data of Table 16 were used with equations (22) and (23), along with the literature value for the third apparent dissociation constant for pyridoxal phosphate, assumed to be that of the second dissociation of the phosphoric acid ester group ($\text{pK}'_3 = 6.2 \pm 0.2$; Table 1, Review of Pertinent Literature), to calculate values for k_{HA} and k_A .

(22) $\text{pH} = \text{pK}'_3 + \log (A)/(HA)$, where (A) and (HA) are concentrations of the dibasic and monobasic forms of the phosphate ester group of pyridoxal phosphate

(23) $k_B(B) = k_{\text{HA}}(HA) + k_A(A)$, where k_{HA} and k_A are catalytic constants for the forms HA and A, respectively

The values calculated for k_{HA} and k_A were 2.11 and 1.15×10^{-4} M/sec., respectively. The monobasic form which has one dissociable proton and a net negative charge on the phosphate group thus had a catalytic constant of about twice that for the dibasic form which has no dissociable protons and two negative charges. It was concluded that the monobasic form of

Table 16. Phosphate catalytic constants (k_B') of pyridoxal phosphate in the nonenzymic B transamination with leucine^{a,b}

pH	4.1	5.0	5.4	6.0	6.5
$k_{obs} \times 10^7/\text{sec.}$ (k_o , for PLP)	34.0	30.0	28.0	23.6	19.0
$k_o \times 10^7/\text{sec.}^c$ (for DPL)	12.6	9.0	8.4	6.2	4.3
$k_B' \times 10^4/\text{M/sec.}$	2.14	2.10	1.96	1.74	1.47

^aReaction conditions in Table 15

^bCalculated from equation (21a)

^cData of Figure 26a, O

the phosphate group in pyridoxal phosphate is a better intramolecular acid-base catalyst in the nonenzymic transamination of leucine to ketoisocaproate than the dibasic form. Furthermore, intermolecular catalysis by a second molecule of PLP and the inductive effect of the phosphate group may be ruled out. Another possible explanation for this effect of the neighboring phosphate ester group is that of lowering the activation energy by stabilization of electronic charges favorable for the transition state.

General Acid-Base Catalysis of Nonenzymic Transaminations of Leucine with Pyridoxal Analogs

Although the intramolecular catalysis of nonenzymic transamination of leucine by phosphate (in PLP) was much more efficient than the intermolecular catalysis of phosphate (in equimolar concentration with deoxypyridoxal), the intermolecular catalysis was increased to an even greater extent by increasing the concentration of phosphate in a model system.

Intermolecular catalysis compared to intramolecular catalysis by phosphate

Catalysis by phosphate buffer seemed to shift the pH optimum from about pH 4 up to about 5.5 (Figure 27). Buffer catalytic constants were calculated from the limited data available at several pH values, near the acidic optimum for the reaction, from equation (21). The phosphate buffer (external or intermolecular) catalytic constants were three to four times greater than the k_o constants for pyridoxal phosphate. At least at pH 5.95, k_B was truly a constant, and the simple forms of equations (21) and (23) were verified because no cross terms, such as $K_{HA} k_A (HA)(A)$ could be justified.

Calculations of catalytic constants for the specific buffer components present in this pH range, $H_2PO_4^-$ and HPO_4^{2-} , were made from the limited data of Table 17, using equations (22) and (23), where pK is pK_2' of phosphoric acid. The calculated constants, k_{HA} and k_A , indicated that most of the

Figure-27. General acid-base catalysis by phosphate buffer of the nonenzymic transamination of leucine with pyridoxal phosphate as a function of pH^a

Pseudo first-order rate constants, $k_1 \times 10^6/\text{sec.}$, vs. pH

1.0 M phosphate

- k_1 from PLP decrease by EOA method
- k_1 from PLP decrease by QH method
- △ k_1 from KIC formation by QH method

0.50 M phosphate

- k_1 from PLP decrease by QH method
- ▲ k_1 from KIC formation by QH method

1.0 M imidazole

- ▲ k_1 from KIC formation by QH method

(Lines extending from points indicate extent of experimental error)

^aInitial conditions: 0.05 M leu, 0.01 M PLP, 25°C

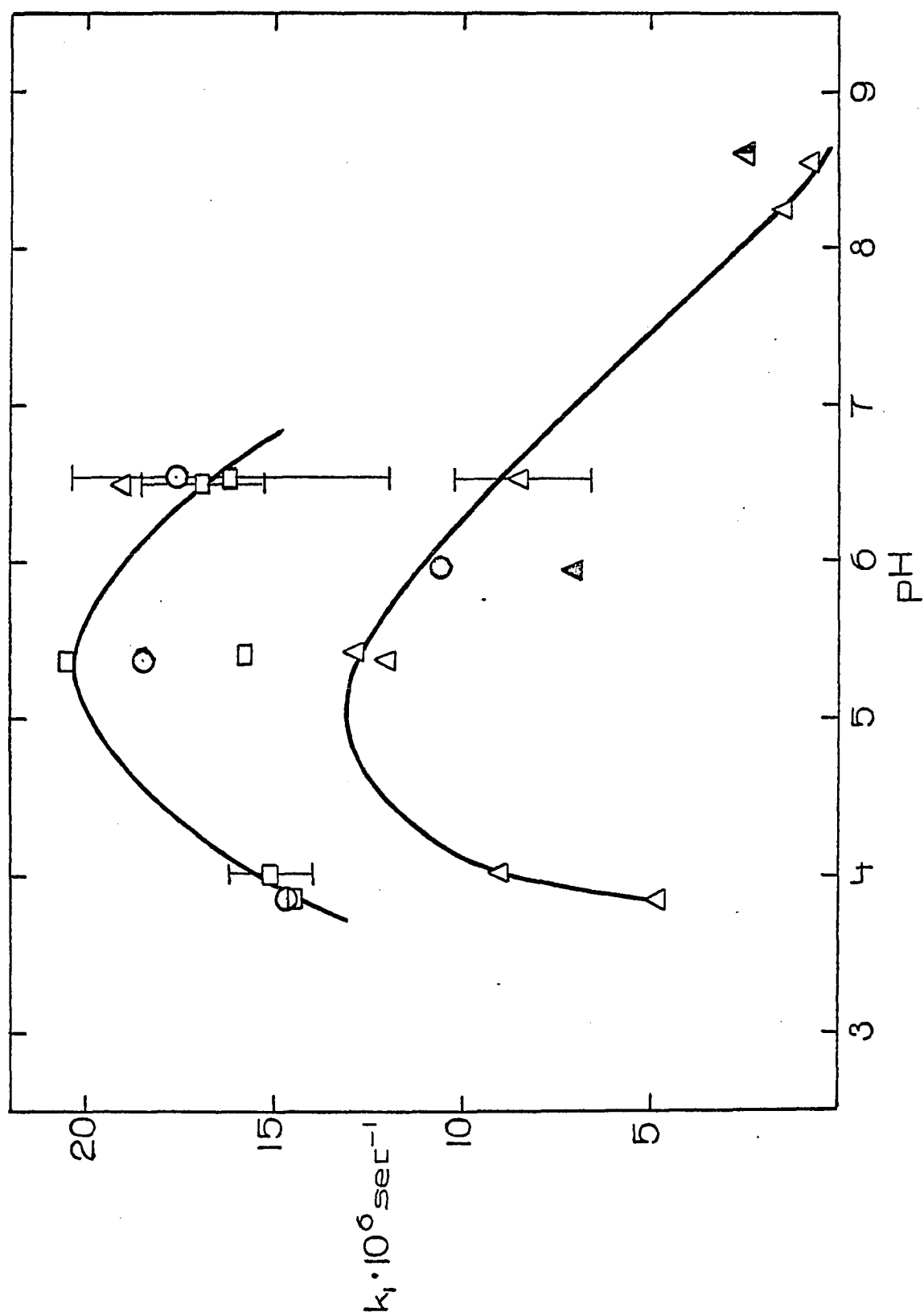


Table 17. Phosphate buffer catalytic constants^a in the non-enzymic transamination of leucine with pyridoxal phosphate^b

pH	(B)	KIC formation			Decrease in PLP		
		k _o	k _{obs}	k _B	k _o	k _{obs}	k _B
5.95	0.0	2.4	2.4	0	2.6	2.6	0
	0.50	2.4	7.0	9.2	2.6	10.6	16.0
	1.00	2.4	11.6	9.2	2.6	18.8	16.2
5.4	1.00	2.8	12.5	9.7			
5.0	1.00	3.0	12.0	9.0			
6.5	1.00	1.9	8.5	6.6			

^aPseudo first-order rate constants ($\times 10^6/\text{sec.}$ for k_o and k_{obs} and k_{obs} ; $\times 10^6/\text{M/sec.}$ for k_B)

^bInitial concentrations: 0.05 M leu, 0.01 M PLP, 25°

buffer catalysis (k_B) is due to the acid component, H_2PO_4^- , in this pH range, and only a very small portion of k_B can be attributed to k_A from HPO_4^{2-} . This contrasts with the finding that the catalytic constant for the monobasic form of the phosphate group in PLP was about twice as great as that for the dibasic form. The catalytic constants for the phosphate group of PLP (1.4 to $2.1 \times 10^{-4}/\text{M/sec.}$) were 20 to 23 times greater than those for phosphate buffer (6.6 to $9.7 \times 10^{-6}/\text{M/}$

sec.) in this pH range. Also, the catalytic constant for the monobasic form of the phosphate group of PLP (2.1×10^{-4} /M/sec.) was about 23 times that of the H_2PO_4^- species (9×10^{-6} /M/sec.) of phosphate buffer, which was the intermolecular catalyst in this pH range.

The apparent second-order rate constant for the decrease in PLP, from 0.02 M, equimolar, PLP and leu, was enhanced by a factor of 6 at pH 5.9 in 0.85 M phosphate buffer (5.5×10^{-5} M/sec. for an unbuffered solution to 33×10^{-5} /M/sec. in 0.85 M phosphate). The catalytic constant was calculated to be 32×10^{-5} /M/sec., using equation (21) under these conditions. Qualitatively the second-order rate constant was decreased at lower buffer concentrations (0.5 and 0.1 M).

It is instructive to ask what concentration of phosphate buffer is required to catalyze the formation of ketoisocaproate from leucine in excess of 0.01 M DPL in order that the rate of this reaction will be the same as that for an unbuffered solution of PLP and leucine under the same conditions. This prediction was made using the available data and equating k_{obs} values of equations (21) and (21a): $k_o + k_B(B) = k_o + k_B'(0.01 \text{ M PLP})$, or $(B) = k_B'/k_B(0.01 \text{ M})$, and since k_B'/k_B is 23, $(B) = 0.23 \text{ M}$, the concentration of phosphate buffer equivalent in catalytic activity to the intramolecular catalytic effect of the phosphate group of 0.01 M PLP, in the pH range of 4 to 6.5. Yet to be taken into account is the efficiency of general acid-base catalysis

by phosphate buffer on the basis of its frequency of collision with intermediate aldimine under these conditions.

Intermolecular catalysis by acetate buffer

The catalytic constant for acetate buffer was about twice the k_0 values for the unbuffered systems. Pseudo first-order rate constants for these reactions were calculated by various kinetic methods from different analytical data and compared in Tables 14 and 18. These values were generally found to decrease with increasing pH in the range of pH 3.9 and 6.8. Above pH 6 acetate had little effect on the rate of the unbuffered reaction. At least at pH 3.9 and 4.6 the rate was proportional to the acetate buffer concentration. The reactions near the pH optimum could be followed practically to equilibrium in two days when the buffer concentration was at least 0.5 M. The equilibrium constant, K_e , was graphically found by trial and error (the best straight line for a first-order plot corrected for equilibrium concentrations) to be 4.1, and this value was used in finding equilibrium concentrations for kinetic methods which required them (Tables 14 and 18).

From the rates determined from the ketoisocaproate formed at pH 3.9 and 4.6 (Table 18), specific catalytic constants for the buffer components were calculated using equations (21), (22), and (23). The constant for acetic acid, $11.2 \times 10^{-6}/M/\text{sec.}$, is twice that for acetate, $5.6 \times 10^{-6}/M/\text{sec.}$, in this

Table 18. Pseudo first-order rate constants for the nonenzymic transamination of leucine with pyridoxal phosphate in unbuffered solutions at 25°C

pH	(B),M	Initial mM		Analytical method	Kinetic method	$k_{\text{obs}} \times 10^6/\text{sec.}$	
		(PLP)	(leu)			X: +KIC	-PLP
3.9	0.5	10	75	QH	$\log\left(\frac{X_e}{(X_e - X_t)^a}\right)$	9.5	----
	1.0	10	75	QH	"	13.8	----
					$\log(A' - A)$	17.1	----
4.6	0.5	10	50	QH	"	13.5	----
					$\log(A_e - A_o)$	9.2	----
					$\left(\frac{A_e - A_t}{A_e - A_o}\right)$	8.7	----
					"	----	14.05
					"	----	12.85
		20	100	QH	$\frac{dA/dt}{.23.5 \text{ mM} \times A - A_o}$		
		10	50	EOA			
				QH			
	1.0	10	50	QH	"	13.1	----
					$\log(A' - A)$	10.8	----
					$\log\left(\frac{X_e}{(X_e - X_t)^a}\right)$	12.2	----
					$\log\left(\frac{A_e - A_o}{(A_e - A_t)}\right)$	11.6	----
					"	12.7	----
6.8	1.0	10	50	QH	"	----	22.2
					"	----	20.4
6.8	1.0	10	50	QH	$(t_y/t_x)k_y$	1.2	0.94

^aUsing $K_e = 4.1 = (KIC)(PMP)/(PLP)(leu)$

pH range, which was evidence for general acid catalysis.

Cross-terms, such as $k_{HA} \cdot k_A (HOAc)(\bar{O}Ac)$, in the expression for the observed rate constant were neglected, since k_{obs} was accounted for by the sum of k_o , $k_{HA} (HOAc)$, and $k_A (\bar{O}Ac)$.

For the nonenzymic transamination of alanine and pyridoxal studied at 100° by Blake et al. (1963), the ratio of the catalytic constant for acetic acid to that in the absence of acetate buffer (k_{HOAc}/k_o) was about 7. The catalytic constant, k_{HOAc} , was calculated to be 0.011/M/sec. under these conditions. These values at 100° were quite a bit greater than those obtained for the transamination of leucine at 25°.

The rate-determining tautomerization of imines was not only dependent upon pH and independent of ionic strength, but was subject to general acid catalysis by several buffers (Banks et al., 1961; Blake et al., 1963; Vernon, 1964).

First-order velocity constants were proportional to acetic acid concentration which indicated general acid catalysis (Blake et al., 1963). The excess alanine used is also an acid, and this requirement of at least 0.2 M alanine for their analytical method prevented detailed experiments at lower alanine concentrations and at constant pH in order to clarify the dependence of the rate on the nature and concentration of the buffer.

In the reaction between pyridoxamine and alanine Banks et al. (1961) observed catalysis by N,N-dimethylethanolamine and by N,N-dimethylglycine. The equilibrium constant for

ketimine formation was unaffected but the rate constant for imine tautomerization was affected by both the nature and concentration of the buffer. Buffer catalytic constants were calculated from an equation entirely analogous to equation (21). The highest rate constant for the non-metal ion-mediated reaction between pyridoxamine and pyruvate was about $2.6 \times 10^{-5}/\text{sec.}$ (pH 10, 25°C , 0.1 to 0.2 M N,N-dimethylglycine buffer) (Banks et al., 1961), and this value was only about ten times lower than that for the reverse reaction (between pyridoxal and alanine) at 100°C , pH 4, acetate buffer ($2.85 \times 10^{-4}/\text{sec.}$) (Blake et al., 1963), but was ten times higher than a rate constant reported for the latter reaction at 25°C , pH 8.0, 0.05 M sodium acetate ($2.6 \times 10^{-6}/\text{sec.}$) (Fleck and Alberty, 1962).

The stoichiometry of the nonenzymic transamination of leucine with pyridoxal phosphate, catalyzed by acetate buffer, was carefully studied. Millimolar (mM) concentrations of pyridoxal phosphate (PLP) and ketoisocaproate (KIC) were measured at various times in solutions near the pH 4 optimum and are compared in Table 19. The total concentration of PLP and KIC seemed to decrease during the reaction, although a solution of just PLP and KIC was stable over a 5-day period (Table 13). The values in Table 19 are average from several experiments and average deviations are indicated. Analyses of PLP by the QH and EOA methods are compared in Table 19 and Figure 28, and were in general agreement. The PLP measured by the QH method was higher than by the EOA method early in the

Table 19. Stoichiometry of nonenzymic transamination of 75 mM leucine with 10 mM pyridoxal phosphate (average values from several experiments)

<u>pH 3.9, 1.0 M acetate buffer</u>					
t hrs.	<u>by EOA</u>	<u>by QH</u>	mM KIC	<u>Total mM PLP & KIC</u>	
	mM PLP	mM PLP		PLP by EOA	PLP by QH
0	8.73 +0.03	10.05 +0.15	0.02 +0.06	8.75 +0.06	10.07 +0.15
6	5.44 +0.11	5.70 +0.14	3.26 +0.36	8.70 +0.36	8.96 +0.36
18.5	2.97 +0.01	2.65 +0.02	5.63 +0.10	8.60 +0.10	8.28 +0.10
24	2.28 +0.01	1.91 +0.02	6.57 +0.03	8.85 +0.03	8.48 +0.03
25	2.32 +0.07	2.02 +0.02	6.63 +0.03	8.95 +0.07	8.65 +0.03
44	1.76 ----	0.65 +0.13	7.31 +0.35	9.07 +0.35	7.96 +0.35
48	0.85 +0.10	0.60 +0.01	7.82 +0.02	8.67 +0.10	8.42 +0.02
156	0.59 ----	0.0 ----	8.25 +0.09	8.84 +0.09	8.25 +0.09
<u>pH 4.1, unbuffered</u>					
0	9.94 +0.04	10.25 +0.03	0.0 ----	9.94 +0.04	10.25 +0.03
6	9.04 +0.04	9.14 +0.04	1.40 +0.01	10.44 +0.04	10.54 +0.04
24	7.03 +0.01	6.77 +0.10	2.15 +0.05	9.18 +0.05	8.92 +0.10
48	5.27	4.75	3.49	10.02	8.24
67.5	----	3.07	4.17	----	7.24

Figure 28. Reaction of pyridoxal phosphate with leucine^a

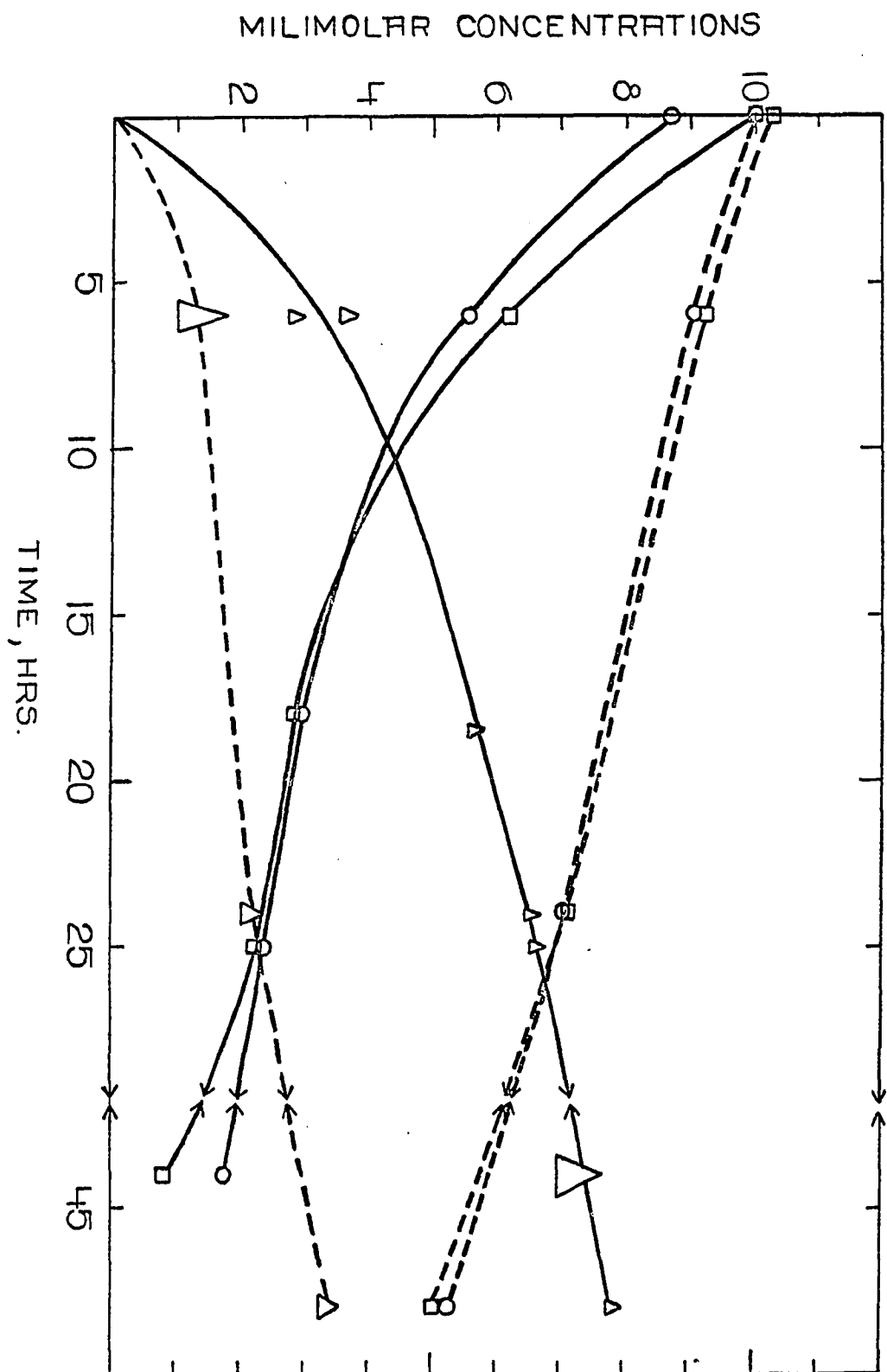
Millimolar concentrations vs. time, hrs.

- △ KIC by QH method
- PLP by QH method
- PLP by EOA method

^a Initial conditions: 75 mM leu, 10 mM PLP, 25°

_____ pH 3.88, 1.0 M acetate buffer

----- pH 4.08, unbuffered



reactions (0 to 40% decrease in PLP, and was lower than by the EOA method in later stages of the reaction (after 40% decrease in PLP). The decrease in PLP generally exceeded the formation of KIC.

Rates based on decrease in PLP generally exceeded corresponding rates for KIC formation (Table 18). Possible side reactions leading to loss of PLP without the corresponding formation of KIC have been previously discussed under stoichiometric and kinetic anomalies in the reaction of leucine with deoxypyridoxal.

Rates of leucine transamination by deoxypyridoxal and pyridoxal phosphate were compared in buffered and unbuffered systems at two pH values, (Table 20). Pseudo first-order rate constants for ketoisocaproate formation determined by the QH method indicated that the phosphate group of pyridoxal phosphate probably enhanced the leucine transamination at pH 4.2 (in unbuffered systems) about three times faster than did deoxypyridoxal. However, in 1.0 M acetate buffer at the same pH, the transamination of leucine with deoxypyridoxal was about two times faster than that with pyridoxal phosphate. In comparing calculated k_o and k_B values, the rate constant for pyridoxal phosphate was about two times that for deoxypyridoxal in unbuffered systems, whereas the acetate buffer catalytic constant for deoxypyridoxal was about three times that for pyridoxal phosphate.

At pH 6.8 pyridoxal phosphate is an even better amino

Table 20. Comparison of rate constants for transamination of leucine with deoxypyridoxal and with pyridoxal phosphate

	pH 4.2			pH 6.8	
	unbuffered 1.0M acetate			unbuffered 1.0M imidazole	
	$k_{\text{obs}} \times \frac{10^6}{\text{sec.}}$	$k_B \times \frac{10^6}{\text{sec.}}$	$k_B \times \frac{10^6}{\text{M}^a}$	$k_{\text{obs}} \times \frac{10^6}{\text{sec.}}$	$k_{\text{obs}} \times \frac{10^4}{\text{M}^b}$
DPL	1.25 ^c	28.0 ^d	24.6 ^d	0.27 ^c	17.5
	(k _o , calc. = 3.4)				
PLP	4.2 ^e	12.8 ^e	8.6 ^e	1.2 ^e	5.4
Rate constant ratios					
DPL/PLP	----	2.2	2.9	---	3.1
PLP/DPL	3.4	---	---	4.4	---

^aPseudo first-order rate constants from Guggenheim plots of KIC formation (by QH method)
0.01 M initial concentration of pyridoxal analog

^bSecond-order rate constants based on loss of pyridoxal analog due to transamination (by EOA method)
0.02 M initial concentration of pyridoxal analog
0.03 M initial concentration of leucine

^c0.05 M initial concentration of leucine

^d0.10 M initial concentration of leucine

^e0.075 M initial concentration of leucine

group acceptor than deoxypyridoxal in unbuffered systems. The observed rate constant for the coenzyme was almost $4\frac{1}{2}$ times greater than that for deoxypyridoxal under these conditions. However, its buffer catalytic constant was again much less than that for deoxypyridoxal at this pH. In comparing the observed second-order rate constants for the loss of pyridoxal analog due to transamination in 1.0 M imidazole buffer, the constant for deoxypyridoxal was about three times that for pyridoxal phosphate.

Intermolecular catalysis by imidazole buffer

A value for the rate catalyzed by 1.0 M imidazole buffer at pH 8.6 was shown in Figure 27 and was about $2\frac{1}{2}$ times greater than the corresponding observed rate catalyzed by phosphate buffer. However, under similar reaction conditions, but with deoxypyridoxal instead of pyridoxal phosphate as the amino group acceptor, phosphate was found by Bresnahan to be about 300 times more effective as a catalyst ($1.4 \times 10^{-3}/\text{sec.}$) than imidazole ($6 \times 10^{-6}/\text{sec.}$) at 1.0 M concentrations, but only 1/3 as effective (1.45 vs. $4.5 \times 10^{-4}/\text{sec.}$) at 0.1 M concentrations of buffer. The rate seemed to be proportional to phosphate concentration but mysteriously decreased with increasing imidazole concentration (Sheryl Bresnahan, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Non-enzymatic transamination reactions of leucine and alanine methyl ester with

pyridoxal and its analogs. Private communication. 1964).

Conditions of pH for the maximum catalytic effect of imidazole were sought. Although Bruice and Topping (1963b) quantitatively measured an increasing rate from pH 7 to 10 in a related nonenzymic transamination system (pyridoxal, phenylglycine, and imidazole), neutral pH seemed to be best for the imidazole-catalyzed reaction of leucine with pyridoxal, followed by the ethanolic imine method. Compared to the rate at pH 8.5, where Bruice and Topping made most of their measurements, the rate at pH 6.8 was 4 times greater for the decrease of pyridoxal, and only $\frac{1}{2}$ as much pyridoxal was lost in the same time intervals in the presence of imidazole and absence of leucine in control solutions. However, the amounts of ketoisocaproate formed were about the same at these pH values. At pH 8.5 there was no increase in the rate when leucine concentration was increased, which would be expected if pyridoxal is completely converted to imine with leucine at this pH. There was also relatively more pyridoxal lost in control solutions than there was in reaction solutions of pH less than 6. It was concluded that the imidazole-catalyzed reaction was too complex at pH less than 6.

Sheryl Bresnahan in this laboratory also found that the rate of the reaction of 0.03 M leucine with 10^{-4} M deoxypyridoxal catalyzed by 1.0 M imidazole was greater at pH 6.6 than at pH 8.6 (pseudo first-order rate constants, calculated by the Guggenheim method: $6.18 \pm 0.87 \times 10^{-5}$ /sec. at pH 6.62 from in-

creasing absorbancy at 325 m μ and decreasing absorbancy at 390 m μ ; $0.57 \pm 0.02 \times 10^{-5}$ /sec. at pH 8.6, from increasing absorbancy at 315 m μ and decreasing absorbancies at 387 and 273 m μ ; 1.37×10^{-5} /sec. at pH 10.0, from decreasing absorbancy at 430 m μ). These findings that a pH optimum for the general acid-base catalysis by imidazole is closer to neutral pH than in the alkaline range, as Bruice and Topping suggested, seem reasonable in the light of the pK value for imidazole being 6.95 at 25°C. Above pH 7, the nonenzymic transamination reaction increased in rate for the unbuffered system of deoxypyridoxal and leucine, and perhaps, Bruice and Topping observed the same effect in the alkaline range with imidazole.

The decreases in deoxypyridoxal and pyridoxal phosphate in systems containing leucine and imidazole or phosphate buffer, for approximately equimolar reactants, followed second-order kinetics. The reactions were followed to 30-45% loss of pyridoxal analog, as measured by its ethanolicimine, to at least 50% approach to equilibrium.

Pyridoxal analogs containing the 5-hydroxymethyl group predominate in aqueous solution as internal hemiacetals instead of as free aldehydes, as observed from spectra (Figure 2; Table 1). Spectra of deoxypyridoxal are evidence that this analog exists in aqueous solution, mainly as a free or hydrated aldehyde, which forms are much more reactive toward amino acids than the internal hemiacetal form of pyridoxal. Transamination between pyridoxal phosphate and glutamate pro-

ceeded closer to completion than that of pyridoxal and glutamate, because of the cyclic hemiacetal form of pyridoxal (Metzler and Snell, 1952b). It was desirable to compare the reactivities of pyridoxal, deoxypyridoxal and pyridoxal phosphate and thus measure the effect of hemiacetal formation in the present nonenzymic transamination systems.

The basic character of the pyridine nitrogen and its role in the catalysis was also considered. Electron withdrawal from the alpha-carbon atom of the amino acid residue in the imine intermediate should be facilitated by a positive charge on the ring nitrogen. The protonated ring nitrogen atom in pyridoxylidene valine was estimated to have a pK of about 5.9 (Figure 14). However, the synthetic N-methyl analog of pyridoxal bears a positively-charged ring nitrogen independent of pH. Its imines were thus expected to react faster than those of pyridoxal, especially at pH values above 6.

It became apparent that these relative rate comparisons depended on the kinetic method used. For example, if based on calculated initial rates of pyridoxal analog lost in 1 M imidazole at pH 6.8, the rate ratios were 22 for DPL, 5.5 for PLP, 3 for PLM, relative to 1 for PL. Whereas, if based on a 3.5% loss of pyridoxal analog (the amount of pyridoxal lost in 5 hours) the rate ratios of inverse reaction times to achieve this amount of reaction were 16 for DPL, 3 to 4 for PLP, 1.6 for PLM, relative to 1 for PL (Figure 29). The equilibrium position seemed to be at about 50% loss of pyridoxal

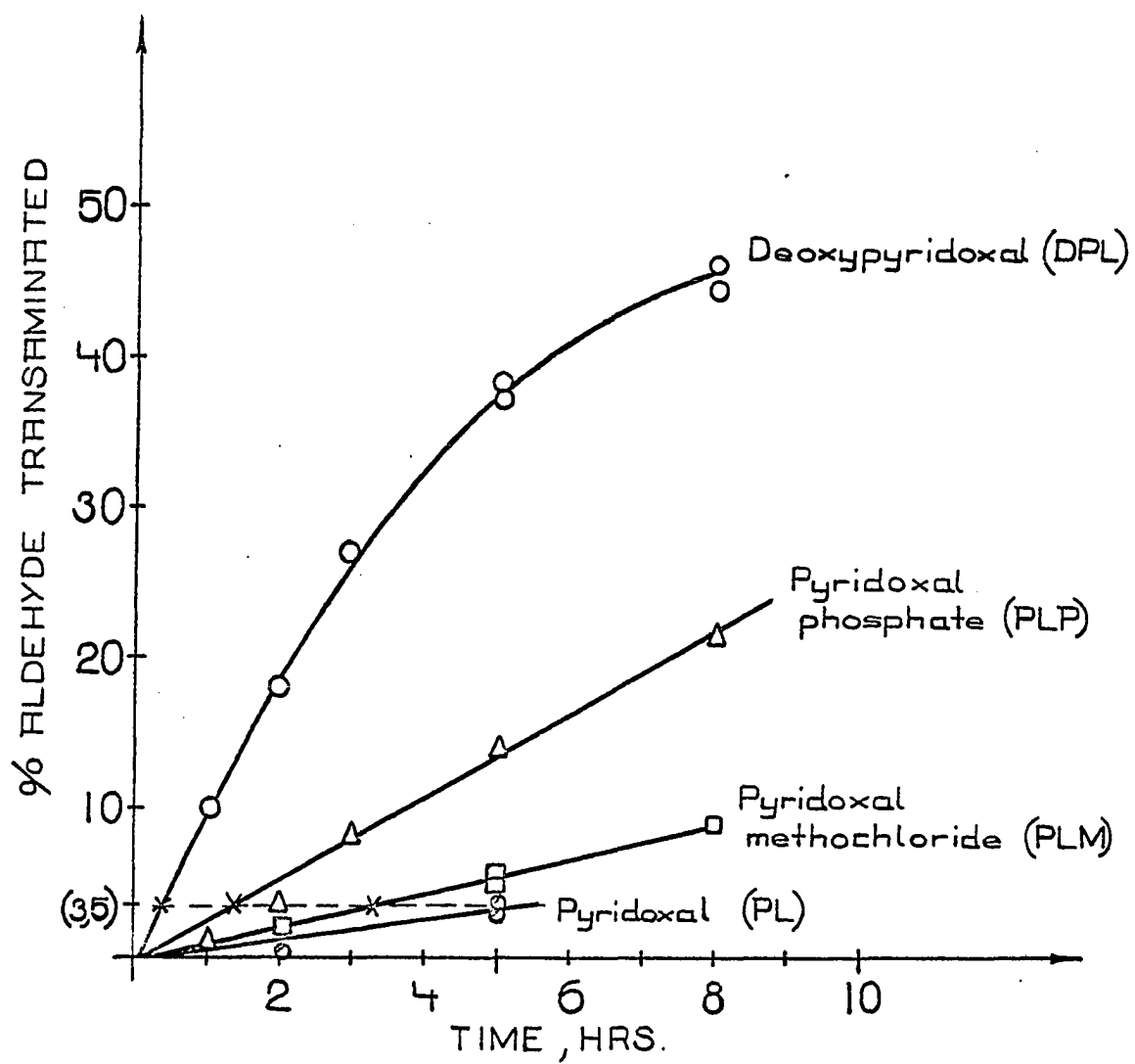
Figure 29. Relative transamination rates of four amino group acceptors with leucine, catalyzed by 1.0 M imidazole buffer^a.

	t hrs. for 3.5% transamination of pyridoxal analog ^{b,c}	Relative rates <u>$5/x = t_{PL}/t_x = k_x/k_{PL}$</u>
DPL	0.30	16
PLP	1.2 - 1.7	3 - 4
PLM	3.0	1.6
PL	5.0	1.0

^aInitial conditions: 0.03 M leu, 0.02 M pyridoxal analog, pH 6.8, 25°

^bAt 3.5% decrease in pyridoxal analog, the amount of pyridoxal lost in 5 hours in the presence of leu and imidazole, minus the amount lost in the same time in the absence of leu and presence of imidazole

^cMeasured decreases in pyridoxal analog by ethanolimine method and, after correcting for loss in PL and PLM due to imidazole interaction, calculated per cent pyridoxal analog lost due to transamination with leucine



analog, under these second-order conditions. Second-order rate constants were calculated for the reactions with DPL ($17.5 \pm 1.1 \times 10^{-4}$ /M/sec.) and with PLP ($5.36 \pm 0.22 \times 10^{-4}$ /M/sec.), and the rate ratio, $k, \text{DPL}/k, \text{PLP} = 3.3$ was obtained under these conditions.

An attempt to compare pyridoxal and its N-methyl analog in an imidazole system at pH 4 indicated a much slower reaction, which was difficult to measure (only 3 to 4% pyridoxal was lost in 84 hours). The best reactions catalyzed by imidazole seemed to be above pH 6, unfortunately.

These results may be explained on the basis that buffer catalysis was a greater effect than the intramolecular catalysis by functional groups substituted at the 5-position of pyridoxal. These intramolecular effects may only be observed in the absence of buffer or in very low buffer concentration. These catalytic groups, such as the phosphate ester in pyridoxal phosphate, interfere with buffer catalysis, resulting in lower buffer catalytic constants than those for deoxypyridoxal, which has no functional group in the 5-position.

Comparisons of transamination rates of pyridoxal phosphate, and other pyridoxal analogs, with deoxypyridoxal should take into account the relative amounts of aldimine intermediates present (from experimentally determined imine formation constants), unless these intermediates are present at low steady state concentrations (but this is not the case for these reaction conditions where relatively high reactant con-

centrations are used). The rates of conversion of the aldimines of different pyridoxal analogs to products would yield a fair comparison of the relative transamination rates effected by intramolecular catalysis.

Significant decreases of pyridoxal and of pyridoxal methochloride in control solutions containing 1.0 M imidazole were measured by the ethanolimine method. These decreases were not reproducible nor could they be predicted. Corrections for these changes caused by imidazole were made to obtain net pyridoxal decreases which were due to transamination with leucine. However, no such effect of imidazole of deoxypyridoxal and pyridoxal phosphate were observed. The stability of these pyridoxal analogs in the presence of imidazole suggests that the hydroxymethyl group in position 5 of pyridoxal and its N-methyl analog is necessary for this effect.

Perhaps, imidazole somehow affects the equilibrium of these latter compounds to favor their hemiacetal forms and to inhibit the reaction with ethanolamine, which presumably reacts with the free aldehyde forms in yielding ethanolimines quantitatively. The gradual decreases of these two pyridoxal analogs in these control solutions could be accounted for on the basis of slow interactions with imidazole, such as a shift in equilibrium or a formation of a condensation product requiring a hydroxymethyl group. As far as this author can determine, Bruice and Topping did not report the use of such control solutions of pyridoxal and imidazole in their studies

of the kinetics of the imidazole - catalyzed reaction between pyridoxal and phenylglycine.

Bruice and Topping, who have reported kinetic studies of the imidazole-imidazolium ion concerted general acid, general base catalysis of the transamination of phenylglycine with pyridoxal in a recent series of publications, have claimed that the uniqueness of imidazole as a catalyst is in its solubilization of amino acids by complexing with them, as well as with the imine intermediates (1963d). Phenylglycine, an unnatural amino acid, is less soluble in water than leucine. But when its solubility increased in solutions of imidazole buffer, Bruice and Topping suggested the formation of a complex between imidazole and the phenyl group of the amino acid (1963c). However, DePrenger, in this laboratory, also found that leucine, an aliphatic amino acid, had greater solubility in solutions of imidazole buffer than in water alone.

No significant difference was found between observed rates of nonenzymic transamination of leucine and of phenylglycine (Donald DePrenger, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. A study of rates of transamination of pyridoxal analogues catalyzed by imidazole. Private communication. 1964). However, the fact that a higher maximum wavelength was found for the solution of leucine, pyridoxal, imidazole (410 to 415 mμ) than the corresponding solution of phenyl-

glycine (395 mμ) under the same conditions indicated a greater extent of imine formation in the leucine solution. Since the rates were the same, the imine of phenylglycine and pyridoxal must be more reactive than that of leucine and pyridoxal. This difference may be explained by the difference in electro-meric effects of a phenyl group and an alkyl group on the bond-breaking process at the alpha-carbon atoms of the amino acid residues in these imines.

However, at high amino acid concentration DePrenger found that the reaction with leucine proceeded much faster, but followed zero-order kinetics compared to the slower reaction with phenylglycine which still followed pseudo first-order kinetics. But when the pyridoxal concentration was tripled so that the molar ratio of leucine to pyridoxal was 67 to 1, instead of 200 to 1, a pseudo first-order rate constant was calculated as 1.5×10^{-5} /sec., significantly smaller than that calculated for the reaction in which only a 2 to 1 molar ratio of leucine to pyridoxal existed at 10^{-4} M concentrations (3.7×10^{-5} /sec.). It is not understood why the observed rate decreased with an increase in leucine concentration.

The reaction of leucine with deoxypyridoxal under these conditions with leucine in excess by a factor of 200 times was found by DePrenger to be further complicated by a slow formation of imine, which was followed by the absorbancy at 410 mμ increasing to a maximum in 12 minutes after mixing the reactants. After this time the rapid decrease in absorbancy

followed zero-order kinetics. Louise Hodgkin and Sheryl Bresnahan in this laboratory also independently obtained zero-order kinetics for the same reaction. (Increasing absorbancy at 317 mμ and decreasing absorbancy at 415 or 387 mμ were directly proportional to time.)

Bruice and Topping (1963a) found that increasing the imidazole concentration from 0.1 to 1.8 M increased the rate of ketimine formation from 10^{-4} M reactants at pH 8.6 by a factor of 200 times (k_{obs} increased from $8 \times 10^{-7}/\text{sec.}$ to $1.5 \times 10^{-4}/\text{sec.}$). Furthermore, the rate was found to depend upon the square of the imidazole concentration up to a maximum of about 1.8 M, supporting their claim that two molecules of imidazole complexed with aldimine in the acid-base catalysis of the rate-limiting step. When the concentration of phenylglycine was doubled for the same reaction conditions in 1.8 M imidazole the observed rate constant was further increased to $2.0 \times 10^{-4}/\text{sec.}$

Under these same conditions DePrenger in this laboratory observed a rate constant of only $0.37 \times 10^{-4}/\text{sec.}$, $5\frac{1}{2}$ times less than that reported by Bruice and Topping. Perhaps, in the calculation of their rate constant, Bruice and Topping only considered the formation of ketimine in "correcting" their observed rate constant, since the relative slowness of the conversion of ketimine to products prevented a precise determination of the final equilibrium owing to complicating side reactions in this final step (1963a; also see rate equa-

tion, Review of Pertinent Literature). But DePrenger simply calculated an observed rate constant for the over-all transamination reaction.

DePrenger also found no significant difference between rates measured at 25° and at 30°C (the latter being the temperature used by Bruice and Topping). Nor was there any effect of ionic strength from 0.05 M KCl, which Bruice and Topping and others so carefully controlled in their model systems.

Finally, Donald DePrenger, Louise Hodgkin and Sheryl Bresnahan in this laboratory compared rate constants for the nonenzymic transamination of these amino acids with several pyridoxal analogs catalyzed by imidazole buffer and followed by spectral methods. In 1.8 M imidazole, pH 8.6, pyridoxal ($3.67 \times 10^{-5}/\text{sec.}$) reacted slightly faster than pyridoxal phosphate ($3.4 \pm 0.2 \times 10^{-5}/\text{sec.}$) with phenylglycine, in which there was a 2 to 1 molar excess of amino acid in both cases. The latter constant may be compared with $3.6 \times 10^{-5}/\text{sec.}$, (as measured by decreasing absorbancy at 278 mμ) in which there was a 67 to 1 molar excess of leucine. Nor did the leucine imine of deoxypyridoxal ($5.8 \times 10^{-6}/\text{sec.}$, as measured by decreasing absorbancy at 426 mμ) react much faster than that of pyridoxal ($5.5 \times 10^{-6}/\text{sec.}$, as measured by decreasing absorbancy at 410 mμ). For this latter comparison there was a 500 to 1 molar excess of amino acid in these reactions. Again, it is not understood why the rate constants have de-

creased with an increase in amino acid concentration. Apparently at pH 8.6 in high imidazole buffer concentration the hemiacetal form of pyridoxal did not decrease the reaction rate, as compared with the rates for deoxypyridoxal and pyridoxal phosphate, although a large effect of the hemiacetal form was observed at pH 6.8 (Figure 29).

Decreasing the concentration of imidazole to 1.0 M an average rate constant for the reaction of PLP with a 200 molar excess of leucine was calculated to be $7.3 \pm 0.3 \times 10^{-5}$ /sec. (as measured by decreasing absorbancies at 414 and 275 m μ and increasing absorbancy at 322 m μ). This was compared with a value of 2.5×10^{-6} /sec. (as measured for KIC formation by the QH method), in which there was only a 5 to 1 molar ratio of leucine to PLP. These values obtained in this laboratory may be compared with 3×10^{-5} /sec. obtained by Bruice and Topping (1963d) for the formation of ketimine from equimolar pyridoxal and phenylglycine under the same conditions of pH and imidazole concentration.

A striking effect of a positive charge on the pyridine nitrogen atom was observed by DePrenger in the laboratory. In 1.8 M imidazole, pH 8.6, the leucine imine of pyridoxal methochloride (58×10^{-6} /sec., as measured by decreasing absorbancy at 385 m μ) reacted ten times faster than the imine of pyridoxal (5.5×10^{-6} /sec., as measured by decreasing absorbancy at 410 m μ). (The wavelengths of maximum absorbancy of these imines differ, as expected from the differences in

ionic forms.) This lends further support to the mechanism which is favored by a positive charge on the pyridine ring nitrogen atom of the imine. At pH 8.6 the imine of pyridoxal and leucine has dissociated the proton from the pyridine nitrogen atom, whereas that of pyridoxal methochloride, of course, retains a positively-charged pyridine nitrogen atom.

However, a quantitative comparison of the reactivities of these pyridoxal analogs should take into account their relative aldimine and hemiacetal formation constants under these conditions. In the latter comparisons DePrenger found that the apparent molar absorbancy index of the pyridoxal methochloride imine was only about $\frac{1}{4}$ that of the imines of pyridoxal or of deoxypyridoxal. This markedly decreased stability of the PLM imine, despite the estimated 1.7 times greater concentration of PLM free aldehyde than that of PL, emphasizes that the positive charge on the pyridine nitrogen atom is important in producing a high degree of reactivity. It is quite likely that the PLP coenzyme may be attached to the protein in such enzymes through hydrogen bonding at the pyridine nitrogen atom. From these results it is suggested that such attachment would have a profound effect on the reactivity of the imines which exist as intermediates in the catalytic process.

Metal Ion Catalysis of Nonenzymic Transamination of Amino Acids with Pyridoxal Analogs

Metal ion enhancement of general acid-base catalysis

The general acid-base catalysis of nonenzymic transamination of leucine with pyridoxal analogs was further enhanced by the addition of metal ions. Near the pH 4 optimum the additive effects of acetate buffer and cupric ion were measured by the following calculations.

The rate constant, k_0' , for the reaction of 0.1 M leucine (leu) with 0.01 M deoxypyridoxal (DPL) in unbuffered solution at pH 4.2, was calculated to be 3.4×10^{-5} /M/sec. from the k_0' value of 2.50×10^{-5} /M/sec. obtained for 0.05 M leu multiplied by the factor 72/53, the ratio of k_0' values for the two leucine concentrations at pH 7.2 (Table 12). The pseudo first-order rate constant for the reaction with 0.1 M leu in unbuffered solution at pH 4.2 is predicted to be 3.4×10^{-6} /sec. by equation (19a).

The observed pseudo first-order rate constant for these reaction conditions in the presence of 1.0 M acetate buffer was 2.8×10^{-5} /sec., which was an eight-fold increase over that of the reaction in unbuffered solution. (These values were for KIC formation measured by the QH method.) The catalytic constant for acetate buffer, obtained by use of equation (20), was 2.46×10^{-5} /M/sec.

The addition of 1.0 mM Cu (II) to this model system almost

doubled the observed rate constant to $4.7 \times 10^{-5}/\text{sec}$. This constant, however, was calculated indirectly from a second-order rate constant of $1.2 \times 10^{-4}/A_{385 \text{ m}\mu}/\text{sec}$. multiplied by the maximum absorbancy at 385 m μ of 0.393. This wavelength is the absorption band for the cupric chelate of the aldimine of leucine and DPL, and loss of absorbancy at this wavelength followed simple second-order kinetics (straight-line slope for $1/A$ vs. t) after it was found that a Guggenheim plot concaved sharply downward. The maximum absorbancy at 385 m μ was reached at about ten minutes after mixing, indicating slow chelate formation with the aldimine. From equation (24), the catalytic constant for cupric ion, $k_{\text{Cu (II)}}$, was calculated as $19 \times 10^{-3}/\text{M}/\text{sec}$. (24) $k_{\text{obs}} = k_o' (\text{leu}) + k_B (\text{B}) + k_{\text{Cu (II)}} (\text{Cu}^{\text{II}})$.

The catalytic constant for cupric ion was about 700 times greater than that for acetate buffer under these conditions. The observed rate constant of $4.7 \times 10^{-5}/\text{sec}$. may be compared with that of $3 \times 10^{-5}/\text{sec}$. for the reaction between pyridoxal and phenylglycine catalyzed by 1.0 M imidazole at pH 8.6 (Bruice and Topping, 1963d), which was not affected by aluminum ion, and with that of $2.6 \times 10^{-5}/\text{sec}$. for the reaction of pyridoxal and alanine in N,N-dimethylglycine buffer at pH 10, which was further catalyzed by cupric ion by a factor of 100 (Banks et al., 1961).

Catalysis of the reaction of pyridoxal with leucine was enhanced by about three times when 0.02 M zinc salt was in-

cluded with the system buffered with 1.0 M imidazole at pH 6.8. This contrasted with the conclusion reached by Bruice and Topping (1963a) that metal ions do not affect imidazole-catalyzed reactions, from their observations that aluminum ion did not affect the observed rate at pH 8.6 catalyzed by 1.8 M imidazole.

Comparisons were made also in the zinc-imidazole system between the reactivities of pyridoxal (PL), pyridoxal methochloride (PLM), deoxypyridoxal (DPL), and pyridoxal phosphate (PLP) as amino group acceptors. Relative rates were based on calculations of initial rates of decrease in pyridoxal analog, as measured by the ethanolimine method. All of these reactions were 0.02 M in pyridoxal analog, 0.03 M in leucine, pH 6.6 to 6.8. In addition, 1.0 M imidazole, 0.02 M zinc ion with ionic strength adjusted to 0.5 M with sodium acetate, or 1.0 M imidazole plus 0.02 M zinc ion were included in these model systems. The relative rates in 1.0 M imidazole buffer were compared in the previous section.

In the systems containing zinc ion under these conditions a white precipitate formed in the reactions with DPL at time zero, and no further attempts to compare the zinc catalysis of the reactions with DPL and PLP were made. It was quite likely that in solutions of relatively high concentrations of imine (DPL or PLP with leucine) the resulting zinc chelate of these imines formed to an extent exceeding their solubility. But in solutions of relatively low concentrations of imine

(PL or PLM with leucine) rate comparisons were made.

Using equation (9), initial rates were converted to second-order rate constants: dP/dt was 3×10^{-8} M/sec. or $k_2 = 5 \times 10^{-5}$ M/sec., for PL and leu, in the zinc ion-sodium acetate system. These values were 1.7 times greater than those for PLM and leu in a corresponding system. But in the zinc ion-imidazole buffer system, dP/dt was 12×10^{-8} M/sec. or $k_2 = 20 \times 10^{-5}$ M/sec. for PL and leu, and the corresponding values for PLM and leu were 1.6 times greater. Reasons for these apparently conflicting results are not at all obvious.

Blake et al., (1963) also observed enhancement of acid-base catalysis by aluminum ion. An acetate buffer (0.1 M acetic acid, 0.1 M acetate) doubled the observed rate constant of the reaction between alanine and pyridoxal at 100°C compared to the reaction in unbuffered solution of the same pH with other conditions held constant. When 5×10^{-5} M aluminum ion was included in this system with the acetate buffer, the rate constant was tripled. Qualitative observations of these effects were made earlier by Metzler and Snell (1952b).

Metal ion catalysis of nonenzymic transaminations of amino acids with pyridoxal and with pyridoxal methochloride

Attempts were made to measure the effect of N-methylation in pyridoxal on the nonenzymic transamination of amino acids catalyzed by metal ions. Rate measurements were based on millimolar concentrations of pyridine aldehyde lost or of keto acid formed after 10 and 30 minutes near the boiling

point of water at two pH values measured at 25° and controlled by 0.2 M buffers. The transamination of leucine with pyridoxal methochloride (PLM) indicated up to $1\frac{1}{2}$ times more changes in concentrations of reactants and products than that with pyridoxal (PL) at pH 4.8 (Table 21).

However, concentration changes in the reactions of serine with PL and PLM were about the same. Rates of formation of products analyzed as keto acid from serine were much greater than the corresponding rates of decrease in pyridine aldehyde. This latter anomaly may be due to the side reactions of serine with pyridoxal, such as β -elimination and aldol cleavage, which lead to different carbonyl products, other than pyruvate via transamination. And the dinitrophenylhydrazone product from serine was assumed to be pyruvate, since the keto acid formation was calculated from a pyruvate standard curve. Analytical methods and procedures of Metzler and Snell (1952b) were used in these experiments.

Under the conditions of a pH near neutrality the reactions are much slower than at pH 4, in agreement with earlier observations that the pH optimum for aluminum ion-catalyzed transamination is about 4.5 (Metzler and Snell, 1952b). However, as expected, the leucine transamination rates were even higher with the N-methyl analog than with pyridoxal at the pH above the pK of the pyridinium group in the pyridoxal imine. In this case, the reaction with the

Table 21. Comparison of reactions of pyridoxal (PL) and pyridoxal-N-methochloride (PLM) with amino acid at 100°C catalyzed by aluminum ion (alum.) at pH 4.8 (20°C): 0.2 M in $\text{NH}_4\text{OAc} + \text{HOAc}$, 0.3 M ionic strength

1 mM alum. +10 mM reactant	mM changes in 10'		in 30'	
	Pyridine aldehyde lost	Keto acid formed	Pyridine aldehyde lost	Keto acid formed
PL + ser	2.3	8.3	2.5	11.8
+ leu	3.9	3.7	5.7	5.4
PLM + ser	2.1	8.9	2.5	10.7
+ leu	6.0	4.7	6.8	5.6

Table 22. Comparison of reactions of pyridoxal (PL) and pyridoxal-N-methochloride (PLM) with amino acid at 100°C catalyzed by aluminum ion (alum.) at pH 6.6 (20°C): 0.2 M in phosphate buffer, 0.3 M ionic strength

1 mM alum. +10 mM reactants	mM changes in 10'		in 30'	
	Pyridine aldehyde lost	Keto acid formed	Pyridine aldehyde lost	Keto acid formed
PL + ser	0.0	0.0-0.1	0.1	0.0
+ leu	0.8	0.3-0.6	1.8	1.0
PLM + ser	0.0	0.0-0.05	0.0-0.3	0.13
+ leu	1.26	0.82	2.7	2.1

positively charged imine of the N-methyl analog proceeded to between $1\frac{1}{2}$ to $2\frac{1}{2}$ times farther in 10 and 30 minutes than with the imine of pyridoxal (Table 22). Also, these rates were clearly greater than those of the serine reactions. These results are also in harmony with the proposal that the N-protonated (or N-methylated) form of the metal chelate is the reactive form (Johnston et al., 1963).

These rates of leucine transamination were expressed as initial rates, dP/dt , for the concentration changes after 10 minutes reaction (Table 23). As usual, the rates of decrease in pyridine aldehyde were greater than the rates of keto acid formation.

Table 23. Comparisons of reactions of pyridoxal (PL) and pyridoxal-N-methochloride (PLM) with amino acid at 100°C catalyzed by aluminum ion (alum.): calculated initial rates, $dP/dt \times 10^6/\text{M}/\text{sec.}$, of leucine transamination from Tables 21 and 22

	pH 4.8		pH 6.6
		(k_2 /M/sec.)	
<u>PL & leu</u>			
PL lost	6.5	(0.18)	1.3
KIC formed	6.2	(0.17)	0.5-1.0
<u>PLM & leu</u>			
PLM lost	10	(0.20)	2.1
KIC formed	7.8	(0.16)	1.4

The extents of these reactions at pH 5 were compared with earlier values (Table 24). It was assumed that the equilibrium values for % pyridine aldehyde lost in the transamination of leucine were 60% for PL and 70% for PLM, in order to estimate the apparent second-order rate constants from the initial rates of Table 23. From equation (9), relating initial rates, dP/dt , to second-order rate constants, k_2 , these constants were calculated using 6 mM for the "effective" or reacting concentrations of PL and leucine and 7 mM for the reacting concentrations of PLM and leucine. These values were near 0.2/M/sec. (Table 23), and may be compared with the highest rate constant obtained by Blake et al., (1963) of 0.0025/M/sec. for the decrease in pyridoxal, starting with 10 mM PL, 0.20 M α alanine, 0.10 M acetate, 0.10 M acetic acid, catalyzed by 5×10^{-5} M alum. at 100°C. This comparison indicates that a 20 times higher concentration of aluminum ion catalyzed the reaction by 100 times under these conditions, if leucine and alanine have about the same reactivities as amino group donors.

The stability of pyridoxal methochloride (PLM) was checked at 100°C, because some of its reactions with amino acids were measured under these conditions. The solutions were 10 mM in PLM, 1 mM in alum., and were buffered with either 0.2 M ammonium acetate at pH 4.7 or with 0.2 M phosphate at pH 6.6, 25°C. A portion of each solution was heated in a boiling water bath for 30 minutes. Aliquots were then

Table 24. Comparison of reactions of pyridoxal (PL) and pyridoxal-N-methochloride (PLM) with amino acid at 100°C catalyzed by aluminum ion (alum.) at pH 5 (20°C): 0.2 M in $\text{NH}_4\text{OAc} + \text{HOAc}$

1 mM alum. + 10 mM reactants	% pyridine aldehyde lost after 30'
PL + ser	25
PLM + ser	25
PL + leu	57
PLM + leu	68
PL + leu	51 ^a
PL + leu	After 120' (equilibrium) 59 ^a

^a Source: Metzler and Snell, 1952b

acidified to pH 0.2 in diluting them to a convenient concentration for spectral analysis. Absorbancies of these solutions were measured at 288 and 293 mμ against appropriate blanks. At either pH, absorbancies of all solutions were greater at 293 than at 288 mμ, the absorption maxima of the hemiacetal cation forms of PLM and PL, respectively. The ratio of absorbancies at these two wavelengths for each solution at pH 4.7 remained constant within one percent, (attributable to experimental error). This constant absorbancy ratio was expected if the heat treatment did not lead to hydro-

lysis or cleavage of the N-methyl group to yield pyridoxal, which has a higher absorbancy than PLM at the lower wavelength.

The ratio of absorbancies for each solution at pH 6.6 indicated either a maximum of three percent loss of PLM due to an opening of the N-methylpyridine ring or to experimental error caused by discrepancies in the absorbancies of the blank solutions. But at least no significant extent of demethylation to pyridoxal occurred under the reaction conditions (Johnston et al., 1963). Under similar reaction conditions the individual stabilities of pyridoxal, pyridoxamine, amino and keto acids were checked previously (Metzler and Snell, 1952b), and remarkable stability was noted for most of these compounds during the relatively short reaction times required (1 to 3 hrs.) at 100°C.

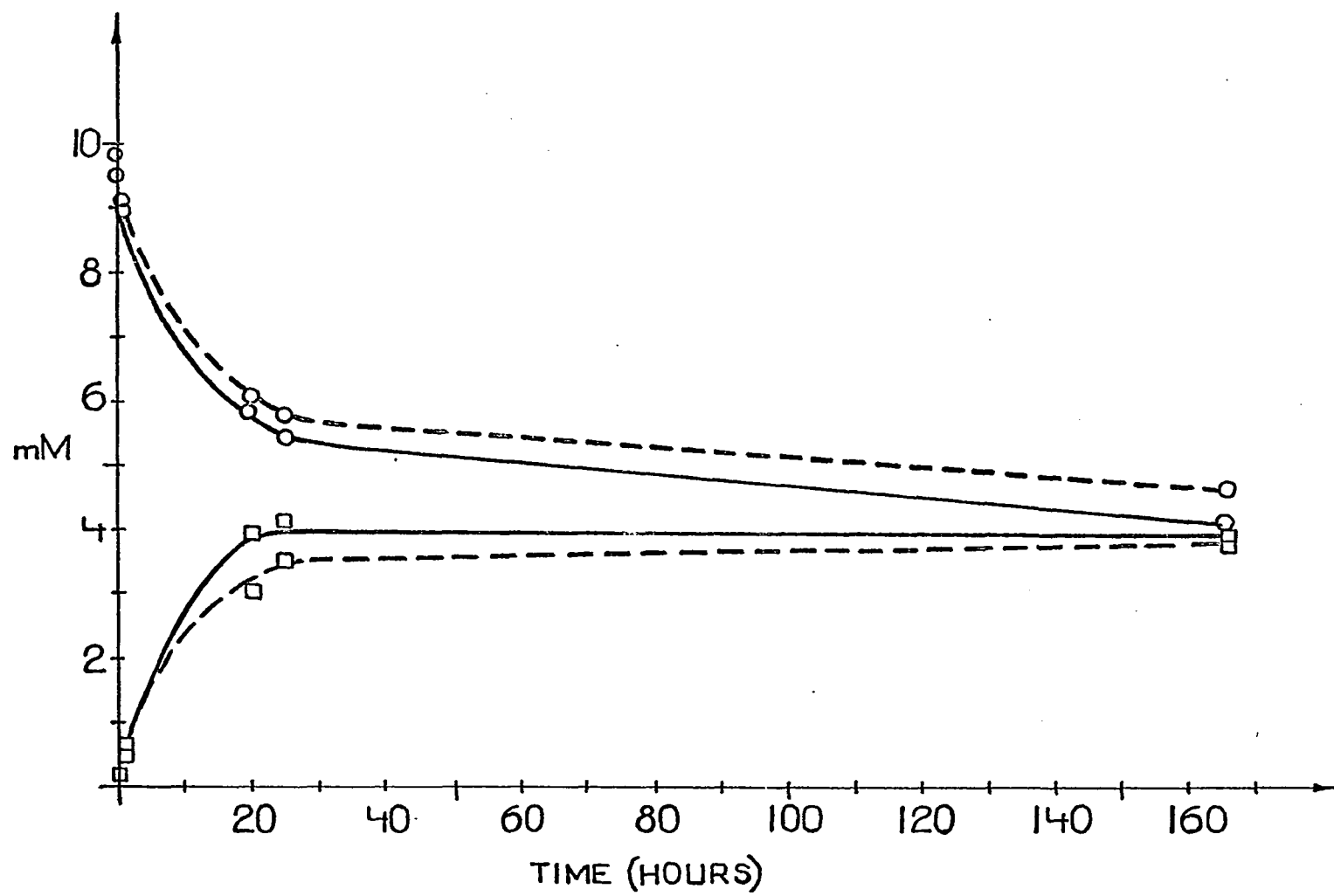
Since this model transamination reaction also increases with an increase in temperature, the rates at 25°C were only about 2% as great as the corresponding rates obtained near 100°C (boiling water). Metal-ion catalyzed reactions were followed at 25°C, using the modified analytical methods adapted from the procedures of Metzler and Snell (1952b). In a comparison between pyridoxal and its N-methyl analog at pH 4 the rates of Al (III)-catalyzed reactions were about the same, although the pyridoxal reaction appeared to be slightly faster under these conditions (Figure 30). Pseudo first-order rate constants, calculated by the Guggenheim method, indicated that the rates of decrease in pyridoxal ($2.7 \times 10^{-5}/$

Figure 30. Comparison of nonenzymic transaminations of leucine with pyridoxal (PL) and with pyridoxal-N-methochloride (PLM)^a

millimolar (mM) concentrations vs. time

- _____ decrease in PL
- ----- decrease in PLM
- _____ formation of KIC from leu and PL
- ----- formation of KIC from leu and PLM

^aInitial conditions: 120 mM leu, 10 mM PL or PLM, 2 mM alum., pH 4.2, 0.1 M acetate buffer, 0.5 M ionic strength, 25°C



sec.) and of formation of ketoisocaproate ($2.85 \times 10^{-5}/\text{sec.}$) were about the same, although initial rates (dP/dt) indicated a significantly greater rate of decrease in pyridoxal. The initial rates were found to be constant over the first five hours at least. These pseudo first-order rate constants were only slightly higher than the best rate constant reported by Banks *et al.* (1961) for the reaction of 0.2 M alanine with 0.01 M pyridoxal at pH 10 in 0.1 M N,N-dimethylglycine buffer ($2.6 \times 10^{-5}/\text{sec.}$).

Figure 10 shows that the rates of reaction between pyridoxal and leucine catalyzed by aluminum ion in 0.1 M acetate buffer were about the same as those for deoxypyridoxal and leucine in 1.0 M acetate buffer without added metal ion. This indicates that catalysis by 2 mM aluminum ion is at least equivalent to that by 1.0 M acetate buffer under these conditions at pH 4.2, since deoxypyridoxal is a much better amino group acceptor than pyridoxal.

In a similar reaction in which there was about $2\frac{1}{2}$ times less initial concentration of leucine the initial rates were about half as great. Equilibrium was approached after 18 days, indicated by a 52% decrease in pyridoxal. A control solution containing sodium alpha-ketoisocaproate, instead of leucine under reaction conditions, indicated no significant changes in pyridoxal or keto acid concentrations, within experimental error, during the first five hours at least.

Studies with zinc ion catalysis under these conditions

indicated a very slow reaction even after 25 hours. A rough comparison between the aluminum and zinc ion catalyses at pH 4 indicated a 14% decrease in pyridoxal in the reaction with leucine and aluminum ion in five hours and 1% decrease in the zinc ion-catalyzed reaction. Furthermore, 8 times more keto acid was produced from the former reaction than from the latter in this time.

However, zinc ion catalysis is probably one of the most favorable metal ion systems for quantitative rate studies, because, in comparison to other metal ions, zinc ion exchanges rapidly with the hydrogen-bonded proton in the aldimine intermediate and it has a relatively simple solution chemistry. Furthermore, oxidation side reactions caused by zinc ion are of little significance, and formation constants of zinc ion with ligands are known. The only disadvantage is that zinc is a mediocre metal ion catalyst, far below the catalytic effectiveness of copper, iron, and aluminum ions (Metzler and Snell, 1952b ; Gregerman and Christensen, 1956; Longenecker and Snell, 1957). Although manganese (II) was recently reported to be one of the best metal ions for promoting the transamination of glutamate with 3-hydroxypyridine-4-aldehyde (Bruice, 1964), it had only mediocre activity in catalyzing the transamination of glutamate with pyridoxal at 100° (Metzler and Snell, 1952b; Longenecker and Snell, 1957).

Zinc ion catalysis was found to be more effective at a higher pH, just below neutrality (Table 25; reactions 1,2,5),

in agreement with the findings of Longenecker and Snell (1957). The reactions proceeded best when the ionic strength was adjusted with sodium acetate than with perchlorate or chloride salts - another indication of general acid-base catalysis. The gradual formation of a white precipitate as the pH was increased and was prevented during the first day of reaction by adding the zinc salt after mixing the other components (forming the imine first). Concentrations of leucine and zinc perchlorate were varied (Table 25; reactions 2-7) to find reaction rates comparable to those of the aluminum ion-catalyzed reactions at pH 4 (Table 25; reactions 2,5,6,8), although reproducibility of the analytical methods used in following these reactions was undesirable.

Under the conditions indicated near neutral pH, initial rates of pyridoxal lost and ketoisocaproate formed were reproducible and fairly constant, within experimental error, during the first 8 hours of reaction (Table 26), after analytical procedures were modified (See Experimental). Formation of some white precipitate, a big problem in metal ion systems especially at pH values above 5, was noticeable after one day and prevented further reliable rate measurements beyond the initial rates, thus making reaction order and rate constants difficult to determine. In 5 hours this reaction proceeded to about $2\frac{1}{2}\%$ and in 8 hours to about $4\frac{1}{2}\%$. Or, about 1% of the reaction, with reference to the equilibrium point, occurred per hour in the initial reaction con-

Table 25. Comparisons of metal ion-catalyzed nonenzymic transamination rates at 25°C

	Initial conditions				dP/dt after 2 hrs. (-moles ₈ PL/ l/sec. X 10 ⁸)
	mM PL	mM Leu	0.5 μ salt (NaOAc, ClO ₄ ⁻ , Cl ⁻)	pH	
1)	10	80	10 mM Zn (II)	4.7	1
2)	20	45	20 mM Zn (II)	6.2	4
	21	45	20 mM Zn (II)	6.5	7
	20	45	20 mM Zn (II)	6.5	12
3)	20	20	20 mM Zn (II)	5.7	3
4)	20	30	10 mM Zn (II)	7.3	10 (ppt.)
5)	20	30	20 mM Zn (II)	6.8	6
	20	30	20 mM Zn (II)	6.9	12
6)	20	30	30 mM Zn (II)	6.6	9
	20	30	30 mM Zn (II)	6.7	10
7)	20	30	40 mM Zn (II)	6.5	4
8)	10	120	2 mM Al (III)	4.2	15
	10	50	2 mM Al (III)	4.3	8
9)	22	45	20 mM Cu (II)	5.8-6.5	65 (ppt.)

ditions.

An approximation of the over-all second-order rate constants, comparing pyridoxal and its N-methyl analog in this zinc system, was made from the initial rates using equation (9). The lower reactivity of pyridoxal methochloride (3 X

Table 26. Zinc ion-catalyzed transamination rates^a

Hours:	5		7		8	
P: ^b	PL lost	KIC formed	PL lost	KIC formed	PL lost	KIC formed
1)	2.9	2.8	3.1	3.8		
	± 0.3	± 0.6	± 0.6	± 2.2		
2)	3.6	4.1			3.1	3.2
	± 0.2	± 0.3			± 0.2	± 0.6

^aInitial concentrations: 20 mM PL·HCl
 30 mM Leu
 20 mM Zn (ClO₄)₂
 0.5 M with NaOAc, pH 6.8, 25°C

^bOverall rates at times indicated: $dP/dt \times 10^8$ M/sec.

10^{-5} /M/sec.) may be due to the lower pH recorded for it (6.2) as compared to the pyridoxal system (5×10^{-5} /M/sec., pH 6.8). However, previous to this, unexplained difficulties were experienced in trying to measure a reaction between pyridoxal methochloride and leucine in this zinc system.

A few experiments with cupric ion catalysis, which was found to be the best metal ion catalyst of these model system reactions (Metzler and Snell, 1952b), indicated that cupric chelates of imines were not completely soluble at pH 6. At this pH, the cupric ion-catalyzed reaction was between 10 and

15 times faster than the zinc ion-catalyzed reaction, without considering that most of the cupric ion probably had precipitated out of solution (Table 25; Reaction 9).

A more thorough study of the cupric ion-catalyzed reaction of pyridoxal with at least a 5-fold excess of leucine was made at pH 5, 25°C by Dr. Keith Schmude in this laboratory. Observed first-order rate constants were obtained from plots of absorbancy of portions of the reaction solution in ethanolamine at 365 mμ vs. time. The appearance of two distinct regions in a plot when Cu (II) and pyridoxal concentrations are approximately equal may be due to slow formation of the imine chelate. The rate constant was not a linear function of the concentration of Cu (II).

The precipitate which formed in cupric ion model systems was probably cupric dileucinate with a solubility product of about 1.6×10^{-8} at pH 5. It was predicted that the maximum Cu (II) concentration permitted would decrease as the square of the leucine concentration to explain why the rate did not increase with increasing concentrations of leucine and pyridoxal. Also, oxidation of pyridoxal was probably catalyzed by Cu (II), since about half of the pyridoxal lost in the presence of leucine was lost in its absence.

Furthermore, the rate seemed to depend on the ratio of concentrations of Cu (II) and pyridoxal and was maximum when this ratio was unity, which indicated that a simple one-to-one imine chelate was the reactive intermediate. This con-

clusion was also reached from kinetic studies of the reverse reaction, that of pyridoxamine with excess ketoisocaproate, catalyzed by cupric ion. Zinc ion was found to be 100 to 200 times less effective as a catalyst than cupric ion under these conditions. However, no deviation from linearity in the first order plots was observed, indicating a rapid formation of zinc chelate. Measurements of absorbancies at 380 mμ as a function of time verified the relative rates of imine chelate formation from these two metal ions. Also, apparently much less zinc chelate was formed as compared to the cupric chelate, from these spectral studies. The reaction rate decreased at lower pH, as expected from the decreased stability of the intermediate (Dr. Keith Schmude, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. More pyridoxalchemistry. Private communication. 1963.)

Nonenzymic Transamination of Leucine with 5-Carboxylate Analogs of Pyridoxal

Attempts were made to determine the effects of the carboxylate group in aliphatic side chains in the 5-position of pyridoxal on the nonenzymic transamination of leucine. The synthetic compounds, 5-"carboxypyridoxal" (CPL), with a carboxylate group substituted for the hydroxymethyl group of pyridoxal, and "alpha⁵-pyridoxalylacetic acid (PLA), with a propionate group substituted for the hydroxymethyl group,

were considered (Figures 1, 17). (As of this date, "alpha⁵-pyridoxalylformic acid," FPL or PLF, having an acetate group substituted for the hydroxymethyl group, was not available in free aldehyde form.)

"Carboxypyridoxal" vs. pyridoxal phosphate in acetate buffer

An estimate on the effectiveness of 5-"carboxypyridoxal" as an amino group acceptor, compared with pyridoxal phosphate, was made using a solution of about 0.01 M CPL, (not yet recrystallized, since recrystallization was a slow process). The nonenzymic transamination of leucine with pyridoxal phosphate proceeded about three times farther in the same time as compared to that with "carboxypyridoxal", under the conditions indicated (Table 27). Under similar conditions of pH and buffer concentration the pseudo first-order rate constants were 12.8×10^{-6} /sec. for pyridoxal phosphate and 28×10^{-6} /sec. for deoxypyridoxal, as amino group acceptors.

The lesser reactivity of "carboxypyridoxal", as compared to pyridoxal phosphate under these conditions, may be partly due to formation of an internal lactal between the adjacent carboxylate and formyl groups on the pyridine ring, especially in an acidic media. Difficulty experienced in this laboratory in preparing the next higher homolog of carboxypyridoxal "alpha⁵-pyridoxalylformic acid," (PLF or FPL) has been attributed to formation of an internal lactal structure (See Figure 36).

Table 27. Changes in millimolar concentrations of pyridoxal analog (PA) and ketoisocaproate (KIC) after 9.5 hours reaction^a

	+ mM KIC ^b	-mM PA
PLP	0.98 ±0.05	1.38 ^c 1.00 ^d
CPL	0.37 ±0.02	0.43 ^c 0.3 (?) ^e

^aInitial conditions: 2 mM PA, 75 mM leu, 1.0 M acetate, pH 3.9, 25°

^bFormation of KIC based on $\Delta A_{305 \text{ m}\mu}$ by QH method and calculated from standard curve (Figure 8)

^cDecrease in pyridoxal analog based on $\Delta A_{400 \text{ m}\mu}$ by QH method and calculation from standard curve (Figure 8)

^dDecrease in pyridoxal phosphate based on $\Delta A_{350 \text{ m}\mu}$ by EOA method and calculation from standard curve (Figure 8)

^eDecrease in carboxypyridoxal based on $\Delta A_{350 \text{ m}\mu}$ by EOA method and calculation from PLP standard curve (Figure 8); however, the spectrum of CPL in EOA had the highest peak at 318 and not near 350 mμ; A_{318} increased, instead of decreased as A_{350}

"alpha⁵-Pyridoxalylacetate" vs. deoxypyridoxal in imidazole buffer

Bresnahan in this laboratory found that "alpha⁵-pyridoxalylacetate" (PLA) reacted 1.7 times faster than deoxypyridoxal with leucine in 1.0 M imidazole buffer (pH 8.6,

14.5×10^{-6} /sec. for PLA, 8.5×10^{-6} /sec. for DPL, 10^{-4} M each with 0.02 M leu). However, these reactions seemed to be zero-order in that absorbancy was directly proportional (linear) with time.

Comparisons of reactions in unbuffered solutions

General acid-base catalysis by buffers has been found to interfere with observation of intramolecular acid-base catalysis by phosphate in pyridoxal phosphate, because buffer catalytic constants for nonenzymic transamination of leucine with deoxypyridoxal are greater than those with pyridoxal phosphate. For this reason measurement of effects of functional groups in the 5-position of pyridoxal on the nonenzymic transamination of leucine should be carried out in unbuffered solutions (prepared by careful adjustment of pH).

The nonenzymic transamination of leucine with "alpha⁵-pyridoxalylacetic acid" (PLA) was compared to that with deoxypyridoxal (DPL) and was found to be between 10 to 20 times faster. Calculation of relative rate constants were based on $\Delta A_{305 \text{ m}\mu}$ by the QH method (data for $\Delta A_{400 \text{ m}\mu}$ was unsuitable due to the slow reaction of PLA with QH); and also on inverse times compared to the same $\Delta A_{305 \text{ m}\mu}$ for DPL, pH 7.2 and 9.2, and the k_{obs} value at pH 8.1 of 3.6×10^{-7} /sec. (Figure 20). The average value of k_{obs} calculated for the reaction with PLA was $6 \pm 1.7 \times 10^{-6}$ /sec. (Figure 31).

Direct spectral data were inconsistent, perhaps due to

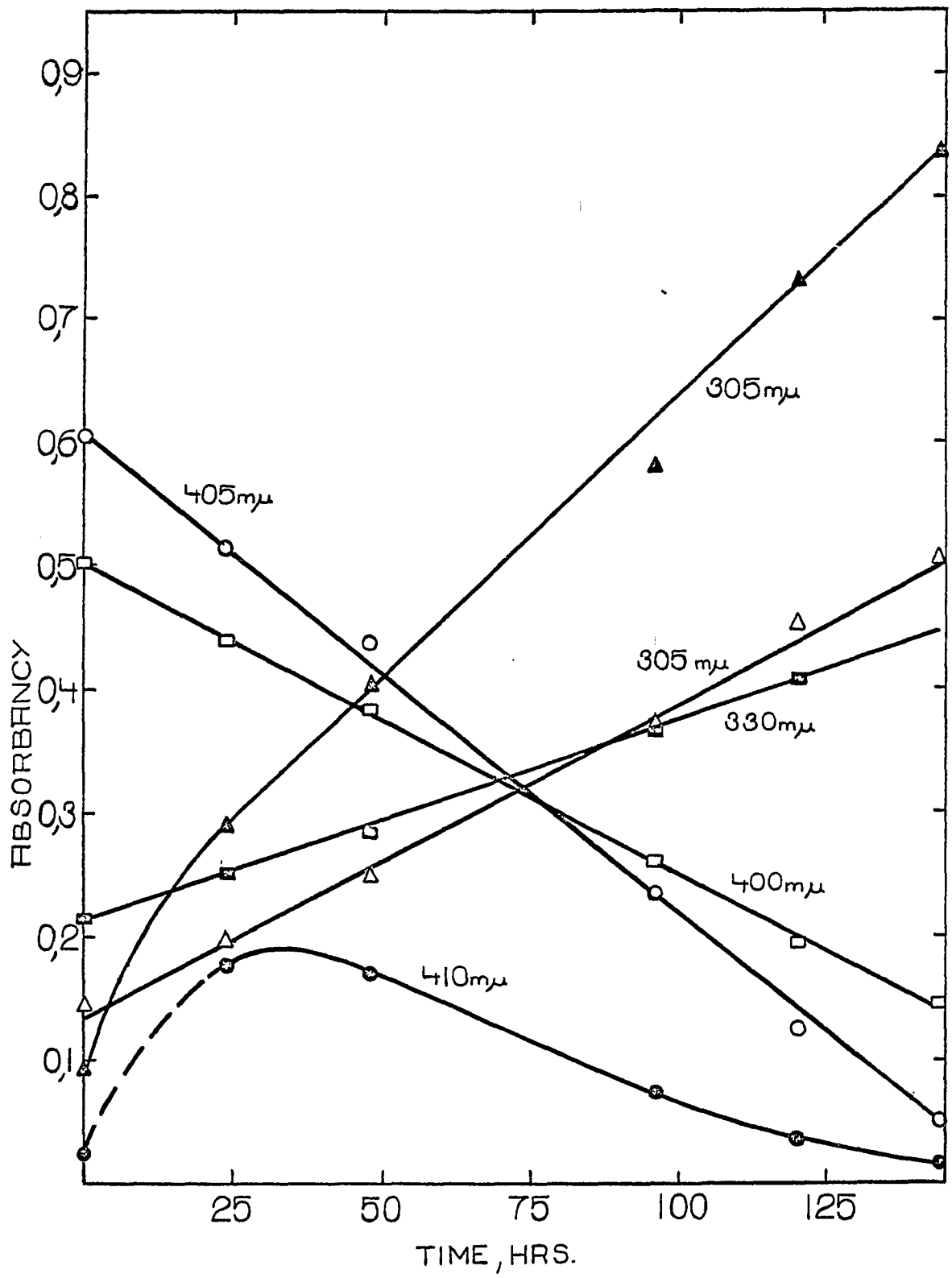
Figure 31. Apparent zero-order kinetics for nonenzymic transaminations of leucine with pyridoxal phosphate (PLP) and with "alpha⁵-pyridoxal-ylacetate" (PLA)^a followed practically to completion

<u>PLP, pH 6.4</u>	<u>$k_{\text{obs}} \times 10^6/\text{sec.}$</u>
△ A vs. (KIC) by QH method	1.6 - 2.8 ^b ; 4.0 ^c
□ A vs. (PLP) by QH method	3.1 - 2.3 ^b
○ A vs. (aldimine) by direct spectra	5.5 ^c
<u>PLA, pH 8.1</u>	
▲ A vs. (KIC) by QH method (zero-order only after 24 hrs.)	6 ± 1.7 ^c
■ A vs. (ketimine or PMA) by direct spectra	2.0
● A vs. (aldimine) by direct spectra (not zero-order)	2.0

^aInitial conditions: 50 mM leu, 10 mM PLP or PLA, unbuffered, 25°C

^bCalculated from initial and final slopes in Guggenheim plots

^cEstimated relative rate compared to reaction with DPL at pH 6.8, under similar conditions, by taking a ratio of inverse times to complete the same amount of reaction: $(k_x = k_y \frac{t}{t_x})$



formation of white precipitate in the dark orange solution of PLA and leucine after twenty four hours (about 25%) reaction. Titration of the same preparation of PLA by Dr. Tomita in this laboratory also resulted in the formation of a brown precipitate above pH 9.5 which seemed to be a decomposition product. As in several of the preceding nonenzymic transamination reactions with other pyridoxal analogs, under different conditions, the data were not consistent with pseudo first-order kinetics in that linear plots of absorbancy vs. time were obtained, from both the QH and the spectral analyses. The QH method suggested slower initial rates. The spectral method, however, gave the same rates for decrease in absorbancy at 405 m μ as for increase in absorbancy at 330 m μ (Figure 31).

The significance and interpretation of apparent zero-order kinetics in nonenzymic transamination systems under a variety of conditions for these reactions followed practically to completion are unclear to this author. Banks et al., (1961) also found zero-order kinetics at high concentrations of alanine in the reaction with pyridoxal, although the reaction did follow first-order kinetics at relatively low concentrations of alanine. Zero-order kinetics suggests the saturation of a catalyst in the rate-limiting step in the reaction, as in the case of enzyme kinetics.

Comparisons of the reactivities of these two carboxylate analogs (PLA and CPL) were made with deoxypyridoxal and pyri-

doxal phosphate at pH 4.5 (near the acid pH optimum), where PLA should be more stable than at high pH. Each reaction was followed for a period of one day with as many different analytical methods as possible. Direct spectral analysis indicated that the reactions could best be followed by changes in concentrations of the hydrogen-bonded aldimine, with decreasing absorbancies at 400 to 415 m μ , and of products (probably pyridoxamine analogs or ketimines) with increasing absorbancies at about 325 m μ . Decreases in absorbancies at 400 to 415 m μ were constant with time (linear plots of A vs. t), so these slopes (-m) were compared as relative reaction rates (Table 28). It was difficult to control the pH of the unbuffered solutions.

Table 28. Spectral results comparing relative reactivities of deoxypyridoxal (DPL), pyridoxal phosphate (PLP), "alpha⁵-pyridoxalylacetate" (PLA), and "5-carboxypyridoxal" (CPL)^a

Pyridoxal analog	-m X 10 ³ A/hr	Initial pH	pH after about (X) days reaction
DPL	5.7	4.6	6.6 (2)
PLP	3.1	4.9	5.4 (1)
PLA	1.0	4.7	4.9 (1)
CPL	0	5.3	-----

^aInitial conditions: 0.10 M leu, 1.0 mM pyridoxal analog, unbuffered, 25°C

The spectrum of leucine and "carboxypyridoxal" was not significantly different from the spectrum of CPL alone, and no changes in either spectrum were detected one day after preparation of the solutions. These spectra had absorption maxima at 320 and 282 m μ , indicating the absence of free aldehyde. Perhaps this preparation of "CPL" crystals was primarily the lactal form, the 4-carboxylic acid or lactone or some other form without a free 4-formyl group. The "carboxypyridoxal" sample had been recrystallized as bright yellow crystals and dried in vacuo. The spectrum of "alpha⁵ pyridoxalylacetate" indicated the presence of about only $\frac{1}{4}$ as much pyridoxal analog as expected from the amount weighed out. This sample had been recrystallized once from water, in the same manner as the sample that had shown a fast reaction at pH 8 (Figure 31). The relative amounts of imine formed were in the order of PLA < PLP < DPL, under these conditions.

Results of analyses by the QH method are indicated by the extents of reaction in Table 29. Also, no reactions were detected with the carboxylate analogs of pyridoxal and ethanolamine. (See Experimental for details.)

Chromatography of reaction solutions

Products of transamination were also identified by thin-layer chromatography of reaction solutions of leucine and pyridoxal analogs, other than deoxypyridoxal (Tables 30, 31).

Table 29. Extent of reaction of leucine with pyridoxal analogs measured by the QH method^a

	A_{305}^b	Relative extent	A_{400}^c	Relative extent	Hours
DPL	0.038	1.0	0.024	1.0	18.5
PLP	0.142	3.7	0.054	2.3	18.25
PLA	0	0	0.008	0.3	18.33
CPL	0	0	0	0	18.0

^a 0.20 ml. aliquots of 1 mM in (PL analog) and (KIC) with 0.5 ml. 1 mM QH in 10 ml. 0.01 N HCl

^b $A_{305 \text{ m}\mu}$, measure of (KIC)(corrected for changes in control sample (-leu))

^c $A_{400 \text{ m}\mu}$, measure of (PL analog) (corrected for changes in control sample (-leu))

These results, in general, for the reactions of leucine with pyridoxal phosphate and with "alpha⁵-pyridoxalylacetate" agreed with those for the reaction of leucine with deoxypyridoxal (Figures 18, 32). However, the pink ninhydrin spots of pyridoxamine phosphate (PMP) and "alpha⁵-pyridoxaminoacetate" (PMA) were more difficult to detect than the orange ninhydrin spot of deoxypyridoxamine, because the former were located just above the purple ninhydrin spot for leucine (See R_F

Table 30. Thin-layer silica gel chromatography of solutions after about 200 hours reaction at 25^{oa}

	R _F values	Spot color
Dinitrophenylhydrazine-treated solutions developed with n-BuOH (5), HOAc (1), H ₂ O (4) :		
DNP-KIC	0.7	yellow
Additional spots after ninhydrin spray:		
leu	0.3	purple
PMP	0.4	pink
PMA	0.4	pink

^aInitial conditions: 50 mM leu, 10 mM PLP, pH 6.4, or 10 mM PLA, pH 8.1, unbuffered

values in Tables 30, 31).

In view of the fact that the more quantitative analytical methods did not detect any reaction of CPL with leucine, the appearance of dinitrophenylhydrazone of ketoisocaproate (along with an unidentified yellow spot) on a chromatogram of this mixture was quite puzzling (Table 31). Failure to sometimes detect transamination products in the other reaction solutions was attributed to insufficient sample size.

Table 31. Thin-layer silica gel chromatography^a of reaction solutions^b

<u>R_F values and spot colors</u>				
Reagents:	Gibbs' + Ninhydrin		DNPH + Ninhydrin	
Spot identity	Sample	Standard	Sample	Standard
<u>PLP + leu</u>	0.22-0.29 brown with pink edges			
PLP		0.31 brown	0.15 yellow	0.36
leu			0.31-0.32 pink	
KIC			0.53 yellow	0.53
PMP (?)	(0.03) brown			
DNPH (2 spots)			0.74-0.83 yellow	0.77-0.81
<u>PLA + leu</u>	0.26-0.30 brown with pink edges			
PLA	0.57 brown	0.57	0.50 (0.31) yellow	0.49(0.28)
leu			0.55 (0.27) pink	
KIC			----- yellow	0.46
DNPH (2)			0.76-0.83 yellow	0.74-0.87
<u>CPL + leu</u>			0.69 yellow	0.73
CPL			0.69 yellow	0.73
leu			0.37 pink	

Table 31. (continued)

Spot identity	R_F values and spot colors			
	Reagents: Gibbs' + Ninhydrin		DNPH + Ninhydrin	
	Sample	Standard	Sample	Standard
KIC			0.43 yellow	0.46
?			0.24 yellow	
DNPH (2)			0.78-0.84	0.80-0.87

^aDeveloped with upper layer of n-BuOH (5), H₂O (4), E OH (1)

^bInitial conditions: 0.10 M leu, 1.0 mM PLP, PLA or CPL, pH 4.7 - 5.4, unbuffered

Nonenzymic Transaminations of Alanine Methyl Ester with Pyridoxal Analogs

The slowness of nonenzymic transaminations of leucine with pyridoxal analogs in unbuffered systems encouraged the search for a more reactive amino group donor (than leucine). Previous studies of nonenzymic transamination of amino acid esters indicated that these amino group donors (in the absence of metal ion catalysts) were at least as effective

Figure 32. Thin-layer silica gel chromatography of reaction solutions of leucine and deoxypyridoxal

Column no.

1	leu
2	DPL
3	KIC
4	DPL + leu near t_e^a
5	DPL
6	DPL + leu near t_e^b

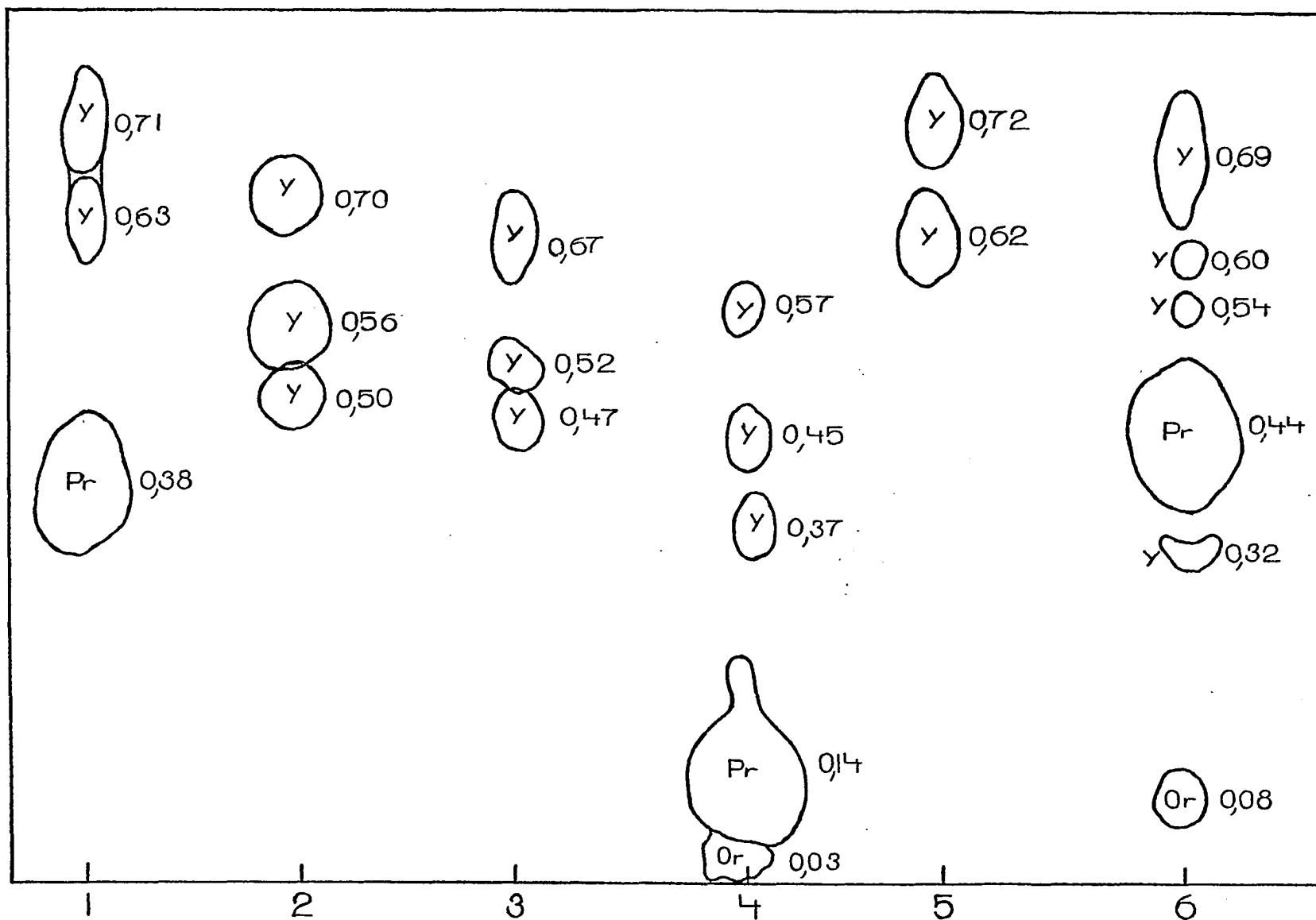
Spots were treated with DNPH before development with n-BuOH (5), water (4), ETOH (1) - upper layer and sprayed with ninhydrin after development

R_F values next to spots calculated for center of spot

Color code: Y - yellow
Pr - purple
Or - orange

^a Initial conditions: 1.0 mM DPL, 0.01 M leu, 0.5 M acetate, pH 4.4, 25°C

^b Same as a, except 0.05 M leu, pH 4.1



as the corresponding amino acids in the presence of metal ion catalysts. Also, metal ions do not catalyze the nonenzymic transamination of amino acid esters (Cennamo, 1964). Alanine methyl ester was chosen because of its greater solubility than leucine esters, as well as leucine, itself. (Higher solubility means a higher ratio of amino group donor to amino group acceptor, resulting in a greater fraction of pyridoxal analog converted to imine, and simplifying the kinetics by imposing pseudo first-order conditions with respect to the pyridoxal analog.)

Stoichiometry

Rates of decrease in concentrations of reactants (free pyridoxal analog plus its imine of alanine methyl ester) followed directly by decreasing absorbancies at 390 to 420 μ were practically equal to rates of increase in concentrations of products (pyridoxamine analogs or ketimines), followed directly by increasing absorbancies at about 325 μ (except in the cases of pyridoxal and pyridoxal methochloride, which already had strong absorption in this region due to the predominance of hemiacetal form). Furthermore, a linear plot of absorbancy at 327 μ against absorbancy at 398 μ throughout the time period of the reaction of 0.10 M alanine methyl ester (AME) with 0.001 M deoxypyridoxal at pH 4.6 was obtained.

The formation of pyruvate methyl ester was not followed by the quinolyldiazotization method because of the very slow

(about four hours compared to one hour for ketoisocaproate) reaction of the keto acid ester with the QH reagent at room temperature. However, the spectrum of the quinolyldiazone of the keto acid ester had a sharp absorption maximum at 300 mμ, which was very similar to that of QH products with free keto acids.

Chromatography of reaction solutions

Evidence for products of nonenzymic transamination of alanine methyl ester with deoxypyridoxal (DPL) and "alpha⁵-pyridoxalylacetate," (PLA) was obtained by chromatography of these reaction solutions on thin-layer silica gel with the developing and spotting techniques previously described for the leucine systems. No such evidence was obtained from a reaction solution of "5-carboxypyridoxal" and AME (Figure 33).

Kinetics

Absorption spectral changes did not follow simple first-order kinetics (although the conditions were pseudo first-order, with respect to deoxypyridoxal), since the reaction proceeded to an equilibrium point at greater than 80% conversion of deoxypyridoxal to deoxypyridoxamine, by an excess of the amino group donor. However, Guggenheim plots were linear and pseudo first-order rate constants were calculated, despite decreases in pH of the unbuffered solutions during the reactions (Table 32). The reactions were followed spec-

Figure 33. Thin-layer silica gel chromatography of reaction solutions^a

Column no.

- | | |
|---|---|
| 1 | AME |
| 2 | DPL |
| 3 | DPL + AME at t_e |
| 4 | PLA |
| 5 | PLA + AME near t_e (after about 1 day reaction) |
| 6 | CPL |
| 7 | CPL + AME near t_e |

DNPH reagent added to each spot before development with n-BuOH (5), water (4), EtOH (1)-upper layer; sprayed with ninhydrin after development

R_F values next to spots calculated for center of spot

Color code: Y - yellow

Pr - purple

Pk - pink

Or - orange

^a Initial conditions: 0.20 M alanine methyl ester (AME), 1.0 mM DPL, PLA or CPL, pH 7.5 unbuffered, 25°C

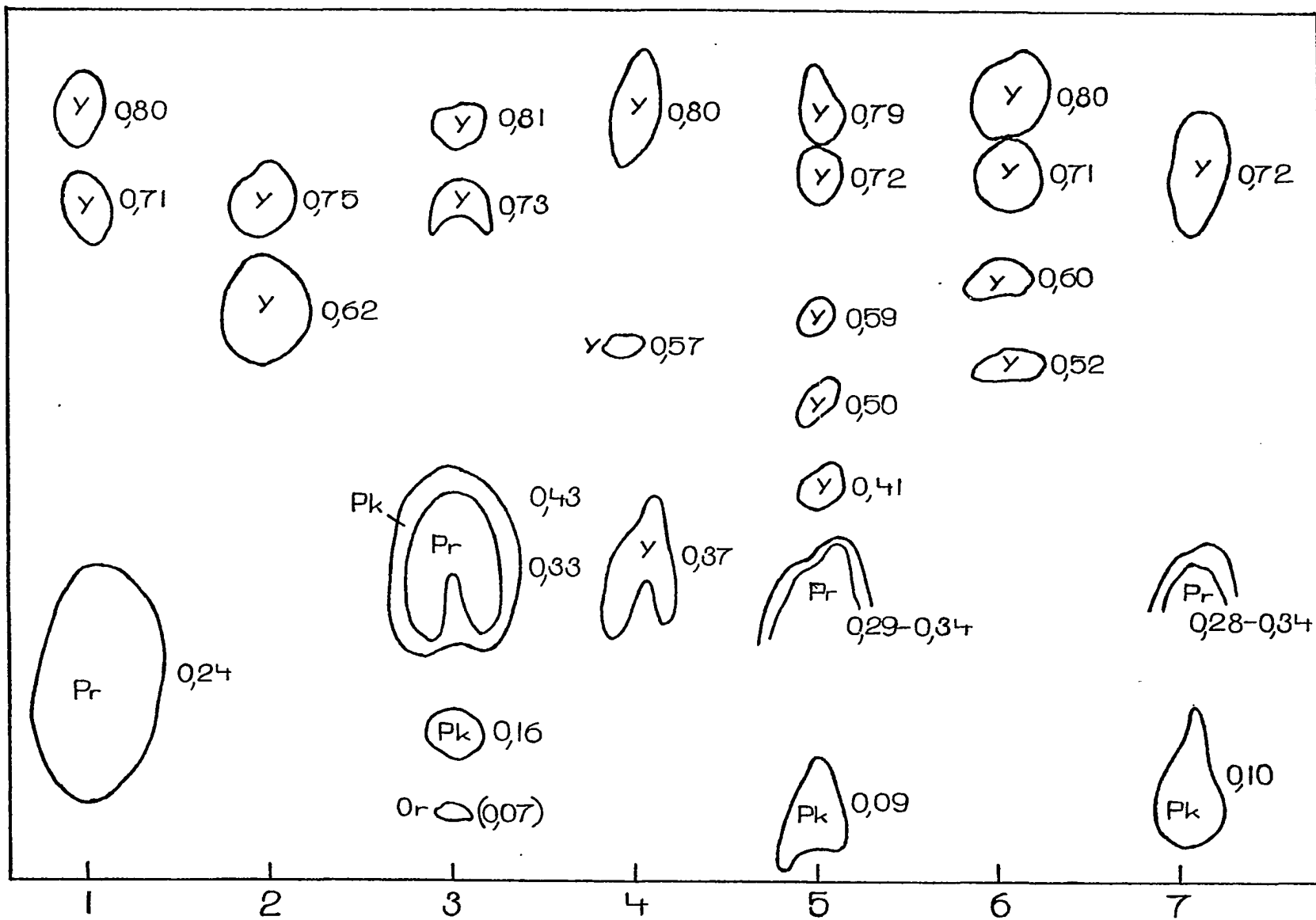


Table 32. Pseudo first-order rate constants (k_{obs}) for non-enzymic transaminations of alanine methyl ester (AME) with pyridoxal analogs in unbuffered aqueous solutions, 25°C^a

No.	Initial conditions		Spectral changes followed at wavelengths (mμ)	$k_{\text{obs}} \times 10^5/\text{sec.}$	pH after t (hrs.)
	pH	(PL analog)			
1	5.1	DPL	-415, -400, +325	10.4 \pm 0.3	4.2 (5)
	4.6	DPL	-398, +327	9.2 \pm 0.2	4.4 (13)
2	4.5	DPL	-415	12.4 \pm 0.5	3.5 (24)
3	4.0	PLP	-420, -415, -400 +325	16.9 \pm 0.2	3.85 (5)
4	4.9	PL	-405	5.6	3.6 (27)
5	4.5	PL	-400	3.6	3.5 (27)
6	4.5	PLM	-405	4.3	3.5 (27)
7	7.5	PL	-400	3.9	7.2 (3)
					5.5 (22)
8	7.5	PLM	-405	25	7.2 (3)
					5.5 (22)
9	6.8	PLP	-400, +325	13.1 \pm 0.5	6.5 (2.5)
10	7.2	PLA	-415, +335	2.4 \pm 0.6	6.65 (6.5)
11	7.2	CPL	-328, +275	negligible	5.6 (29)

^a Initial conditions: 0.50 M AME, 1.0 M PL analog, except for 0.10 M AME in reactions 1 and 0.5 mM PL in reaction 4

trally in about 5 to 30 hours to completion at 25°C.

Rates of reaction started at pH 4 to 5

The pseudo first-order rate constant for the reaction of 0.5 M alanine methyl ester (AME) with 1.0 mM deoxypyridoxal (DPL) was not much greater than that for the reaction of 0.1 M AME with 1.0 mM DPL (Table 32; reactions 1, 2). Pyridoxal phosphate (PLP) is a better amino group acceptor than deoxypyridoxal under similar conditions, since the rate constant for the reaction of PLP with AME was about 1.3 to 1.4 times greater than that for the reaction of DPL with AME (Table 32; reactions 2,3). This may be further evidence for intramolecular acid-base catalysis of aldimine to ketimine conversion by the phosphate group of PLP. Pyridoxal (PL) and its N-methyl analog (PLM) are poorer amino group acceptors under similar conditions, since the rate constants of their reactions with AME are only about one-third as great as that of the reaction of DPL with AME (Table 32; reactions 2,4-6). This is further evidence that pyridoxal and analogs with 5-hydroxymethyl groups have decreased reactivity toward amino group donors, because their aldehyde groups are largely tied up as internal or cyclic hemiacetals.

Rates of reactions started at neutral pH

The comparison of pseudo first-order rate constants for the reactions of pyridoxal (PL) and pyridoxal methochloride (PLM) with alanine methyl ester, which were started at pH 7.5,

was striking evidence that the aldimine protonated at the pyridine ring nitrogen atom (PLM) is much more reactive (by a factor of 6 times under these conditions) than the aldimine which is unprotonated (PL) (Table 32; reactions 7, 8). However, the rate constant for the pyridoxal reactions did not decrease significantly at this pH from that at the lower pH. The rate constant for the reaction with PLP in neutral solution did decrease somewhat, as expected from dissociation of the pyridinium group of the most reactive imine, even though more imine was formed as expected from the formation constant as a function of pH (Figure 15), and as evidenced by the 15 μ higher absorption band (Table 32, reaction 9).

The rate constant for the reaction of PLA with AME was less than one-fifth as great as that of the reaction of PLP with AME under these conditions. The reaction of CPL with AME was negligible after one day, indicating negligible amounts of free aldehyde in the CPL preparation that was used. It was likely also that the preparation of PLA was of questionable purity, since greater reaction rates were observed with PLA than with PLP and leucine. These preparations had been stored as aqueous stock solutions in the frozen state, and these solutions were adjusted to about pH 8 to favor the free aldehyde form, by opening up the lactal ring, after thawing and before adjusting the pH of the reaction solution with AME to neutrality. This procedure is expected to be successful in yielding a high proportion of

reactive free aldehyde, if the preparation contains potential aldehyde bound in the form of an internal lactal with the adjacent carboxylate group.

In general, the rate constants for the reaction of pyridoxal with alanine methyl ester were only about one-third as great as those obtained for the nonenzymic transamination of L-alanine with pyridoxal at 100°C in unbuffered solutions (Table 33; Blake *et al.*, 1963). They were about $1\frac{1}{2}$ times greater than that of the reaction of pyridoxal with alanine at 25°C (Banks *et al.*, 1961), although the latter were measured at pH 10 in N,N-dimethylglycine buffer, which catalyzed the reaction. And they were about one-third as great as those for the reaction of phenylglycine and pyridoxal, catalyzed by 1.8 M imidazole at pH 8.6 but slightly higher than that for the same reaction in 1.0 M imidazole (Bruce and Topping, 1963a), and 15 times greater than those for the reaction of alanine and pyridoxal, unbuffered at pH 8. (Fleck and Alberty, 1962).

Furthermore, alanine methyl ester systems were more reactive than leucine model transamination systems (Table 34). The unbuffered system of the ester and pyridoxal had a pseudo first-order rate constant of about $1\frac{1}{2}$ times greater than that of leucine and pyridoxal catalyzed by aluminum ion and acetate buffer. The rate constant for the reaction of the ester with pyridoxal phosphate was about 17 times greater than that for leucine and PLP (both reaction solutions being unbuffered).

Table 33. Comparison of selected rate constants for non-enzymic transamination of pyridoxal with amino acids under various conditions

Initial conditions	$k_{\text{obs}} \times 10^5 \text{ sec.}^{-1}$	Reference
0.20 M L-ala		Blake <u>et al.</u> , 1963
0.01 M PL	14.8	
pH 4.15, unbuffered		
100°C		
+ 0.10 M HOAc + 0.10 M NaOAc	28.5	
+ 5×10^{-5} M Alum	50	
0.17 M ala	0.26	Fleck and Alberty, 1962
10^{-4} M PL		
pH 8.0, 0.05 M NaOAc		
25°C		
0.2 M Ala	2.6	Banks <u>et al.</u> , 1961
0.01 M PL		
pH 10, 0.1 M N, N- dimethylglycine		
25°C		
10^{-4} M = ϕ gly = PL	14.7	Bruice and Topping, 1963a
pH 8.6, 1.8 M Im		
10^{-3} M = ϕ gly = PL, 1.0 M Im	3.0	
0.1 M Im	0.08	
30°C		

Table 33. (continued)

Initial conditions	$k_{\text{obs}} \times 10^5 \text{ sec.}^{-1}$	Reference
0.50 M Ala methyl ester	3.6-3.9	(Bresnahan and Albert, 1964) ^a
1.0 mM PL		
pH 7.5 or 4.5, unbuffered		
25°C		

^a Most of these results with AME (Tables 32 and 34) were reported at the Seventh West Central States Biochemistry Conference, November 7, 1964, Iowa City Iowa, Sheryl Bresnahan, Jerry Albert, and David E. Metzler. Non-enzymic transamination of alanine methyl ester and leucine with pyridoxal analogs

Table 34. Comparison of pseudo first-order rate constants for nonenzymic transamination of alanine methyl ester and of leucine with pyridoxal analogs

Leu Initial Conditions	$k_{\text{obs}} \times 10^5 \text{ sec.}^{-1}$ 25°C	AME Initial conditions
0.120 M Leu		0.50 M AME
10 mM PL	2.7	1.0 mM PL
2.0 mM alum		pH 4.5, unbuffered
pH 4.2, 0.1 M acetate buffer		

Table 34. (continued)

Leu Initial conditions	k_{obs}	$\times 10^5 \text{ sec.}^{-1}$ 25°C	AME Initial conditions
0.10 M leu			0.50 M AME
20 mM PLP			1.0 mM PLP
pH 3.9, unbuffered	0.95	16.9 ± 0.2	pH 4.0, unbuffered
0.05 M leu			
10 mM PLP			
pH 8.6, 1.0 M imidazole	0.25		
pH 8.3, 1.0 M phosphate	0.14		
0.10 M leu			0.50 M AME
10 mM DPL			1.0 mM DPL
pH 4.2, unbuffered	0.34	12.4 ± 0.5	pH 4.5, unbuffered
pH 4.2, 1.0 M acetate buffer	2.8		
pH 4.2, 1.0 M acetate buffer and 1.0 mM Cu(II)	4.7		
0.01 M leucine ethyl ester ^a			
0.1 mM DPL			
0.10 M phosphate, pH 6.0	2.1-		
	4.3		
1.0 M phosphate	5.2		

^aRobert Johnson, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Non-enzymatic transamination of leucine ethyl ester with pyridoxal analogs. Private communication. 1964.

And the rate constant for the reaction of the ester with deoxypyridoxal was about 37 times greater than that for leucine and DPL (both reaction solutions being unbuffered). The ester reaction in unbuffered solutions is still about 4 to 5 times greater than the leucine reaction in the presence of 1.0 M acetate buffer and about $2\frac{1}{2}$ to 3 times greater than the latter with an additional cupric ion catalyst. The rate constant for the reaction of DPL with AME was about 3 times greater than that with leucine ethyl ester buffered with phosphate (Table 34).

Alanine methyl ester has been probably one of the best amino group donors (for which quantitative rate constants have been obtained) for comparing reactivities of pyridoxal analogs in unbuffered solutions at 25°C. Not only were the reactions fast enough to be practically followed to completion in 5 to 30 hours, but contaminating metal ions do not interfere (a problem in unbuffered amino acid systems). Other advantages of the alanine methyl ester system include its high solubility, permitting measurements of pseudo first-order rate constants (first-order with respect to small amounts of pyridoxal analogs, which conserves the precious synthetic compound), and the ease of measurement of the rate constant by direct absorption spectral data (using cell spacers, if necessary) with the Guggenheim method, without apparently any side reactions or unexplainable stoichiometry (which may be observed with the slower reacting amino acid

systems).

The enhanced reactivity of amino acid esters over free amino acids as amino group donors in nonenzymic transamination systems has been explained on the basis of eliminating the negative charge of the carboxyl group (Cennamo, 1964; Guirard and Snell, 1964). Metal ion chelation is prevented by loss of the carboxylate ligand, but inductive withdrawal of electrons around the alpha-carbon in the amino acid residue of the aldimine is enhanced by loss of the negative charge on the carboxyl group in amino acid esters. It has further been suggested that transaminases enhance the transamination of amino acids in a similar manner, by blocking the negative charge of the carboxyl group.

Increases and decreases in absorption maxima of imines of pyridoxal phosphate and peptides, amino acid esters or proteins were observed within about an hour after mixing (Christensen, 1958). These spectral changes were incorrectly attributed to formation of certain hydrated and ionic forms of the imines. More recent evidence has been presented for the assignment of these same absorption bands to the tautomeric ketoenamine and enolimine forms (A, B-Figure 4; Review of Pertinent Literature; Martell, 1963). Although the spectral-time changes observed by Christensen have been confirmed (William P. Jencks, Ithaca, New York, Cornell University, Department of Chemistry. Spectral-time changes for imines of pyridoxal phosphate and substituted amino acids.

Private communication. 1964), a satisfactory interpretation has not been presented.

These findings cast some doubt on the reported nonenzymic transaminations of pyridoxal analogs with glycine ethyl ester (Tomita and Metzler, 1964), leucine esters (Johnson, 1964; Cennamo, 1964), and with alanine methyl ester in this laboratory. However, in contradiction to this possibility, Christensen recovered pyridoxal completely by the ethanolimine method after his spectral changes approached equilibrium, whereas decreases of pyridoxal or of pyridoxal analogs were followed by this same method (Johnson, 1964; Cennamo, 1964). In addition, keto acid was detected in the reaction mixture at 100°C by the dinitrophenylhydrazine method (Cennamo, 1964). Products of nonenzymic transamination of pyridoxal analogs and alanine methyl ester also were detected by thin-layer chromatography and by the quinolyhydrazine method.

At present this author believes that, in general, either nonenzymic transamination or at least ketimine formation reactions were followed, but that the spectral phenomena observed by Christensen may be side reactions that might account for the stoichiometric and kinetic anomalies observed during some of these transamination reactions. Ketimine formation and the slow breakdown of ketimine to products could explain loss of pyridine aldehyde, the spectral changes, and the difficulty experienced in detecting transamination products by thin-layer chromatography. Perhaps, the ketimines of

amino acid esters and pyridoxal analogs are more stable than those of free amino acids, because a free carboxylate group catalyzes or enhances the hydrolysis of ketimine to products. This would imply that imidazole had a similar effect on the free carboxylate group of phenylglycine in its ketimine with pyridoxal since an unusually slow hydrolysis to products was reported (Bruice and Topping, 1963a).

Deuterium Isotope Effect on the Rate-Limiting Step in Nonenzymic Transamination

The tri-deuterated isotope of leucine containing deuterium atoms instead of the protium atoms in the alpha- and beta-positions (D_3 -leu) was compared with protio-leucine (H_3 -leu) in two different model transamination systems. With deoxypyridoxal as the amino group acceptor and imidazole as the buffer and catalyst the reaction with H_3 -leucine was about fifteen times faster than that with D_3 -leucine based on the ratio of second-order rate constants, and about ten times faster, based on the inverse ratio of times for 10% decrease in deoxypyridoxal (Table 35). The reaction of D_3 -leucine proceeded about $1\frac{1}{2}$ times faster in approximately 50% D_2O . Acid-base catalysis by D_2O is suggested, since the dissociation constant for D_2O is less than that for H_2O .

With pyridoxal phosphate as the amino group acceptor and acetate as the buffer and catalyst the reaction with H_3 -leucine was about 8 times faster than that with D_3 -leucine

Table 35. Deuterium isotope effect in transamination of leucine with deoxypyridoxal^a

Apparent second-order rate constants X 10 ⁻⁴ /M/sec.		Relative rates k _H /k _D	
H ₃ -leu	17.5 ±1.1		15.3
D ₃ -leu (in H ₂ O)	1.14 ±0.14		1.0
D ₃ -leu (in 50% D ₂ O)	1.95 ±0.13		1.7
	<u>Time for 10% reaction^b</u>	<u>% DPL lost/ hrs.</u>	
H ₃ -leu	1.0 hr.	45%/8 hrs.	9.7
D ₃ -leu (in H ₂ O)	9.7 hrs.	18%/24 hrs.	1.0
D ₃ -leu (in 50% D ₂ O)	6.6 hrs.	25%/24 hrs.	1.4

^aInitial conditions: 30 mM leu, 20 mM DPL, 1.0 M imidazole, pH 6.8 - 7.0, 25°

^bFrom plots of % decrease in deoxypyridoxal vs. time in hours, followed by the ethanolimine method

(Table 36; Figure 34), based on the ratio of inverse times to complete a certain percentage of the reaction between 26 and 38%. The ratio of rate constants or initial rates, substantiated by a Guggenheim plot of the data from the protio-

Table 36. Deuterium isotope effect in transamination of leucine with pyridoxal phosphate^a

A) Decreases in (PLP) were followed by ethanolimine (EOA) method and calculations were made from plots of A_{350} vs. t hrs.; for H_3 -leu, assumed A_o (A at t_o) = 1.32 and A_e (A at t_e) = 0.09; for D_3 -leu assumed $A_o - A_e = 1.23$ and $A_o = 1.34$; then chose points:

For D_3 -leu	A_{350}	$(A_o - A_t)$	% $(A_o - A_t)$	t hrs.	$\frac{(1/t_H)}{(1/t_D)} = k_H/k_D$
	1.00	0.37	30.0	30	7.5
	1.05	0.32	26.0	24	7.5
For H_3 -leu	0.95	0.37	30.0	4.0	
	1.00	0.32	26.0	3.2	

B) Decreases in (PLP) were followed by QH method and calculations were made from plots of A_{400} vs. t hrs.; for H_3 -leu, assumed $A_o = 0.465$, $A_e = 0.105$; for D_3 -leu, assumed $A_o - A_e = 0.36$ and $A_o = 0.450$: then chose points:

For D_3 -leu	A_{400}	$(A_o - A_t)$	% $(A_o - A_t)$	t hrs.	k_H/k_D
	0.315	0.135	37.5	30	7.5
	0.340	0.110	30.5	24	8.6
For H_3 -leu	0.330	0.135	37.5	4.0	
	0.355	0.110	30.5	2.8	

^aInitial conditions: 75 mM leu, 10 mM PLP, 1.0 M acetate buffer, 25°, pH 3.88 for H_3 -leu, pH 3.78 for D_3 -leu

Table 36. (continued)

C) Formation of ketoisocaproate was followed by QH method and calculations were made from plots of $A_{305 \text{ m}\mu}$ vs. t hrs.: for H_3 -leu, assumed $A_o = 0.120$, $A_e = 0.475$; for D_3 -leu, assumed $A_o - A_e = 0.355$ and $A_o = 0.115$; then chose points:

For D_3 -leu	A_{305}	$(A_o - A_t)$	$\%(A_o - A_t)$	t hrs.	k_H/k_D
	0.230	0.115	32.4	30	6.7
	0.185	0.070	19.8	24	12
	0.160	0.045	11.3	6	6

For H_3 -leu

0.235	0.115	32.4	4.5
0.190	0.070	19.8	2.0
0.165	0.045	11.3	1.0

Average $k_H/k_D = 8.0 \pm 1.3$

leucine reaction, was in agreement with the estimated relative rates (Table 37).

This deuterium isotope effect on the rate of nonenzymic transamination of leucine with pyridoxal analogs directly confirmed the previous kinetic findings of others that the rate-determining step involves the cleavage of the alpha-carbon-hydrogen bond in the amino acid-alimine of pyridoxal. The over-all rate of transamination of glutamate was reduced, as predicted by the binary mechanism and by model system studies, in which the tautomerization of alimine to ketimine

Figure 34. Deuterium isotope effect in transamination of leucine with pyridoxal phosphate^a

- PLP_H, decrease in (PLP) from reaction with H₃-leu followed by EOA method
- PLP_H, decrease in (PLP) from reaction with H₃-leu followed by QH method
- △ KIC_H, increase in (KIC) from H₃-leu followed by QH method
- PLP_D, decrease in (PLP) from reaction with D₃-leu followed by EOA method
- PLP_D, decrease in (PLP) from reaction with D₃-leu followed by QH method
- ▲ KIC_D, increase in (KIC) from D₃-leu followed by QH method

^aReaction conditions: Table 36

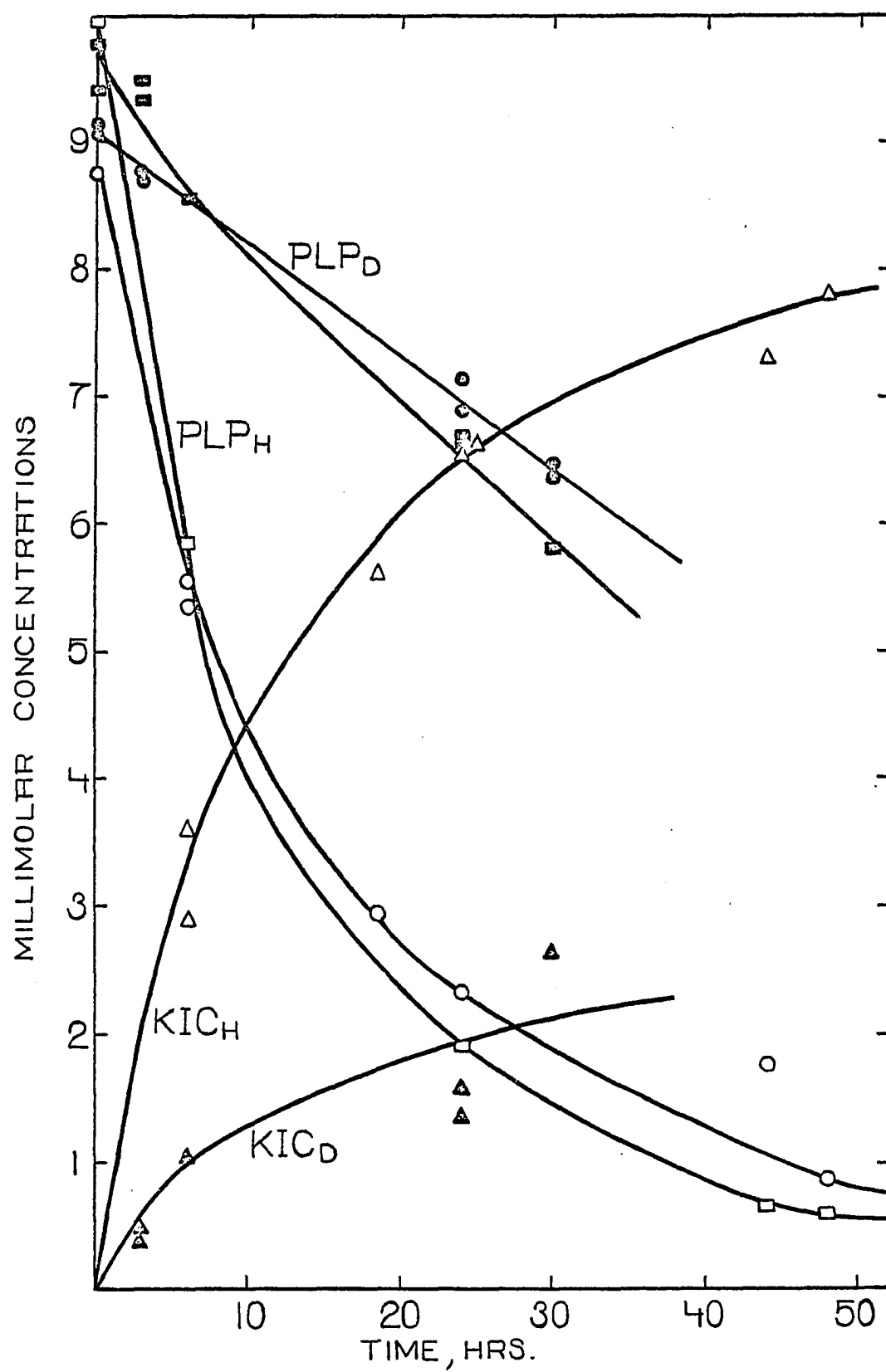


Table 37. Comparison of H₃-leu and D₃-leu based on calculated rate constants from data of Figure 34

	$k_{\text{obs}} \times 10^6/\text{sec.}$	from $\log (A' - A)$ vs. t (Guggenheim plots) ^a
H ₃ -leu	16.6	350 mμ
	16.6	305
	17.2	400
$k = (dp/dt)^b / (0.01 \text{ M PLP}) \times 10^6/\text{sec.}$		$k_{\text{H}}/k_{\text{D}}$
	22	22/3.2 = 6.9
D ₃ -leu	3.2	16.6/3.2 = 5.2

^aBest straight lines through points, discarding initial and final points which "curve off"

^bInitial rates obtained graphically from $A_{350 \text{ m}\mu}$ vs. t

intermediate is the rate-determining step (Review of Pertinent Literature), when alpha-deuterio-glutamate was a substrate for glutamic-aspartic transaminase (Banks et al., 1963; Vernon, 1964).

Smaller isotope effects were observed by Blake et al. (1963) for the nonenzymic transamination of L-deuterio-alanine (containing deuterium atoms in the alpha-and-beta-positions) with pyridoxal at 100°C. However, the isotope

rate ratio k_H/k_D of 2.3 increased with an increase in catalyst concentration (buffer or metal ion); e. g., k_H/k_D was found to be 3.5 in the presence of aluminum ion (Table 38). General acid-base catalysis by D_2O was also suggested from the latter results: $k(\text{H-ala in } H_2O)/k(\text{H-ala in } D_2O) = 3$ and k_H/k_D in $H_2O = 2.2$, but in $D_2O = 2.9$.

Table 38. Deuterium isotope effects in transamination of L-alanine and pyridoxal, 100°C (Blake et al., 1963)

Additional conditions ^a	k_H		k_H/k_D
	$k_1 \times 10^4/\text{sec.}$	$k_2 \times 10^4/\text{M/sec.}$	
unbuffered	1.48	7.40	2.2 ± 0.1
with 0.10 M HOAc, 0.10 M NaOAc	2.85	14.25	2.4 ± 0.06
with 5×10^{-5} M alum.	5.0	25	3.5 ± 0.02
in D_2O (99%)	4.25	21.25	2.9 ± 0.02

^a Initial conditions: 0.01 M PL, 0.20 M L-ala, pH 4.15

The surprisingly large isotope effects observed in the nonenzymic transamination of D_3 -leucine with pyridoxal phosphate and with deoxypyridoxal in 1 M buffers are probably due to general acid-base catalysis by the high concentrations of acetate and imidazole buffers. The effect of buffers on the

isotope ratio may be checked by varying the buffer concentration. The fact that the isotope effect was found to be two times greater for deoxypyridoxal than for pyridoxal phosphate, as the amino group acceptor, may be due to the greater buffer catalytic constants found for deoxypyridoxal compared to pyridoxal phosphate. Also, the nature of the buffer and the pH differed in the two experiments.

Furthermore the isotope ratio could be increased by an increase in concentration of aldimine according to Blake et al. (1963). This would qualitatively account for the differences in the observed isotope ratios in the nonenzymic transaminations of D₃-leucine with deoxypyridoxal and pyridoxal phosphate and of D₄-alanine with pyridoxal, because the imine formation constant for deoxypyridoxal and leucine is about 2 to 3 times greater than that for pyridoxal and leucine (Figure 15), and about two orders of magnitude (100 times) greater than that for pyridoxal and alanine.

A value of k_H/k_D of 7.4 at 25°C is expected if only the stretching frequencies of the carbon-protium and carbon-deuterium bonds are considered: $k_H/k_D = e^{(hf_H - hf_D)/2kT}$. This however, is only a simplification which ignores many possible effects in solution other than the immediate bonds which are broken (Levine, 1963).

"For acid-base reactions of hydrogen bound to carbon, the ratio k_H/k_D has always been found to be as large as 2.7 and sometimes as large as 10. Such large effects can be used

as evidence that a hydrogen atom transfer is rate-determining.²⁴ (Frost and Pearson, 1961, p. 344). The prediction of such isotope rate constant ratios depends on the theoretical equation for the rate and on the approximations included. For the most exact equation, $k_H/k_D = 1/0.058 = 16.7$, at 25°, in the gas phase, neglecting solvent effects. Furthermore, large deuterium-isotope effects have been reported ranging from 10 to 26, for k_H/k_D (Melander, 1960).

Future Pyridoxal Analogs Possibly Capable of Enhanced Intramolecular Catalysis of Nonenzymic Transamination

Nonenzymic transamination reactions between pyridoxal phosphate and amino acids and between pyridoxamine phosphate and keto acids are in progress (Vernon, 1964). Another recent report on studies of general-base catalysis in model transamination reactions has revealed that the reactions of other (simpler) pyridoxal analogs, such as 3-hydroxypyridine-4-aldehyde, with amino acids are being measured in model systems (Bruice, 1964). Although these studies with relatively common or simple pyridoxal analogs are necessary to firmly establish the characteristics of nonenzymic transamination quantitatively, the author believes that the most exciting approach to enzymic catalysis is the study of synthetic pyridoxal analogs with side chains having functional groups capable of intramolecular acid-base catalysis of the intermediate imines with amino acid esters.

Studies of such pyridoxal analogs depend upon their successful syntheses. Syntheses of the "classical" pyridoxal analogs, such as deoxypyridoxal and pyridoxal phosphate, and other vitamin B₆ compounds--are outlined in Figure 35. Presumably, the N-methyl analog of deoxypyridoxal, XIII, could be synthesized also, in a simpler manner than the synthesis of the N-methyl analog of pyridoxal (II), as depicted in this Figure. From the model system studies it became apparent that the effect of a positively-charged pyridine nitrogen atom on the rate of nonenzymic transamination could best be observed with the N-methyl analogs of deoxypyridoxal, XIII, or of pyridoxal phosphate XII, than with pyridoxal-N-methochloride. These two N-methyl analogs, XII and XIII, would not only be useful for study in model systems but also would be interesting for comparison of their spectra, their binding, and activity with apoenzymes of pyridoxal phosphate-dependent enzymes.

The 5-carboxylate analogs of pyridoxal, IX and XI, were more difficult to obtain and keep as the free aldehyde forms, probably due to internal or cyclic lactal formation (Figure 36). It seems likely that these compounds would react best with amino group donors in model transamination systems in neutral or alkaline solution, since acidic conditions favor lactal formation. These reaction rates were sometimes higher and sometimes lower than those for reactions with deoxypyridoxal and pyridoxal phosphate, depending upon the conditions

Figure 35. Syntheses of some pyridoxal analogs, indicating commercially available form (C-)::

- XIV pyridoxol (PO) (C-hydrochloride) from pyridoxamine (PM), VI (C-dihydrochloride), (Wagner and Folkers, 1964)
- I pyridoxal (PL) (C-hemiacetal hydrochloride) from pyridoxal (Brooks, 1960)
- IV 5-deoxypyridoxal (DPL) from pyridoxal (Heyl et al., 1953), from pyridoxal (by Dr. Isao Tomita in this laboratory), or from pyridoxamine, via deoxypyridoxamine (DPM), VIII (not shown), (Kuroda, 1964)
- V pyridoxal-5-phosphate (PLP) (C-monohydrate) from pyridoxamine, via pyridoxamine-5-phosphate (PMP), VII, (C-dihydrochloride), (Heyl et al. 1951; Peterson et al., 1953; Peterson and Sober, 1954; Kuroda, 1963a)
- II pyridoxal-N-methochloride (PLM), (Brooks, 1960; Heyl et al., 1951)
- XII N-methopyridoxal-5-phosphate (MPLP), from pyridoxal (Heyl et al., 1951)
- XIII 5-deoxypyridoxal-N-methochloride (DPLM), from deoxypyridoxal (proposed)

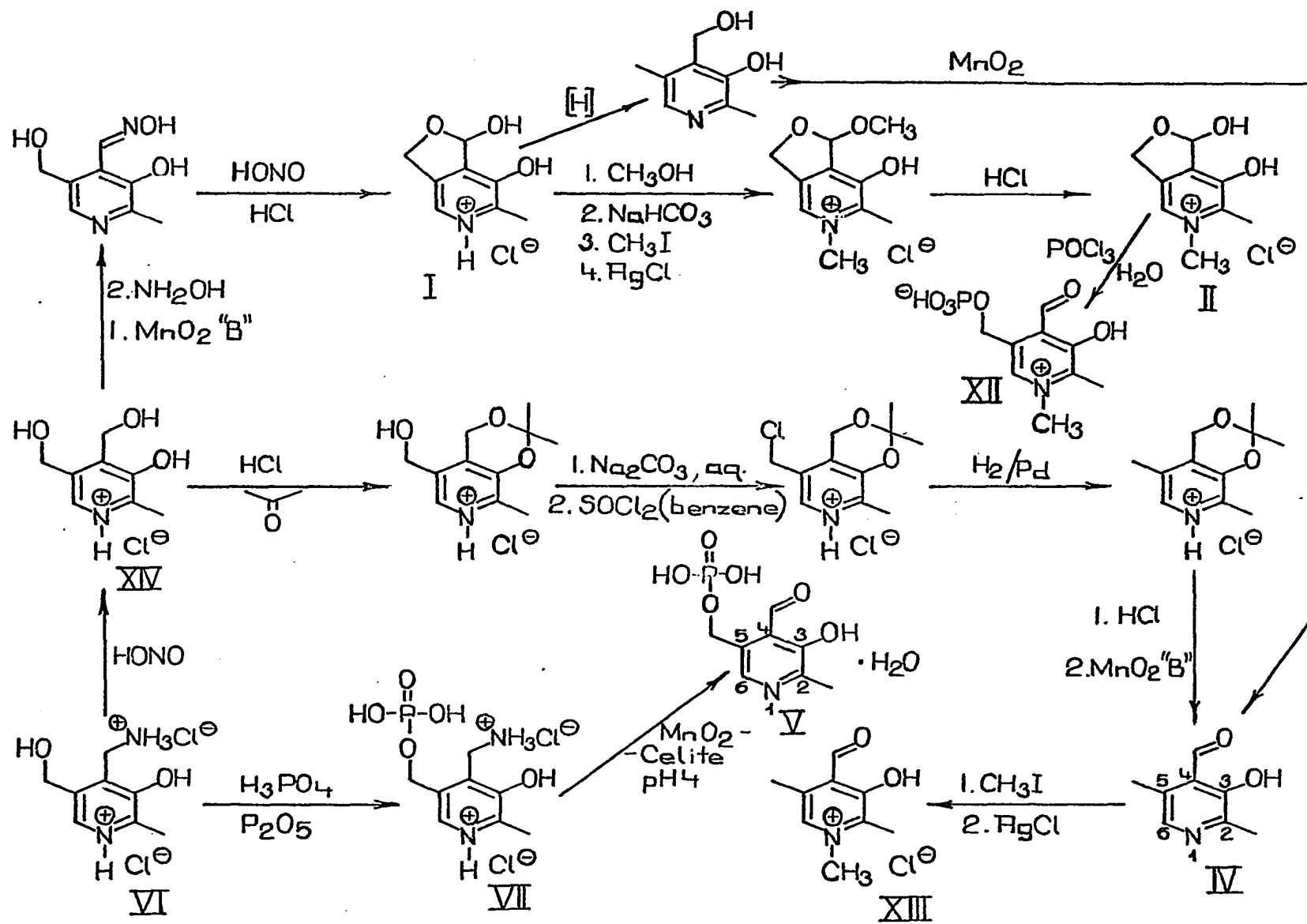
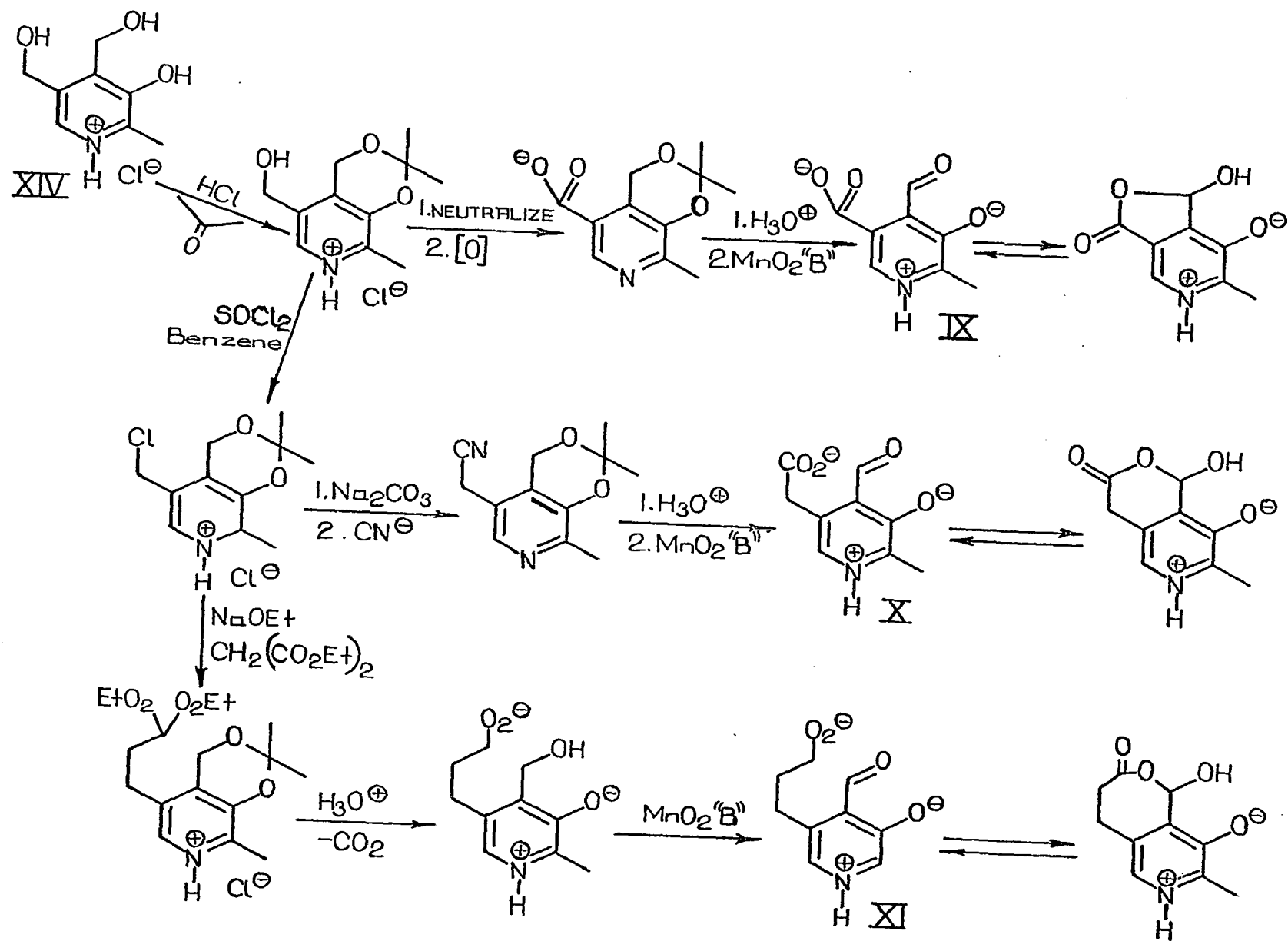


Figure 36. Syntheses of 5-carboxylate analogs of pyridoxal from pyridoxol:

- IX "5-carboxypyridoxal" (CPL) via an isopropylidene pyridoxol
- X " α^5 -pyridoxalylformate" (FPL or PLF) via a 5-chloromethyl isopropylidene pyridoxal (Tomita et al., 1964)
- XI " α^5 -pyridoxalylacetate" (APL or PLA) via a 5-chloromethyl isopropylidene pyridoxal

(Isao Tomita, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Syntheses of 5-carboxylate analogs of pyridoxal. Private communication. 1964).

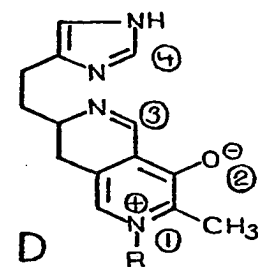
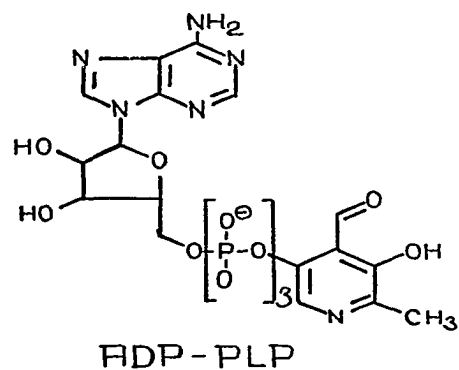
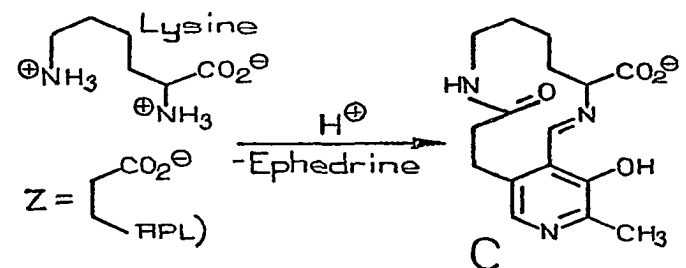
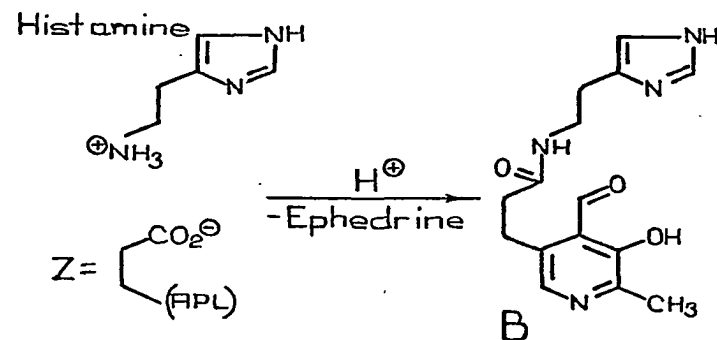
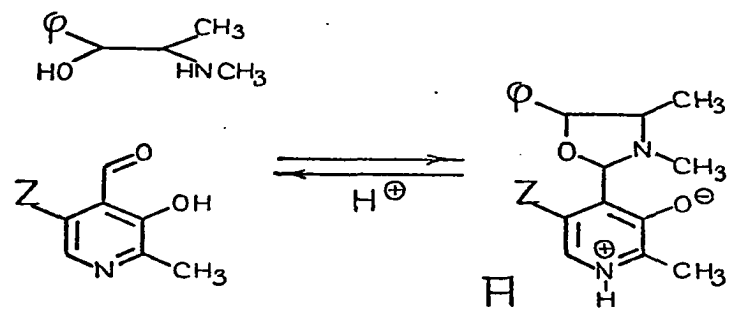


of pH and concentration and relative amount of free aldehyde in the preparation of the carboxylate analog used. More quantitative measurements await further purification of these analogs.

These carboxylate analogs may be considered more valuable as intermediates en route to pyridoxal analogs with substituents at the 5-position capable of carrying out acid-base catalysis of their intermediate imines with amino acids. For example, amide linkages may be formed between the carboxylic acid side chain and bases such as histamine, which would yield a pyridoxal analog with an imidazole group at the end of this side chain. Intermolecular, general acid-base catalysis of nonenzymic transamination by imidazole buffer has been studied and discussed. An imidazole group in a side chain attached to pyridoxal may be even more efficient as an intramolecular acid-base catalyst of the nonenzymic transamination (Figure 37-B). Synthesis of such pyridoxal analogs may begin with protection of the 4-formyl group, with ephedrine, which is easily removed by a pH adjustment (Figure 37-A).

In this scheme, an ester linkage may also be formed with the carboxylate side chain, and other acidic or basic functional groups at the other end of these alcohol side chains may be introduced such as the guanidino, amino, triazolic or tetrazolic groups. Attention may have to be given to determining the appropriate atomic length of the side chain which

Figure 37. Possible coenzyme analogs or model active sites of transamination of amino acids; syntheses suggested starting with ephedrine as protective agent of the 4-formyl group of pyridoxal analogs with 5-carboxylate side chains



will be capable of acting upon the proton-transfer sites of the imine with maximum catalytic enhancement. Particular side chains with such functional groups may give specific catalytic activities to the corresponding pyridoxal analogs.

Transaldimination may enhance the rate of the nonenzymic transamination by activating the formyl carbon atom through a preformed intramolecular aldimine. An example of such a pyridoxal analog may be model compound C-Figure 37. By substituting transaldimination for imine formation between a pyridoxal analog and an amino acid, the enzymic environment in transaminases is approached even more closely.

A methyl substituent at the pyridine ring nitrogen atom may be all that is necessary to maintain a strong positive charge on the nitrogen atom and to increase its electrophilicity, and thus, enhancement of the catalysis of the bond-breaking process in the imine. All of these above aspects of intramolecular catalytic functions are incorporated into model compound D-(Figure 37): 1) substituent on pyridine ring nitrogen atom resulting in increased electrophilicity, 2) unsubstituted phenolate group, 3) activated formyl carbon atom due to preformed intramolecular aldimine, requiring a transaldimination reaction with an amino acid substrate, 4) imidazole or other acid-base group with appropriate side chain length to effect intramolecular catalysis of the reaction of an amino acid imine intermediate.

A possible PLP coenzyme analog with a nucleotide "handle" is the synthetic PLP-ADP compound of Kuroda (1963b). This analog may be interesting to study in nonenzymic as well as in enzymic systems. Such pyridoxal analogs suggested here may also prove to be potent inhibitors of PLP-dependent enzymes and may possess pharmacological activities.

SUMMARY OF CONCLUSIONS

Certain pyridoxal analogs have been studied in aqueous solution, and the extents and rates of their reactions with amino acids have been measured. Rate constants for these non-enzymic transamination reactions are compared and correlated with results reported in the literature.

The absorption spectrum of pyridoxal-N-methochloride at pH values near 4 was found to represent a mixture of two ionic forms, and measurements of the absorbancies at 293.5 and 323 m μ at several pH values near 4 permitted a precise evaluation of the apparent pK_a value of 4.05 ± 0.03 for dissociation of the phenolic group. This was compared to the corresponding value of 4.20 for pyridoxal, suggesting the effect of N-methylation on the acidity of this group.

The equilibrium constant for the formation of the hydrogen-bonded aldimine of this pyridoxal analog with valine was quantitatively measured and the $\log K_1'$ value of -0.02 ± 0.1 was found. This was compared to the corresponding value of -0.27 for pyridoxal. Similarly, relatively less imine was formed from pyridoxal methochloride and leucine than from pyridoxal and leucine in neutral or alkaline solution. The explanation is that a positive charge on the pyridine ring nitrogen atom inhibits resonance structures with a positive charge in positions on the ring needed to stabilize the polarization of the carbonyl group, since the formyl carbon atom

is the nucleophilic site of attack by the amino group of an amino acid.

Also, the rates of nonenzymic transamination of leucine and alanine methyl ester with pyridoxal methochloride were greater than those with pyridoxal, especially at pH values above the pK_a of the pyridinium group for the pyridoxal imines. These findings are consistent with the reactive intermediate having a positive charge on the pyridine ring nitrogen atom as a result of N-methylation. The pyridoxal methochloride imine was more unstable than the pyridoxal imine in that the positive charge on the pyridine ring nitrogen atom enhanced aldimine to ketimine tautomerization, since the quinoid structure intermediate to the aldimine and ketimine forms is more favorable by resonance stabilization in the case of pyridoxal methochloride than of pyridoxal.

The nonenzymic transamination of leucine with 5-deoxypyridoxal was studied stoichiometrically and kinetically; deoxypyridoxal was considered as a reference compound to which other pyridoxal analogs with functional groups in the 5-position may be compared. Products of the nonenzymic transamination were identified by thin-layer chromatography. Ketoisocaproate and deoxypyridoxal were quantitatively measured by a modified method of analysis using quinolyldiazine. Deoxypyridoxal, as well as pyridoxal, pyridoxal methochloride, and pyridoxal phosphate were also quantitatively measured in portions of reaction solutions by the ethanol-

mine method. The pH profile for the rate in the acid range, in the absence of added catalysts, and for the imine formation constants of leucine and deoxypyridoxal, also suggested that the reactive intermediate has a positive charge on the pyridine ring nitrogen atom as a result of N-protonation.

The phosphate group of pyridoxal phosphate enhanced the nonenzymic transamination of leucine between 5 and 11 times, compared with the methyl group of deoxypyridoxal, in the acid pH range in the absence of added buffer. From the pH-rate profile of this reaction the catalytic constant for the monobasic form of this phosphate group was calculated to be twice as great as that of the dibasic form. However, intermolecular catalysis by phosphate buffer as a function of pH indicated that almost all of the catalytic constant for this buffer could be attributed to the monobasic form.

The hydroxymethyl group at position 5 of pyridoxal had a negative effect on nonenzymic transamination rates because of hemiacetal formation blocking the essential formyl group. Substitution of this group with carboxylate side chains, such as propionate, indicated rate enhancement, possibly as a result of intramolecular acid-base catalysis.

Intramolecular effects by substituents in the 5-position of pyridoxal analogs in catalysis of nonenzymic transamination were probably best demonstrated in unbuffered solutions, with a good amino group donor, such as alanine methyl ester. Blocking of the negative charge of the carboxyl group of an

amino acid, as in alanine methyl ester, led to rapid reactions with pyridoxal analogs.

These nonenzymic transamination reactions were also catalyzed by acetate buffer. The catalytic constant for acetic acid was calculated to be about twice as great as that for acetate. Imidazole buffer was also found to be a good general acid-base catalyst. However, metal ions, such as copper (II) and aluminum (III), were found to be good intramolecular catalysts, compared to zinc (II), as chelates of the imines in forming more of these intermediates in the nonenzymic transamination, and thus relatively small amounts were as effective as much larger amounts of buffer.

Finally, the deuterium isotope effect in the nonenzymic transamination of deuterio-leucine, (under different conditions, average k_H/k_D was about 8), confirmed the rate-determining step to be the breaking of the alpha-hydrogen-carbon bond of the pyridoxylidene amino acid.

LITERATURE CITED

- Albert, A. and J. N. Phillips
1956 Ionization constants of heterocyclic substances. II. Hydroxyderivatives of nitrogenous six-membered ring-compounds. Chemical Society (London) Journal 1956: 1294-1304.
- Anderson, F. J. and A. E. Martell
1964 Pyridoxal phosphate: molecular species in solution. American Chemical Society Journal 86: 715-720.
- Banks, B.E.C., A. A. Diamantis, and C. A. Vernon
1961 Transamination. II. The non-enzymic reactions between pyridoxamine and pyruvic acid and between pyridoxal and alanine. Chemical Society (London) Journal 1961: 4235-4247.
- Banks, B.E.C., A. J. Lawrence, C. A. Vernon, and J. F. Wootton
1963 Kinetic studies of glutamic-aspartic transaminase (pig heart muscle). International Union of Biochemistry Symposium Series 30: 197-215.
- Blake, M. I., F. P. Siegel, J. J. Katz, and M. Kilpatrick
1963 Deuterium isotope effects in transamination: L-alanine and pyridoxal. American Chemical Society Journal 85: 294-297.
- Block, R. J., E. L. Durrum, and G. Zweig
1955 A manual of paper chromatography and paper electrophoresis. New York, New York, Academic Press, Inc.
- Bonavita, V. and V. Scardi
1959 Studies on glutamic-oxalacetic transaminase. II. The properties of two derivatives of pyridoxal-5-phosphate. Archives of Biochemistry and Biophysics 82: 300-309.
- Braunstein, A. E.
1957 Pyridoxal phosphate. In P. D. Boyer, H. Lardy, and K. Myrbäck, eds. The enzymes. 2nd ed. Vol. 2. pp. 113-184. New York, New York, Academic Press, Inc.

- Braunstein, A. E.
 1960 Pyridoxal phosphate. In P. D. Boyer, H. Lardy, and K. Myrbäck, eds. The enzymes. 2nd ed. Vol. 2. pp. 113-184. New York, New York, Academic Press, Inc.
- Braunstein, A. E., und M. G. Kritzmann
 1937 II. Die Gleichgewichtsreaktion zwischen 1 () Glutaminsäure und Brenztraubensäure, bzw. 1 () Alanin und alpha-Keto glutarsäure. Enzymologia 2: 138-146.
- Braunstein, A. E., and M. M. Shemyakin
 1952 A theory of amino acid metabolism processes catalyzed by pyridoxal phosphate enzymes. (Translated title) Akademiia Nauk SSSR Doklady 85: 1115-1118.
- Braunstein, A. E. and M. M. Shemyakin
 1953 A theory of amino acid metabolism processes catalyzed by pyridoxal phosphate enzymes. (Translated title) Biokhimiia 18: 393-411.
- Brooks, H. G., Jr.
 1960 Vitamin B₆ derivatives and related compounds. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.
- Bruice, T. C.
 1964 Studies of general-base catalysis in model transamination reactions. International Union of Biochemistry Symposium Series 32: 265-266.
- Bruice, T. C. and T. C. French
 1964 Rates and equilibrium constants of imine formation with pyridine-4-aldehyde and various amino acids. Biochemical and Biophysical Research Communications 15: 402-408.
- Bruice, T. C. and R. M. Topping
 1962 The imidazole-catalyzed (non-metal ion mediated) transamination of phenylglycine by pyridoxal: a reaction occurring at ambient temperatures by way of Michaelis-Menten kinetics. American Chemical Society Journal 84: 2448-2450.
- Bruice, T. C. and R. M. Topping
 1963a Catalytic reactions involving azomethines. I. The imidazole catalysis of the transamination of pyridoxal by alpha-aminophenylacetic acid. American Chemical Society Journal 85: 1480-1485.

- Bruice, T. C. and R. M. Topping
 1963b Catalytic reactions involving azomethines. II. pH dependence of the imidazole catalysis of transamination of pyridoxal by alpha-aminophenylacetic acid. American Chemical Society Journal 85: 1486-1492.
- Bruice, T. C. and R. M. Topping
 1963c Catalytic reactions involving azomethines. III. Influence of morpholine upon the imidazole catalysis of transamination of pyridoxal by alpha-aminophenylacetic acid: transamination of the morpholine imine of pyridoxal. American Chemical Society Journal 85: 1493-1496.
- Bruice, T. C. and R. M. Topping
 1963d The imidazole-imidazolium ion concerted general acid, general base catalysis of the transamination of pyridoxal by alpha-aminophenylacetic acid. International Union of Biochemistry Symposium Series 30: 29-56.
- Cattaneo, J., J. C. Senez, et P. Beaumont
 1960 Transamination non-enzymatiques du pyridoxal phosphate et du pyridoxamine phosphate a temperature physiologique et en presence de metaux. Biochimica et Biophysica Acta 44: 543-553.
- Cennamo, C.
 1954 Non-enzymatic transamination between peptides and pyridoxal. Naturwissenschaften 41: 39.
- Cennamo, C.
 1958 Sui meccanismo della transaminazione non enzimatica dei peptidi. Societa Italiana di Biologia Sperimentale Bollettino 34: 214-217.
- Cennamo, C.
 1961 Transaminazione non enzimatica tra piridossale e esteri di aminoacidi. Societa Italiana di Biologia Sperimentale Bollettino 37: 183-187.
- Cennamo, C.
 1962 Ricerche sui composti intermedi delle reazioni non enzimatiche tra aminoacidi e piridossale. Societa Italiana di Biologia Sperimentale Bollettino 38: 1037-1040.
- Cennamo, C.
 1963 Intermediate compounds of non-enzymic transaminations. International Union of Biochemistry Symposium Series 30: 83-90.

- Cennamo, C.
 1964 Metal ion-independent non-enzymic transamination between pyridoxal and amino acid esters. (to be published in *Biochimica et Biophysica Acta* Nov., 1964.)
- Christensen, H. N.
 1958 Three Schiff base types formed by amino acids, peptides and proteins with pyridoxal and pyridoxal-5-phosphate. *American Chemical Society Journal* 80: 99-105.
- Christensen, H. N.
 1959 Cupric chelates of pyridoxylvaline and pyridoxylidenevaline. *American Chemical Society Journal* 81: 6495-6498.
- Cordes, E. H. and W. P. Jencks
 1962a General acid catalysis of semicarbazone formation. *American Chemical Society Journal* 84: 4319-4328.
- Cordes, E. H. and W. P. Jencks
 1962b Semicarbazone formation from pyridoxal, pyridoxal phosphate and their Schiff bases. *Biochemistry* 1: 773-778.
- Cordes, E. H. and W. P. Jencks
 1963 The mechanism of hydrolysis of Schiff bases derived from aliphatic amines. *American Chemical Society Journal* 85: 2843-2848.
- Dancis, J., J. Hutzler, and M. Levitz
 1963 Thin-layer chromatography and spectrophotometry of alpha-keto acid hydrazones. *Biochimica et Biophysica Acta* 78: 85-90.
- Davis, L., F. Roddy, and D. E. Metzler
 1961 Metal chelates of imines derived from pyridoxal and amino acids. *American Chemical Society Journal* 83: 127-134.
- Dempsey, W. B. and H. N. Christensen
 1962 The specific binding of pyridoxal 5'-phosphate to bovine plasma albumin. *Journal of Biological Chemistry* 237: 1113-1120.
- Eichorn, G. L. and J. W. Dawes
 1954 The metal complexes of vitamin B₆ and Schiff's base derivatives. *American Chemical Society Journal* 76: 5663-5667

- Evangelopoulous, A. E. and I. W. Sizer
1963 Pig heart glutamic-aspartic transaminase. Mechanism of transamination. National Academy of Sciences Proceedings 49: 638-643.
- Fasella, P., G. G. Hammes, and B. L. Vallee
1962 Concerning the role of metals in enzymic transamination. Biochimica et Biophysica Acta 65: 142-143.
- Fasella, P., H. Lis, N. Siliprandi, and C. Baglioni
1957 Electrophoretic and chromatographic study of some chemical transamination reactions involving vitamin B₆. Biochimica et Biophysica Acta 23: 417-428.
- Fasella, P., H. Lis, N. Siliprandi, and C. Baglioni
1958 On the formation of intermediate chelate compounds in model reactions involving vitamin B₆. Journal of Inorganic and Nuclear Chemistry 8: 620-624.
- Fleck, G. M. and R. A. Alberty
1962 Kinetics of the reaction of pyridoxal and alanine. Journal of Physical Chemistry 66: 1678-1682.
- Frost, A. A. and R. G. Pearson
1961 Kinetics and mechanism. 2nd ed. New York, New York, John Wiley and Sons, Inc.
- Gonnard, P.
1962 Acquisitions récentes sur le rôle coenzymatique de la vitamin B₆. Cosmétologie 45: 19-23.
- Gonnard, P.
1963 Sur les hydrazones de phospho-5-pyridoxal, rôle coenzymatique et stabilité. International Union of Biochemistry Symposium Series 30: 307-312.
- Gonnard, P., J. Duhalt, M. Camier, C. Nguyen-Philippon, and N. Biogne
1964 Action of phospho-5'-pyridoximinotriazole on pyridoxalo-enzymes. Biochimica et Biophysica Acta 81: 548-559.
- Gregerman, R. I. and H. N. Christensen
1956 Enzymatic and non-enzymatic dehydrochlorination of β - chloro - L - alanine. American Chemical Society Journal 220: 765-774.

- Guggenheim, E. A.
1926 On the determination of the velocity constant of a unimolecular reaction. *Philosophical Magazine* 2: 538-543.
- Guirard, B. M. and E. E. Snell
1964 Vitamin B₆ function in transamination and decarboxylation reactions. In M. Florkin and E. H. Stotz, eds. *Comprehensive biochemistry: group-transfer reactions*. Vol. 15. pp. 138-174. New York, New York, American Elsevier Publishing Co., Inc.
- Gustafson, R. I. and A. E. Martell
1957 Stabilities of metal chelates of pyridoxamine. *Archives of Biochemistry and Biophysics* 68: 485-498.
- György, P.
1934 Vitamin B₂ and the pellagra-like dermatitis in rats. *Nature (London)* 133: 498-499.
- Hammes, G. G. and P. Fasella
1963a The mechanism of enzymatic transamination. *International Union of Biochemistry Symposium Series* 30: 185-195.
- Hammes, G. G. and P. Fasella
1963b The interaction of glutamic-aspartic transaminase with pseudo substrates. *American Chemical Society Journal* 85: 3929-3932.
- Harbury, H. A. and K. A. Foley
1958 Molecular interaction of isovalloxazine derivatives. *National Academy of Sciences Proceedings* 44: 662-668.
- Heinert, D. and A. E. Martell
1962 Pyridoxine and pyridoxal analogs. V. Syntheses and infrared spectra of Schiff bases. *American Chemical Society Journal* 84: 3257-3263.
- Heinert, D. and A. E. Martell
1963a Pyridoxine and pyridoxal analogs. VI. Electron absorption spectra of Schiff bases. *American Chemical Society Journal* 85: 183-188.
- Heinert, D. and A. E. Martell
1963b Pyridoxine and pyridoxal analogs. VII. Acid-base equilibria of Schiff bases. *American Chemical Society Journal* 85: 188-193.

- Heinert, D. and A. E. Martell
1963c Pyridoxine and pyridoxal analogs. VIII.
Synthesis and infrared spectra of metal chelates.
American Chemical Society Journal 85: 1334-1337.
- Henson, C. P. and W. W. Cleland
1964 Kinetic studies of glutamic oxalacetic trans-
aminase isozymes. Biochemistry 3: 338-345.
- Herbst, R. M.
1944 The transamination reaction. Advances in
Enzymology 4: 75-97.
- Heyl, D., S. A. Harris, and K. Folkers
1953 Chemistry of vitamin B₆. IX. Derivatives of 5-
deoxypyridoxine. American Chemical Society
Journal 75: 653-665.
- Heyl, D., E. Luz, S. A. Harris, and K. Folkers
1951 Phosphates of the vitamin B₆ group. I. The
structure of codecarboxylase. American Chemical
Society Journal 73: 3430-3433.
- Ikawa, M. and E. E. Snell
1954a Benzene analogs of pyridoxal: the reactions of
4-nitrosalicylaldehyde with amino acids. American
Chemical Society Journal 76: 653-665.
- Ikawa, M. and E. E. Snell
1954b Oxidative deamination of amino acids by pyridoxal
and metal salts. American Chemical Society Journal
76: 4900-4902.
- Irvin, J. L. and E. M. Irvin
1947 Spectrophotometric and potentiometric evaluation
of apparent acid dissociation exponents of various
4-aminoquinolines. American Chemical Society
Journal 69: 1091-1099.
- Isenberg, I. and A. Szent-Györgi
1958 Free radical formation in riboflavin complexes.
National Academy of Sciences Proceedings 44:
857-862.
- Jencks, W. P.
1963 Mechanism of enzyme action. Annual Review of
Biochemistry 32: 648-650.

- Jencks, W. P. and E. Cordes
1963 Transaldimination reactions of pyridoxal and related compounds. International Union of Biochemistry Symposium Series 30: 57-67.
- Jenkins, W. T.
1961 Glutamic-aspartic transaminase. V. The reaction with L-alanine. Journal of Biological Chemistry 236: 474-478.
- Jenkins, W. T.
1963 Binary complexes involved in enzymic transamination. International Union of Biochemistry Symposium Series 30: 139-148.
- Jenkins, W. T. and I. W. Sizer
1960 Glutamic-aspartic transaminase. IV. The mechanism of transamination. Journal of Biological Chemistry 235: 620-624.
- Jenkins, W. T., D. A. Yphantis, and I. W. Sizer
1959 Glutamic-aspartic transaminase. I. Assay, purification, and general properties. Journal of Biological Chemistry 234: 51-57.
- Johnson, R.
1964 A study of non-enzymatic transamination reactions of amino acid esters with pyridoxal and its analogs. Unpublished paper presented to Iowa Academy of Sciences, April, 1964. Ames, Iowa, Department of Biochemistry and Biophysics, Iowa State University of Science and Technology.
- Johnston, C. C., H. G. Brooks, J. D. Albert, and D. E. Metzler
1963 Reactions of pyridoxal-N-methochloride with amino acids. International Union of Biochemistry Symposium Series 30: 69-81.
- Junk, G. A. and H. J. Svec
1964 Deuterium exchange in the pyridoxal leucine system. Journal of Organic Chemistry 29: 944-946.
- Kalyankar, G. D. and E. E. Snell
1957 Differentiation of alpha-amino-acids and amines by non-enzymatic transamination on paper chromatograms. Nature (London) 180: 1069-1070.

- Ketelaar, J.A.A., C. van de Stolpe, and H. R. Gersmann
1951 Spectrophotometric studies of the solvation of iodine in dioxan solution. *Recueil des Travaux Chimiques des Pays-Bas* 71: 499-508.
- Ketelaar, J.A.A., C. van de Stolpe, A. Goudsmit, and W. Dzcubas
1952 Spectrophotometric study of the solvation of iodine in dioxan solution. [II] *Recueil des Travaux Chimiques des Pays-Bas* 71: 1104-1114.
- King, H. K.
1963 Studies on leucine decarboxylase. *International Union of Biochemistry Symposium Series* 30: 253-266.
- King, H. K. and N. Lucas
1959 Interaction between pyridoxal phosphate and amino acids. *Biochemical Journal* 72: 18P.
- Kuroda, T.
1963a Synthetic studies of vitamin B₆ derivatives. [I] Synthesis of pyridoxal-5-phosphate and its related compounds. *Vitamins* 28: 211-220.
- Kuroda, T.
1963b Synthetic studies of vitamin B₆ derivatives. [II] Synthesis of adenosine di phosphate of vitamin B₆ group. *Vitamins* 28: 354-361.
- Kuroda, T.
1964 Synthetic studies of vitamin B₆ derivatives. [III] Synthesis of 5-deoxy-derivatives of vitamin B₆ group. *Vitamins* 29: 116-118.
- Levine, L. H.
1963 Some aspects of L-amino acid oxidase catalysis. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.
- Lis, H., P. Fasella, C. Turano, and P. Vecchini
1960 On the mechanism of action of glutamic-aspartic transaminase: intermediate steps in the reaction. *Biochimica et Biophysica Acta* 45: 529-536.
- Longenecker, J. B. and E. E. Snell
1957 The comparative activities of metal ions in promoting pyridoxal-catalyzed reactions of amino acids. *American Chemical Society Journal* 79: 142-145.

- Lucas, N., H. K. King, and S. J. Brown
1962 Substrate attachment in enzymes: the interaction of pyridoxal phosphate with amino acids. *Biochemical Journal* 84: 118-124.
- Lunn, A. K. and R. A. Morton
1952 Ultra-violet absorption spectra of pyridoxine and related compounds. *Analyst* 77: 718-731.
- Mackay, D.
1962 The mechanism of the reaction of cysteine with pyridoxal 5'-phosphate. *Archives of Biochemistry and Biophysics* 99: 93-100.
- Makino, K., Y. Ooi, M. Matsudo, and T. Kuroda
1963 Some notes on the coenzyme activity of phosphopyridoxal derivatives for the glutamic decarboxylase and glutamic aspartic transaminase. *International Union of Biochemistry Symposium Series* 30: 291-306.
- Makino, K., Y. Ooi, M. Matsuda, M. Tsuji, M. Matsumoto, and T. Kuroda
1962 Some notes on the coenzyme activity of phosphopyridoxal derivatives for the brain glutamic decarboxylase. *Biochemical and Biophysical Research Communications* 9: 246-251.
- Martell, A. E.
1963 Schiff bases of pyridoxal analogs: molecular species in solution. *International Union of Biochemistry Symposium Series* 30: 13-28.
- Martin, R. B.
1964 Reactions of carbonyl compounds with amines and derivatives. *Journal of Physical Chemistry* 68: 1369-1377.
- Marvel, C. S. and N. Tarköy
1957 Heat stability studies on chelates from Schiff bases of salicylaldehyde derivatives. *American Chemical Society Journal* 79: 6000-6002.
- Mason, S. F.
1958 The tautomerism of N-hetero-aromatic hydroxy-compounds. III. Ionisation constants. *Chemical Society (London) Journal* 1958: 674-685.

- Matsuo, Y.
1957a Formation of Schiff bases of pyridoxal phosphate: reaction with metal ions. American Chemical Society Journal 79: 2011-2015.
- Matsuo, Y.
1957b Pyridoxal catalysis of non-enzymatic transamination in ethanol solution. American Chemical Society Journal 79: 2016-2019.
- Meister, A.
1953 Sodium alpha-ketoisocaproate. In E. E. Snell, ed. Biochemical preparations. Vol. 3. pp. 66-70. New York, New York, John Wiley and Sons, Inc.
- Meister, A.
1962 Amino group transfer. In P. D. Boyer, H. Lardy, and K. Myrback, eds. The enzymes. 2nd ed. Vol. 6. pp. 193-217. New York, New York, Academic Press, Inc.
- Meister, A. and P. A. Abendschein
1956 Chromatography of alpha-keto acid 2,4-dinitrophenylhydrazones and their hydrogenation products. Analytical Chemistry 28: 171-173.
- Melander, L. C.
1960 Isotope effects on reaction rates. New York, New York, Ronald Press Company.
- Metzler, D. E.
1957 Equilibria between pyridoxal and amino acids and their imines. American Chemical Journal 79: 485-490.
- Metzler, D. E., M. Ikawa, and E. E. Snell
1954a A general mechanism for vitamin B₆-catalyzed reactions. American Chemical Society Journal 76: 648-652.
- Metzler, D. E., J. Olivard, and E. E. Snell
1954b Transamination of pyridoxamine and amino acids with glyoxylic acid. American Chemical Society Journal 76: 644-648.
- Metzler, D. E. and E. E. Snell
1952a Deamination of serine. I. Catalytic deamination of serine and cysteine by pyridoxal and metal salts. Journal of Biological Chemistry 198: 353-361.

- Metzler, D. E. and E. E. Snell
1952b Some transamination reactions involving vitamin B₆. American Chemical Society Journal 74: 979-983.
- Metzler, D. E. and E. E. Snell
1955 Spectra and ionization constants of the vitamin B₆ group and related 3-hydroxypyridine derivatives. American Chemical Society Journal 77: 2431-2437.
- Nakamoto, K. and A. E. Martell
1959a Pyridoxine and pyridoxal analogs. III. Ultra-violet absorption studies and solution equilibria of 2- and 4-hydroxymethyl-3-hydroxypyridines and pyridine-2,3- and 4-aldehydes. American Chemical Society Journal 81: 5857-5863.
- Nakamoto, K. and A. E. Martell
1959b Pyridoxine and pyridoxal analogs. IV. Ultra-violet spectra and solution equilibria of 3-methoxypyridine-2 (and 4-)-aldehydes and of 3-hydroxypyridine-2 (and 4-)-aldehydes. American Chemical Society Journal 81: 5863-5869.
- Neish, W.J.P.
1957 Alpha-Keto acid determinations. In D. Glick, ed. Methods of biochemical analysis. Vol. 5. pp. 107-179. New York, New York, Interscience Publishers.
- Olivard, J. and E. E. Snell
1955a Growth and enzymatic activities of vitamin B₆ analogues I. D-Alanine synthesis. Journal of Biological Chemistry 213: 203-214.
- Olivard, J. and E. E. Snell
1955b Growth and enzymatic activities of vitamin B₆ analogues II. Synthesis of miscellaneous amino acids. Journal of Biological Chemistry 213: 215-228.
- Olivo, F., C. S. Rossi, and N. Siliprandi
1963 Non-enzymic transaminations of gamma-aminobutyric acid. International Union of Biochemistry Symposium Series 30: 91-101.
- Ooi, Y.
1964 Coenzymatic activity of pyridoxal phosphate derivatives on glutamic-oxaloacetic transaminase. Vitamins 29: 101-108.

- Perault, A. M., B. Pullman, and C. Valdemoro
1961 Electronic aspects of the reactions of pyridoxal phosphate enzymes. *Biochimica et Biophysica Acta* 46: 555-575.
- Peterson, E. A. and H. A. Sober
1954 Preparation of crystalline phosphorylated derivatives of vitamin B₆. *American Chemical Society Journal* 76: 169-175.
- Peterson, E. A., H. A. Sober, and A. Meister
1953 Pyridoxamine phosphate; pyridoxal phosphate. In E. E. Snell, ed. *Biochemical preparations*. Vol. 3. pp. 29-39. New York, New York, John Wiley and Sons, Inc.
- Pullman, B.
1963 A quantum-mechanical investigation of pyridoxal-dependent reactions. *International Union of Biochemistry Symposium Series* 30: 103-121.
- Rabinowitz, J. C. and E. E. Snell
1947 The vitamin B₆ group. XII. Microbiological activity and natural occurrence of pyridoxamine phosphate. *Journal of Biological Chemistry* 169: 643-650.
- Robins, E., N. R. Roberts, K. M. Eydt, O. H. Lowry, and D. E. Smith
1956 Microdetermination of alpha-keto acids with special reference to malic, lactic, and glutamic dehydrogenases in brain. *Journal of Biological Chemistry* 218: 897-909.
- Roze, U.
1964 The reaction between pyridoxal phosphate and cycloserine. Unpublished Ph.D. thesis. Saint Louis, Missouri, Library, Washington University.
- Scardi, V., e. V. Bonavita
1957 Determinazione spettrofotometrica del piridossal -5-fosfato. *Societa' Italiana di Biologia Sperimentale Bollettino* 33: 1701-1702.
- Siegel, F. P. and M. I. Blake
1962 Spectrophotometric determination of pyridoxal in the presence of pyridoxamine and pyridoxine. *Analytical Chemistry* 34: 397-398.

- Sizer, I. W. and W. T. Jenkins
1963 The reactivity of the pyridoxal phosphate group of glutamic aspartic aminotransferase. International Union of Biochemistry Symposium Series 30: 123-138.
- Snell, E. E.
1944 The vitamin activities of "pyridoxal" and "pyridoxamine". Journal of Biological Chemistry 154: 313-314.
- Snell, E. E.
1945a The vitamin B₆ group. IV. Evidence for the occurrence of pyridoxamine and pyridoxal in natural products. Journal of Biological Chemistry 157: 491-505.
- Snell, E. E.
1945b The vitamin B₆ group. V. The reversible interconversion of pyridoxal and pyridoxamine by transamination reactions. American Chemical Society Journal 67: 194-197.
- Snell, E. E.
1958 Chemical structure in relation to biological activities of vitamin B₆. In R. S. Harris, G. F. Marrian, and K. V. Thimann, eds. Vitamins and hormones. Vol. 16. pp. 77-125. New York, New York, Academic Press, Inc.
- Snell, E. E.
1962 A comparison between some pyridoxal-dependent enzymatic and non-enzymatic reactions. Brookhaven Symposia in Biology 15: 32-51.
- Snell, E. E.
1963 Non-enzymatic reactions of pyridoxal and their significance. International Union of Biochemistry Symposium Series 30: 1-12.
- Snell, E. E., P. M. Fasella, A. E. Braunstein, and A. Rossi-Fanelli, eds.
1963 Chemical and biological aspects of pyridoxal catalysis. International Union of Biochemistry Symposium Series 30: 1-599.
- Snell, E. E., and W. T. Jenkins
1959 The mechanism of the transamination reaction. Journal of Cellular and Comparative Physiology 54, Supplement 1: 161-177.

- Snell, E. E. and D. E. Metzler
1956 Water soluble vitamins. II. Annual Review of Biochemistry 25: 435-443.
- Snell, E. E. and A. N. Rannefeld
1945 The vitamin B₆ group. III. The vitamin activity of pyridoxal⁶ and pyridoxamine for various organisms. Journal of Biological Chemistry 157: 475-489.
- Spikner, J. E. and J. C. Towne
1962 Fluorometric microdetermination of alpha-keto acids. Analytical Chemistry 34: 1468-1471.
- Tomita, I., H. G. Brooks, Jr., and D. E. Metzler
1964 Synthesis of vitamin B₆ derivatives. II. 3-Hydroxy-4-(hydroxymethyl)-2-methyl-5-pyridine acetic acid and related substances. Unpublished manuscript submitted to Journal of Medicinal Chemistry. Ames, Iowa, Department of Biochemistry and Biophysics, Iowa State University of Science and Technology.
- Tomita, I. and D. E. Metzler
1964 A new analogue of pyridoxal phosphate; 3-hydroxy-2-methyl-5-propionic acid-4-pyridine aldehyde. Unpublished paper presented at the 148th national meeting of the American Chemical Society, Chicago, Illinois, September 1964. Ames, Iowa, Department of Biochemistry and Biophysics, Iowa State University of Science and Technology.
- Umbreit, W. W., D. J. O'Kane, and I. C. Gunsalus
1948 Function of the vitamin B₆ group: mechanism of transamination. Journal of Biological Chemistry 176: 629-637.
- Velick, S. F. and J. Vavra
1962a Glutamic-oxalacetate transaminase. In P. D. Boyer, H. Lardy, and K. Myrback, eds. The enzymes. 2nd ed. Vol. 6. pp. 219-246. New York, New York, Academic Press, Inc.
- Velick, S. F. and J. Vavra
1962b A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. Journal of Biological Chemistry 237: 2109-2122.

- Vernon, C. A.
1964 Glutamic-aspartic transaminase (pig heart muscle). A comparison of the enzymic and non-enzymic reactions. International Union of Biochemistry Symposium Series 32: 259-260.
- von Viscontini, M., C. Ebnöther, und P. Karrer
1951 Konstitution und Synthese der Codecarboxylase. Helvetica Chimica Acta 34: 1834-1842.
- Wada, H. and E. E. Snell
1962 Enzymatic transamination of pyridoxamine I. With oxaloacetate and alpha-ketoglutarate. Journal of Biological Chemistry 237: 127-132.
- Wagner, A. F. and K. Folkers
1964 Vitamins and coenzymes. New York, New York, Interscience Publishers.
- Westheimer, F. H.
1960 Enzyme models. In P. D. Boyer, H. Lardy, and K. Myrbäck, eds. The enzymes. 2nd ed. Vol. 1. pp. 261-268. New York, New York, Academic Press, Inc.
- Wiegand, R. G.
1956 The formation of pyridoxal and pyridoxal 5-phosphate hydrazones. American Chemical Society Journal 78: 5307-5309.
- Williams, V. R. and J. B. Neillands
1954 Apparent ionization constants, spectral properties and metal chelation of the cotranaminases and related compounds. Archives of Biochemistry and Biophysics 53: 56-70.
- Willstadter, E., T. A. Hamor, and J. L. Hoard
1963 Stereochemistry of the manganese (II) - pyridoxylidenevaline chelate. American Chemical Society Journal 85: 1205-1206.

ACKNOWLEDGEMENTS

The author acknowledges the technical help and exchange of information related to this research from a number of persons, especially Miss Erika Rommel, Miss Louise Hodgins, Mr. Donald DePrenger, Dr. Isao Tomita, Miss Sheryl Breshahan, Mr. Robert Johnson, Dr. Keith Schmude, and Mr. F. Scott Furbish.

Previous to this work on model reactions of pyridoxal analogs for the past two years, the author was struggling with the nature of the active site of threonine dehydrase from sheep liver. One aspect of this enzyme work was included in a publication from this laboratory, and several other preliminary findings have since been confirmed and reported by Dr. Willie McLemore. However, this research project was not given up by the author until he was convinced that results from investigation of the active site of this enzyme might be difficult to obtain unless further purification was accomplished. The author thanks Dr. Donald J. Graves for his interest and helpful discussions in this respect. The author also congratulates Dr. Willie McLemore for his accomplishments in this enzyme project, which the author has followed with interest during his graduate studies.

The author is grateful to Dr. David E. Metzler, his major professor, for helping to make the change in research projects with not too much loss in time. The constructive crit-

icisms, patience, and guidance which Dr. Metzler provided not only during the laboratory work, but especially during the writing of this thesis, have established a background of training which will be a valuable asset to the author throughout his scientific career.

The author has appreciated and depended on the moral support and encouragement of his family, relatives, teachers, and friends, in addition to that of Judy. Financial support of graduate assistantships through Iowa State University's Agricultural Experiment Station is gratefully acknowledged.

DEDICATION

This thesis is humbly dedicated to the praise and glory of the living God who has sustained the author in times of trial and forgiven him in times of temptation throughout his life, and Who has made all things possible and continues to make all things work together for good to those who love Him. The witness of many scientists dedicated to Jesus Christ has convinced the author that any apparent conflicts, past, present, or future, between science and Christianity are really only imaginary or at least paradoxical and are due either to poor science, inadequate religion, or both. For the understanding that what people can learn through God's Word and through God's world is complementary, not contradictory, the author especially acknowledges the witness of Dr. Frank L. Lambert, Professor of Chemistry, Occidental College; Dr. Ray Fahien, Professor of Chemical Engineering, University of Florida; Dr. John A. Effenberger, Senior Physical Chemist, DuPont Research Laboratories, Wilmington; Pastor W. J. Fields, Memorial Lutheran Church of Ames; Dr. Walter R. Hearn, Professor of Biochemistry, Iowa State University, and the American Scientific Affiliation.

VITA

The author was born to Mildred Lydia (Voissem) and Leo Paul Albert in Milwaukee, Wisconsin, June 6, 1937. He attended General Douglas MacArthur and Morgandale elementary schools. He had many interests and his particular interest in science did not emerge until his high school years. He was presented the Bausch and Lomb Science Award upon graduation from Pulaski High School in 1955, ranking third in a class of about 415.

After his family moved to California he majored in chemistry at Occidental College, Los Angeles. He started his laboratory experience in organic chemistry in a NSF research project, studying halogenation of organic compounds, directed by Dr. Frank Lambert. He was active in the Beta Mu chapter of Alpha Chi Sigma, the national professional chemistry fraternity, and in the Theta Chapter of Kappa Mu Epsilon, the national honorary mathematics society. Industrial experience during summer vacations included syntheses of organic phosphorus compounds, ultraviolet and infrared spectrophotometry, and inorganic analytical chemistry. He received the American Institute of Chemists Award upon graduation with a Bachelor of Arts degree from Occidental College in 1959, ranking 26th in a class of 287.

During his graduate studies in biochemistry at Iowa State

University he also had minors in organic chemistry and cell biology. He became a candidate for the Ph.D. degree in 1961, and was married to Judith J. Brown at the end of that year.

The committee in charge of his Ph.D. program included:

chairman: Dr. David E. Metzler, Professor of Biochemistry

major : Dr. R. Scott Allen, Professor of Biochemistry
and Animal Science

1 st minor, organic chemistry: Dr. Glen A. Russell,
Professor of Chemistry

2 nd minor, cell biology: Dr. L. Evans Roth, Professor
of Biophysics and Assistant
Dean, Graduate College

member-at-large: Dr. Charlotte E. Roderuck, Professor
of Food and Nutrition

He became a member of Phi Lambda Upsilon, the national honorary chemistry society, through the Theta chapter at Iowa State in 1963. He is also a member of the American Chemical Society and the American Scientific Affiliation. His biochemical interests have been the mechanisms and functions of naturally-occurring compounds in living cells.

Farewell to Iowa! The author hopes that he will not be considered a former Iowan by the local mass media like many others have been so branded for merely passing through this weather-beaten and provincial land.