A MODEL SYSTEM ANALYSIS OF THE MECHANISM OF 2-KETO-3-DEOXY-6 PHOSPHOGLUCONIC ACID ALDOLASE: THE CATALYTIC ROLE OF THE SCHIFF BASE INTERMEDIATE

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MARK A. ROSEMAN

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This is to certify that the

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A MODEL SYSTEM ANALYSIS OF THE MECHANISM OF
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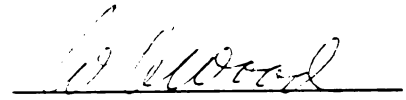
presented by

Mark A. Roseman

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry



Major professor

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ABSTRACT

A MODEL SYSTEM ANALYSIS OF THE MECHANISM OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONIC ACID ALDOLASE: THE CATALYTIC ROLE OF THE SCHIFF BASE INTERMEDIATE

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The rate constants of enamine formation from ketimines of pyruvate, and the cyclic ketimine Δ^1-piperidine-2-carboxylic acid, were determined in aqueous solution using rapid iodination of the enamine to measure the reactions. The constant for ketimines of pyruvate was determined indirectly by comparing the kinetics of iodination of pyruvate in primary amine buffers to the kinetics in imidazole and phosphate buffers. In all buffers, the following rate law was obtained: $k_{obs} = k_o + k_b B + k_a A$. The constant k_a was greater for primary amine catalysis than for imidazole or phosphate catalysis. This result suggested that the $k_a A$ term results from water-catalyzed tautomerization of the protonated ketimine to the enamine. From this, the constant for enamine formation was calculated; $k_e = 0.304\ sec^{-1}$.

The tautomerization of Δ^1-piperidine-2-carboxylic acid could be measured directly. The kinetics indicated that tautomerization of the protonated ketimine predominates, and is strongly catalyzed by general bases. The rate constants obtained with this compound differ markedly with those of the ketimines of pyruvate.

The discrepancies are discussed as well as the application of these results in determining the catalytic role of the ketimine intermediates in enzyme-catalyzed enolizations.

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By

Mark A. Roseman

A THESIS

Submitted to
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DEDICATION

To my junior high school teachers who predicted I would be a high school drop-out;

To my high school counselor who predicted I would never go to college;

To Dean Anderson who predicted I would never graduate from the University of Michigan;

To my father who thought they may be right;

To my mother who knew better;

To my mother-in-law who wanted me to become a doctor;

To my father-in-law who wonders when I'll get a job;

To my wife, the overseer, who kept me working; and

To my son, whose financial support encouraged hasty completion of this work.

ACKNOWLEDGMENTS

I would like to extend my sincere gratitude to Dr. W. A. Wood for the patience he showed me and the courage to support a research project in an area far removed from the general program of his laboratory. I wish to acknowledge financial support as a trainee under the Training Grant (GM 1096) awarded to the Department of Biochemistry.

I also extend my lasting appreciation to my wife Jo Ellen, for her understanding and encouragement throughout the course of this work, and assistance in the preparation of this manuscript.

VITA

Mr. Roseman was born October 30, 1944, in Brooklyn, New York. Shortly thereafter, he was moved to the Interior, where he has lived ever since.

His formative years were spent in Ann Arbor, Michigan, where he attended Ann Arbor High School and the University of Michigan. He majored in Cellular Biology at the University of Michigan, and graduated with a Bachelor of Science degree in May, 1965. Since that time he has attended Michigan State University, where he has received his graduate education in biochemistry.

He is married and has a son.

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CHAPTER I

INTRODUCTION

Enzyme catalysis is unquestionably the most fundamental and most important biological process. Still there is no adequate explanation for it; it is generally acknowledged that the catalytic process has not been satisfactorily described for even a single enzyme.

However, as Koshland points out (1), one important conclusion has been reached: enzyme catalysis can probably be explained in terms of processes familiar to the organic chemist. This conclusion has been the stimulus for the "model system" approach to enzyme catalysis.

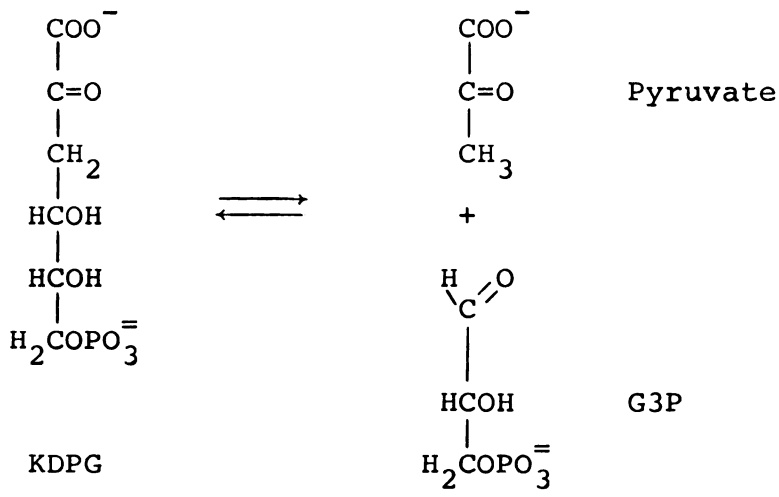
A model system is essentially a nonenzymatic analog of an enzyme-catalyzed reaction. Usually, the model reaction is studied under a number of conditions in order to determine the conditions and catalysts which efficiently accelerate the rate of reaction. In this way, one gathers a list of potential catalytic forces which might be utilized by an enzyme. Indeed, a number of effective catalytic forces have been discovered and characterized this way. These include proximity effects, electrostatic interactions, hydrophobic interactions, general acid-general base effects, charge-transfer-complex formation,

hydrogen-bond formation, bond strain, facilitated diffussion, and orbital steering.

It should be quite evident that if enzyme catalysis operates by processes familiar to organic chemistry, we shall never understand enzyme catalysis any better than we do simple nonenzymatic catalysis. For this reason, model system studies are essential. Unfortunately, they have one serious limitation: it is very difficult to show that a catalytic process which works for a model system actually exists for its enzymatic counterpart. For example, it is easy to show that bond strain accelerates a nonenzymatic reaction; but it is not so easy to show that a particular bond in a substrate is strained in the active site complex. Obviously, meaningful comparisons between model and enzymatic reactions can only be made if a fairly accurate description of enzyme-substrate complexes is at hand. In the absence of such information, model systems often seem to be an endless variety of hypothetical situations.

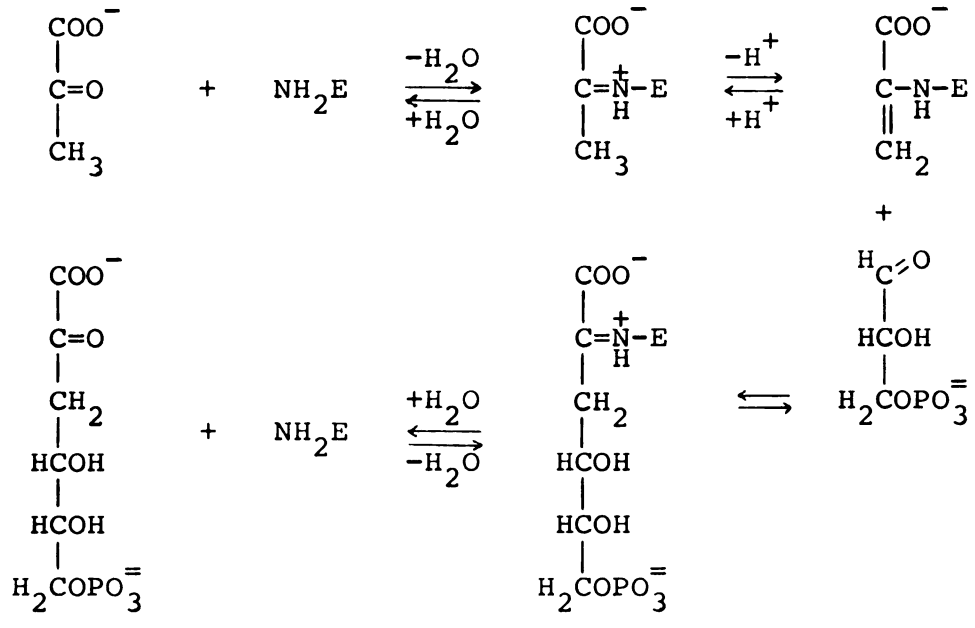
Fortunately, one class of enzymes provides some exception to this limitation--those which form covalent complexes with substrate. In such cases we have a fairly accurate picture of at least one of the interactions between enzyme and substrate. Undoubtedly, the covalent bond is not the only interaction, but it does provide a reasonably firm starting point for a model system analysis.

The research presented here seeks to determine the catalytic effect of Schiff base formation in the bacterial enzyme 2-keto-3-deoxy-6-phosphogluconic acid aldolase (KDPG aldolase). This enzyme catalyzes the reversible aldol cleavage of KDPG to pyruvate and glyceraldehyde-3-phosphate (G3P):



Mechanistically, the reaction has been shown to proceed through an obligatory Schiff base intermediate (2, 3) as illustrated on the next page in the direction of condensation. If pyruvate is incubated with enzyme in the absence of G3P, a rapid exchange of the methyl hydrogens with water is observed. That is, the aldolase catalyzes the enolization of pyruvate. In every sense, this simple "partial" reaction is an enzyme-catalyzed process.

Since it is simple, and proceeds through a covalent intermediate, this reaction is ideal for model



system studies. For these reasons, the Schiff base-catalyzed enolization of pyruvate is the subject of this research.

The fundamental problem then, and the purpose of this research is determining the contribution of the Schiff base to the overall catalysis. This would be done by comparing the enzymatic rate of enolization to that of pyruvate and a Schiff base of pyruvate.

If Schiff base formation is not sufficient to account for the catalytic rate, it is important to consider the popular viewpoint that enzyme catalysis results from several catalytic forces working together. If so, how readily can the tautomerization of the Schiff base itself be catalyzed? All too often the argument is made that if a single catalytic force accelerates a reaction

by a factor, x, and another force independently accelerates the reaction by a factor, y, the combined effect of both forces working together will be an acceleration (x)(y). Is this necessarily correct? All these questions shall be considered.

To determine the contribution of the Schiff base to the overall catalyses, the rate constant for the tautomerization of a ketimine of pyruvate[1] to the enamine in aqueous solution had to be determined:

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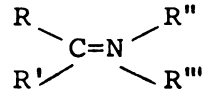
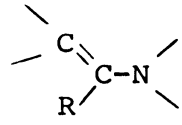
[1]At this point, the standard nomenclature for Schiff base compounds should be presented.

The generic term for a Schiff base is <u>imine</u> for which the general structure is

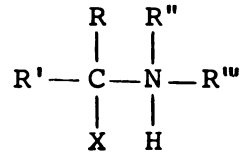


If R or R' is hydrogen, the imine has been formed from an aldehyde and is termed an <u>aldimine</u>; likewise, if R and R' are alkyl groups, the imine has been formed from a ketone and is termed a <u>ketimine</u>.

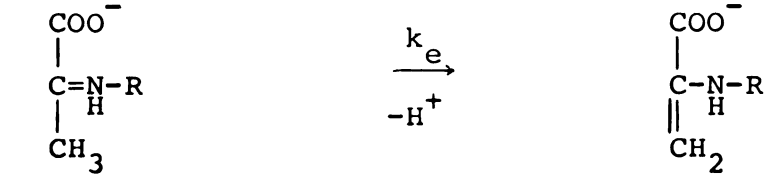
Both aldimines and ketimines can tautomerize to an enol-like structure called an <u>enamine</u>:



Imines can also form addition compounds similar to those formed by aldehydes and ketones:

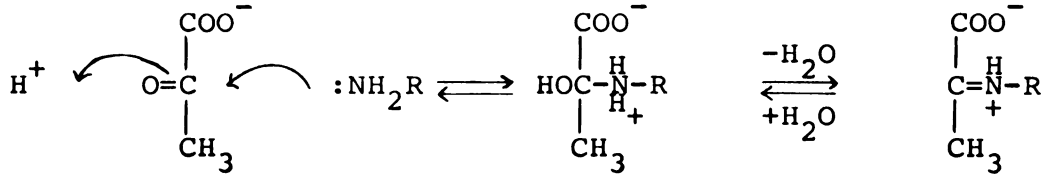


where X is some nucleophile (such as OH, CN, S, etc.). If X is OH, the addition compound is a carbinolamine; otherwise it is usually a substituted imine.



ketimine enamine

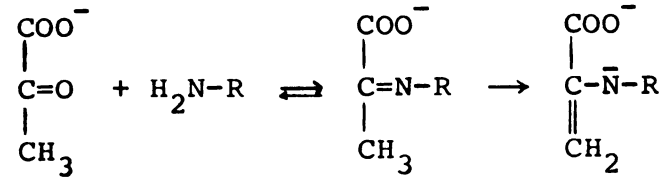
Assuming that the ketamine can be produced, this seems to be a relatively straightforward affair. However, a consideration of the mechanism of Schiff base formation reveals the complications:



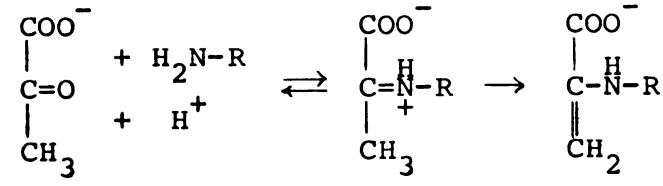
pyruvate amine carbinolamine ketimine

It is seen that Schiff base formation involves a dehydration; consequently, the Schiff base is unstable in aqueous solution. In fact, Schiff base hydrolysis is usually much faster than enamine formation. The equilibrium constant for Schiff base formation is large enough, however, that a significant amount of Schiff base can be formed in rapid equilibrium with pyruvate if fairly high levels of amine are used. Unfortunately, such an equilibrium mixture causes another serious problem: the amine can function effectively as a general acid-base catalyst for the enolization of free pyruvate. Therefore, the enolization of pyruvate occurs by two routes functioning simultaneously:

1. ketimine catalysis

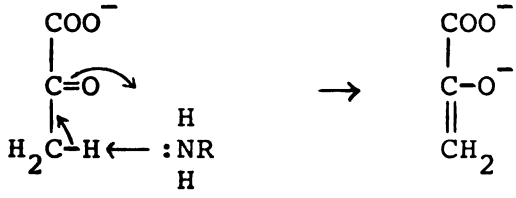


(via neutral imine)

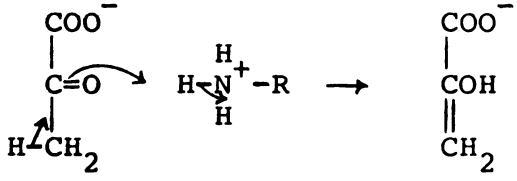


(via protonated imine)

2. general acid-base catalysis



(general base)



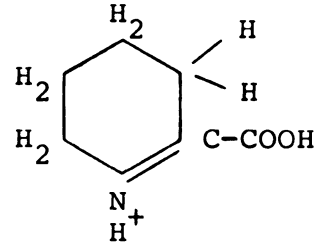
(general acid)

Any method used to measure enolization will not distinguish between the enamine and enol of pyruvate.

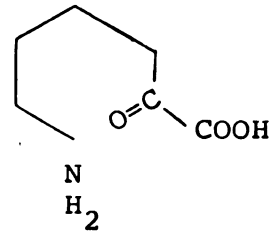
Kinetically, the two mechanisms are equivalent; that is they both obey the same experimental rate equation. Consequently, a certain amount of kinetic gymnastics is required to distinguish one from the other.

A complete description of such analyses will be given in the Literature Review.

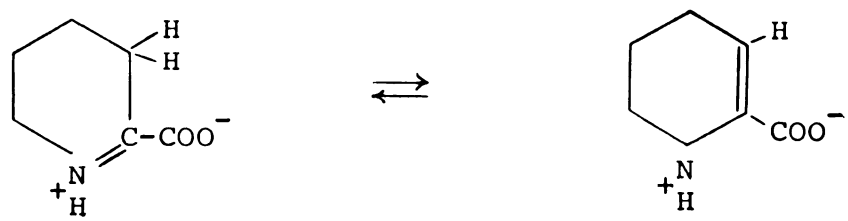
It is only necessary at this point to say that any such analysis has weaknesses. For this reason, an independent method for measuring ketimine-enamine conversion was sought to supplement the method described above. It is clear that all the complications of this system arise from the inescapable requirement to work with equilibrium mixtures. What is needed, then, is a ketimine which is similar to the pyruvyl ketimine but stable to hydrolysis in aqueous solution. Fortunately, such a compound exists, namely Δ^1-piperidine-2-carboxylic acid:



Conceivably, this compound could exist in equilibrium with the open chain form:



However, Macholàn and Svàtek (4) have shown that the compound exists almost exclusively in the ring form over the entire pH scale. Furthermore, they have demonstrated the ketimine-enamine conversion:



While the use of this compound has the advantage of providing a direct measure of ketimine-enamine conversion, there is now the disadvantage of not knowing the effect the ring might have on enamine formation. Therefore, the effect of the ring on enamine formation will be seriously considered when comparing the reactivity of this compound to the acyclic ketimines of pyruvate.

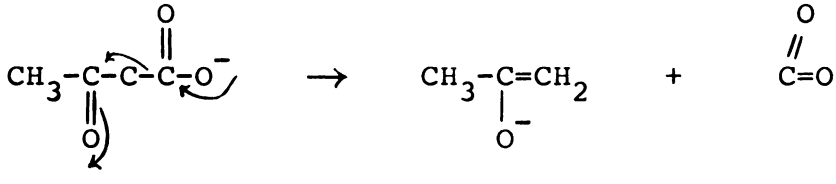
CHAPTER II

LITERATURE REVIEW

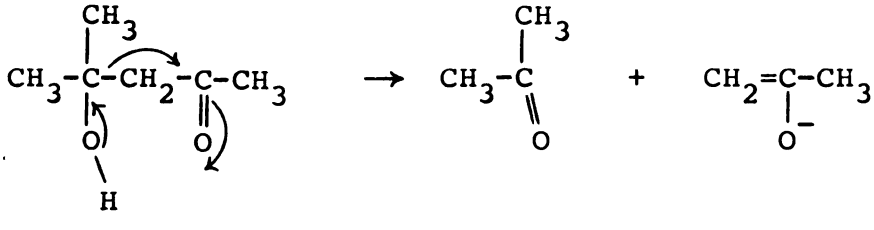
Schiff Base Catalysis: A Perspective

The reactions of carbonyl-containing compounds are numerous and apparently diverse. There is, however, a satisfying mechanistic consistency to them all: the carbonyl group is an electrophilic center for nucleophilic attack or intramolecular electron rearrangement. Two examples of intramolecular rearrangement shown below are β-decarboxylation and dealdolization:

1. β-decarboxylation



2. dealdolization



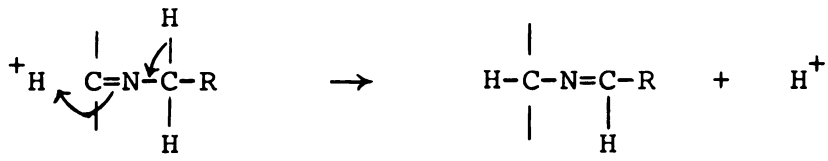
As early as 1932 Pedersen recognized that the protonated form of the carbonyl group ($>C=\overset{+}{O}H$) should serve this electrophilic function far more effectively than the unprotonated form. While this is quite true, protonation is significant only at low pH, since the pK_a of the protonated carbonyl is quite low. There are, however, two means for increasing the electrophilic character of the carbonyl under neutral or alkaline conditions--metal ion activation and Schiff base formation. Only Schiff base formation will be discussed here.

The Schiff base can exist in either neutral or protonated form with a pK_a of 7.00-8.00:

$$>C=N-R + H^+ \rightleftharpoons >C=\overset{H}{\overset{|}{N}}-R^+$$

The similarity to the neutral and protonated forms of the carbonyl group is clear; however, unlike the carbonyl, a significant fraction of Schiff base is protonated under neutral conditions. Schiff base activation of the carbonyl is therefore a particularly attractive mechanism for enzymes which normally operate near neutrality.

There is one other important property of the Schiff base which distinguishes its chemistry from that of the carbonyl. Under appropriate conditions, the position of the carbon-nitrogen double bond can shift:



This double bond rearrangement is an essential feature of many reactions involving pyridoxal phosphate.

A large number of enzymatic and nonenzymatic Schiff base-catalyzed reactions have now been studied in detail. These include: aldol reactions, α-decarboxylations, β-decarboxylations, and enolizations.

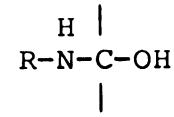
Perhaps the most important class of Schiff base reactions are those involving pyridoxal phosphate. This review, however, is intended to cover only those areas of Schiff base catalysis which are applicable to the present research. Two excellent reviews of pyridoxal catalysis have been published by Bruice (5) and Snell (6).

The Mechanism of Schiff Base Formation

Although the mechanism of Schiff base formation has been exhaustively studied, only those aspects which are relevant to this research will be discussed. Comprehensive reviews on the subject have been published by Bruice (7) and Jencks (8).

Early studies of the kinetics of oxime formation showed the reaction to be catalyzed by acid and alkali

(9, 10). However, the rate showed a peculiar dependence on acid concentration: there appeared to be an optimum acidity between the one extreme where hydroxylamine is totally unionized and the other where it is totally protonated. Although the explanations put forth are not correct in detail, they did suggest the intermediate formation of carbinolamine species



which could have different ionic forms at different levels of acid.

The first definitive evidence for the carbinolamine intermediate came from Bodforss's work on phenylhydrazone formation (11). He found that the disappearance of phenylhydrazine occurs faster than formation of phenylhydrazone; this indicates rapid accumulation of an intermediate.

Later, Olander (12) also concluded from the kinetics of acetoxime formation that a carbinolamine must be an obligatory intermediate.

Bartlett and Conant (13) also described a maximum in the pH-rate profile of semicarbazone formation. Furthermore, they demonstrated that the reaction is catalyzed by general acids. Unfortunately, they failed to carry out the experiments necessary to distinguish the effect of pH from the effect of buffer catalysis. For

this reason their interpretations of the pH-rate profile (which will not be discussed here) are somewhat inconclusive.

The overall features of the mechanism were substantially clarified by Jencks (14). Using spectral techniques he found that at the alkaline side of the pH-rate profile a rapid decrease of carbonyl absorbance is first observed, followed by the slow appearance of imine absorbance. This means that carbinolamine is formed in a rapid equilibrium followed by the rate determining dehydration to imine. Under these conditions, he also showed the dehydration of carbinolamine to be catalyzed by general acids (which contrasts with the previous interpretations that carbinolamine formation is general acid-catalyzed).

On the acid side of the pH-rate profile, the situation is reversed: dehydration is fast and carbinolamine formation is rate determining. The reason for the change in rate determining step is then quite clear. Under acid conditions the acid-catalyzed dehydration is, as one would expect, fast; whereas the rate of carbinolamine formation, which depends on the concentration of the conjugate base of the amine, is slow. Conversely, under alkaline conditions the concentration of conjugate base is high so that carbinolamine formation is fast; whereas the rate of acid-catalyzed dehydration is low.

The maximum rate occurs between these extremes where a suitable compromise is achieved.

Aldol Reactions: Nonenzymatic

The first and perhaps most definitive demonstration of Schiff base catalysis was accomplished by Westheimer and Cohen in 1938 (15). These workers sought to resolve an apparent discrepancy in the literature concerning the dealdolization of diacetone alcohol [$(CH_3)_2$-COH-CH_2COCH_3]. Previously, French (16) had studied the effect of phenol-phenolate buffer on the dealdolization reaction. He found the reaction to be dependent only on the hydroxide ion concentration of the buffer and not the concentration of buffer components. In other words, the reaction appeared to be insensitive to general acid-base catalysis.

On the other hand, Miller and Kilpatrick (17) found the reaction to be catalyzed not only by hydroxide ion, but also primary and secondary amines according to the rate law

$$\text{rate} = k_{obs}(\text{diacetone alcohol})$$

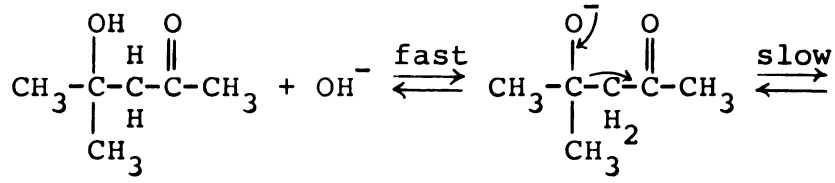
$$= [k_o + k_{OH}(OH) + k_b(B)] \text{ diacetone alcohol}$$

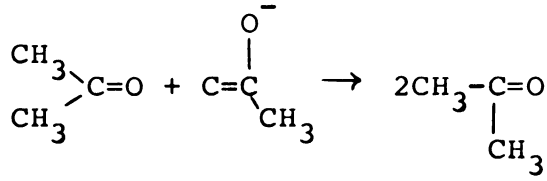
where (B) is the concentration of the basic form of the amine, k_b, k_{OH^-}, k_o are the constants for catalysis by amine, hydroxide ion, and water, respectively, and k_{obs} is the pseudo first order rate constant at constant pH

and buffer concentration. This linear dependence of the rate on [B] is characteristic of general-base catalysis.

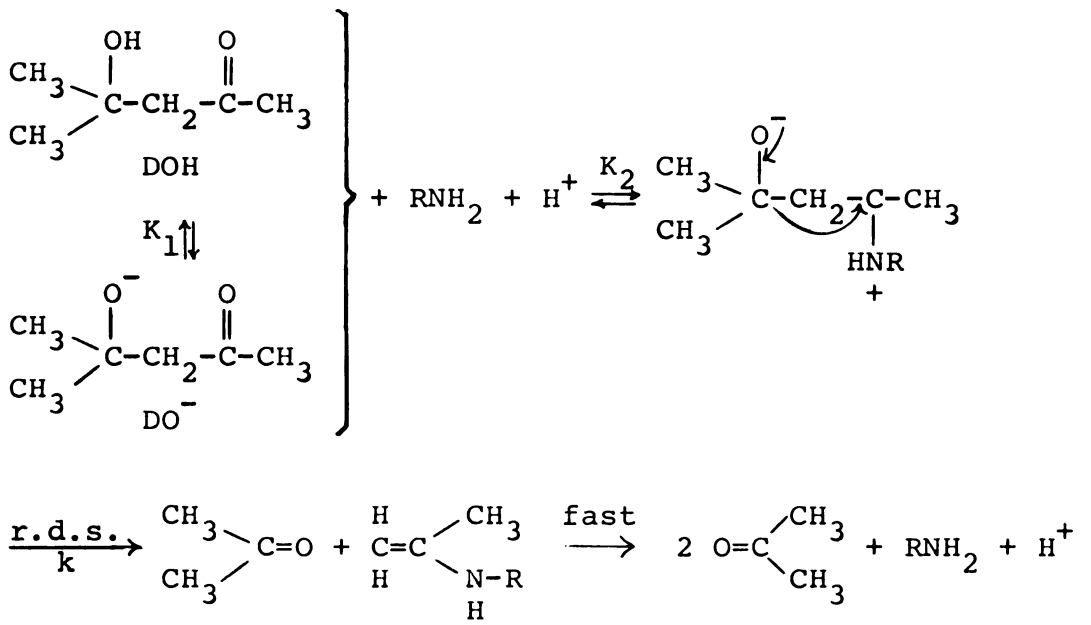
Westheimer and Cohen confirmed this effect of primary and secondary amines but then showed the reaction to be insensitive to tertiary amines. Since tertiary amines should be as effective as general bases, the only reasonable explanation for these results is that primary and secondary amines catalyze the reaction through an intermediate Schiff base mechanism rather than a general-base mechanism; of course, tertiary amines are incapable of forming Schiff bases.

The mechanism for hydroxide-catalyzed dealdolization is shown below. This is typical specific-base catalysis:





The Schiff base-catalyzed mechanism is probably the following:



where DOH is diacetone alcohol; DO^-, the ionized diacetone alcohol; I, the Schiff base intermediate; K_1, the ionization constant of diacetone alcohol; K_2, the equilibrium constant for Schiff base formation; and k, the rate constant for carbon-carbon bond cleavage. Notice that I is the protonated Schiff base of the ionized form of diacetone alcohol.

Since the problem of kinetically equivalent mechanisms occurs throughout this thesis, it is important to show mathematically why this mechanism would obey a rate equation identical to that for general-base catalysis. For simplicity, assume DOH, DO^-, and I are in rapid equilibrium and DO^- and I are in much smaller concentrations than DOH.

Then

$$K_1 = \frac{(DO^-)(H^+)}{(DOH)}$$

$$K_2 = \frac{I}{(RNH_2)(DO^-)(H^+)}$$

The overall rate is, rate = k(I). Rearranging the equilibrium equations

$$(I) = K_2(RNH_2)(DO^-)(H^+)$$

$$(DO^-) = \frac{K_1(DOH)}{H^+}$$

Substituting for DO^-

$$(I) = K_1K_2(RNH_2)(DOH)$$

$$\text{rate} = k(I) = kK_1K_2(RNH_2)(DOH)$$

which is identical in form to that for general base catalysis. It should be pointed out that catalysis through the carbinolamine intermediate rather than the Schiff base is an equally valid interpretation for primary and secondary amine catalysis. However, this does not explain the lack of catalysis by tertiary amines.

Before leaving this topic, Westheimer and Cohen's kinetic interpretations should be put in some perspective. Westheimer himself considered these results "elegant proof"

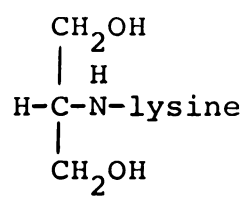
that the amine catalysis was something other than general base catalysis. However, there is one important feature of the dealdolization system that permitted the interpretations to be relatively straightforeward; the insensitivity of the dealdolization to general base catalysis left the Schiff base catalysis naked, so to speak. It will be shown later that general acid-base catalysis often occurs simultaneously with Schiff base catalysis. Had this been the case for the dealdolization of diacetone alcohol, the Schiff base catalysis would probably have been obscured.

Aldol Reactions: Enzymatic

Speck and Forist (18) extended Westheimer's studies of the catalytic effect of amino acids on the dealdolization of diacetone alcohol. These workers considered the possibility that enzyme-catalyzed dealdolizations might proceed through a Schiff base mechanism. They did in fact find that glycine, alanine, and β-alanine catalyzed the reaction.

The strongest experimental evidence for a role of a Schiff base intermediate in an enzymatic mechanism came from inactivation studies with sodium borohydride. This reagent is capable of reducing the Schiff base linkage to a stable amine; therefore, $NaBH_4$ should be able to trap any Schiff base intermediate formed during catalysis.

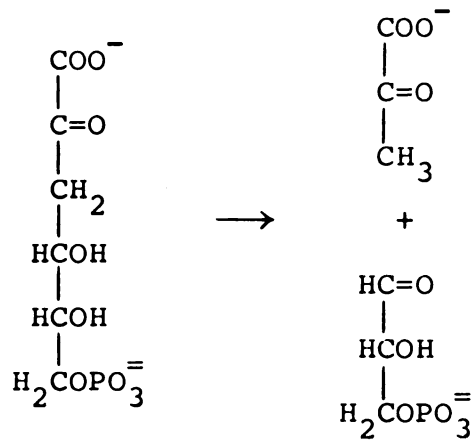
Pontremoli (19) first demonstrated this borohydride inactivation with transaldolase in the presence of substrate, fructose-6-phosphate. Similarly, Grazi, et al. (20) found that muscle aldolase could be inactivated with $NaBH_4$ in the presence of dihydroxyacetone phosphate (DHAP). These workers went on to identify the stably reduced adduct by using $DHAP^{32}$ followed by complete hydrolysis of the protein and subsequent isolation of the radioactive adduct. By chemical degradation and comparison to the authentic compound synthesized by Speck (21) the adduct was identified as N^6-β-glyceryl-lysine:



These results indicate that a Schiff base intermediate is formed from DHAP and an active site lysine residue. Since that time, a number of aldolases--notably KDPG aldolase--have been shown to exhibit the same properties.

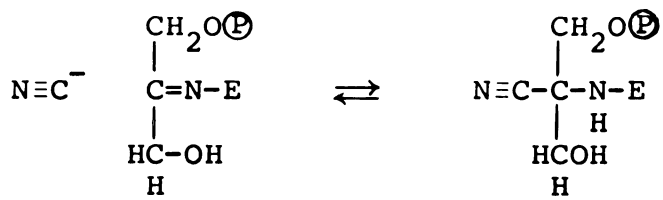
Nonetheless, Rose has raised the objection that borohydride inactivation does not prove that the Schiff base is an obligatory intermediate; conceivably, inactivation could result from fortuitous Schiff base reduction at a residue close to the active site. In order to dispel this objection, he carried out an O^{18} exchange experiment with KDPG aldolase (22). As described in the Introduction

this enzyme catalyzes the aldol cleavage of KDPG to pyruvate and G3P:



When KDPG labeled with O^{18} in the carbonyl oxygen was irreversibly converted to products, no O^{18} appeared in pyruvate. This result strongly supports the Schiff base mechanism.

Finally, cyanide inhibits muscle aldolase in the presence of DHAP (23), presumably by forming an addition compound with the Schiff base (sbustituted ketimine) intermediate:



β-Decarboxylation: Nonenzymatic

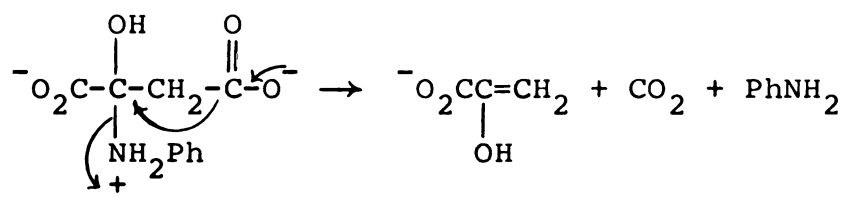
Pedersen (24) found the decarboxylation of α, α, dimethylacetoacetic acid in aniline or o-chloroaniline buffer followed a pH-rate profile corresponding to the rate equation

$$v = k[RNH_2][-\overset{O}{\overset{\|}{C}}-COO^-][H^+].$$

From previous discussions, it is clear that this term is consistent with Schiff base-catalyzed decarboxylation.

In a later study of the amine-catalyzed decarboxylation of oxalacetic acid Pedersen (25) found the reaction to be catalyzed by ammonium, ethylammonium, and anilinium ions. Of these, the anilinium ions were most effective in acid solution. Again, he attributed the catalysis to an intermediate Schiff base. However, this interpretation was convincingly challenged by Hay (26) who showed with model compounds that Schiff base formation between oxalacetate and aniline does not occur at all in aqueous solution. Surprisingly, a stable carbinolamine is formed rapidly and in high concentration. Catalysis is then seen as a concerted elimination of the anilinium ion:

$$PhNH_2 + {}^-O_2CCOCH_2CO_2H \rightleftharpoons$$



Hay went on to show however that Schiff base formation is favorable in ethanol and probably accounts for the catalysis under these conditions.

On the other hand, Westheimer (27) showed that in aqueous solution cyanomethylamine catalyzes the decarboxylation of acetoacetate at the same rate as it forms Schiff base with ethylacetoacetate. Here the ester was used to measure Schiff base formation independently of decarboxylation. Assuming that the acid forms a Schiff base at the same rate as the ester, these results support the Schiff base mechanism.

This reviewer agrees with Jencks's view (28) that there is no real contradiction in all these findings, but merely that different mechanisms operate with different substrates and conditions. Indeed, the carbinolamine-catalyzed pathway may be peculiar to aniline; otherwise it is difficult to see why tertiary alkyl amines are ineffective catalysts.

β-Decarboxylation: Enzymatic

In a study of the acetoacetic acid decarboxylase reaction, Westheimer (29) followed the loss of O^{18} from the C-3 carbonyl oxygen of acetoacetic acid upon conversion to acetone. He found none of the label retained in acetone, which suggests a Schiff base mechanism for decarboxylation. (This complete exchange also argues against a carbinolamine mechanism by which some O^{18} should be retained.)

These interpretations were supported by subsequent borohydride inactivation of the enzyme in the presence of substrate followed by isolation of the expected adduct ε-N^6-isopropyllysine (30, 31).

Furthermore, cyanide was shown to inhibit the enzyme in the presence of substrate, presumably by forming a substituted ketimine.

It has also been shown that certain Schiff base-forming aldolases are capable of catalyzing decarboxylation reactions (32, 33).

Enolization: Nonenzymatic

The only significant investigations in this area have been Bender's studies on the amine-catalyzed enolization of acetone (34) and Hine's studies of the amine-catalyzed enolization of isobutyraldehyde (35). Since these investigations bear most directly on the research presented here, they will both be discussed in some detail.

Bender measured the enolization of acetone in the presence of a large number of amines. The kinetics followed a simple rate law:

$$\text{rate of enolization} = k_{obs}(\text{acetone})$$

$$= [(k_o + k_a(A) + k_b(B) + k_{ab}(A)(B)](\text{acetone})$$

where k_{obs} is the pseudo first order rate constant under conditions of constant buffer concentration and pH; k_o is the constant for catalysis by water, hydroxide ion, and hydronium ion at fixed pH; k_a is the constant for catalysis by the acid species of the buffer; k_b is the constant for catalysis by the basic species of the buffer; k_{ab} is the constant for "concerted" catalysis by both the acid and base species; (A) and (B) are the concentrations of acid and base species, respectively. Only some of the amines showed a k_{ab} term, but all showed k_a and k_b terms. Since the important interpretations were done with methylamine buffer which does not show such a k_{ab} term, only the simplified rate law

$$k_{obs} = k_o + k_aA + k_bB$$

shall be considered for the moment.

All the constants in this equation can be determined as follows. k_{obs} is determined from a plot of acetone concentration versus initial rate at fixed pH and buffer concentration; k_o is determined from the intercept of a plot of buffer concentration versus k_{obs} at fixed pH; k_a and k_b are determined from the following algebraic manipulations:

$$rak = k_{obs}(acetone) = [k_o + k_aA + k_bB](acetone)$$

$$k_{obs} = k_o + k_aA + k_bB$$

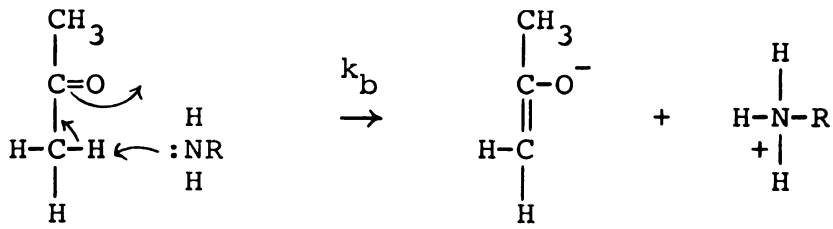
Dividing by A, defining the variable r = A/B, followed by inconsequential rearrangements gives:

$$k' = \frac{k_{obs} - k_o}{A + B} \frac{r + 1}{r} = k_a + k_b(\frac{1}{r})$$

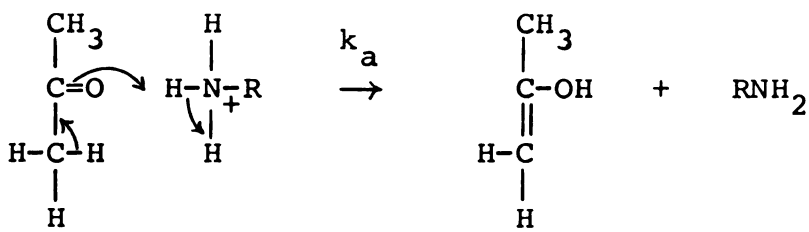
Thus, a plot of k' versus 1/r gives k_b as the slope and k_a as the intercept.

The overall form of the rate law is apparently uninformative since it is identical to that for simple general acid-base catalysis; the effect of Schiff base catalysis is not immediately evident. (As a matter of review, the mechanisms for general acid and base catalysis by amine is shown below:

general base



general acid



However, Bender utilized the magnitude of the constants k_b and k_a rather than the form of the equation to determine the existence of Schiff base catalysis. This was done by use of the Brönsted relation, which relates the strength of an acid or base (by pK_a or pK_b) to its catalytic capacity, k_a or k_b. When the k_b values for the various amines were compared to those for other general bases, the catalytic ability of the basic form of the amine corresponds very well to that predicted for general base catalysis; therefore, the term k_b (B)(acetone) in the rate law was interpreted as simple base catalysis.

However, when the k_a values for the amines were compared in the same way it was found that k_a was as much as one million times larger than the value predicted by the acid strength of the protonated amine. Bender interpreted these results to mean that the k_a (acetone)(A) term in the rate equation actually represents water-catalyzed enolization of the protonated ketimine. This mechanism is, of course, kinetically equivalent to general acid catalysis insofar as the overall form of the rate law is concerned.

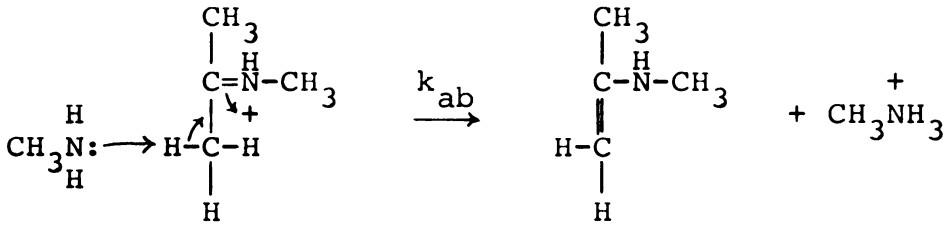
In support of this interpretation, Bender found that the rate law with trimethylamine, which cannot form a Schiff base, contains a k_b term but no k_a term. Furthermore, with methylamine buffer the plot of k' versus 1/r deviates from linearity at lower pH. Further experiments

showed that this deviation could be accounted for by a change in the rate determining step from enolization to Schiff base formation itself.

The rate constant for the enolization of the protonated ketimine can then be calculated if the following constants are known: k_a; the equilibrium constant for Schiff base formation; and the pK_a for the protonated ketimine. Bender found the enolization constant to be 2.6×10^{-2} $M^{-1}sec^{-1}$. This value is approximately four hundred times less than the aldolase-catalyzed enolization of dihydroxyacetone phosphate, but 10^9 times greater than the water-catalyzed enolization of acetone.

While the basic interpretations are probably correct, there are a few criticisms that could be made about this work. First, the pK_a for the protonated ketimine could not be experimentally determined and was therefore approximated as 7.6. Second, it would have been most desirable to measure the enolization on both sides of the pK_a, as is usually done, to show that one ionized form of a substrate is active. Bender apparently tried this, but the system was intractable. Third, the method for determining the important constant k_a requires that k_a be measured <u>relative</u> to k_b (i.e., as the intercept of the k' versus 1/r plot). It turns out that the value of k_a is only 1.5% the value of k_b; that is, a small change in the slope of the k' versus 1/r plot results in a large change in the intercept. Conceivably, an unknown systematic

error in the determination of k_b could result in a positive intercept in such a plot where none should really exist. Fourth, it is a bit strange that in methylamine buffer no term in the rate equation was seen for base-catalyzed enolization of the protonated ketimine:



$$\text{rate} = k_{ab}(\text{acetone})(RNH_3^+)(RNH_2)$$

The absence of such a term implies that the protonated ketimine is not susceptible to further catalysis. This creates the puzzling question as to how an aldolase makes up the four hundred-fold difference in rate. Bender touches on this question but not satisfactorily. Furthermore, this result contrasts with Hine's studies on the enolization of isobutyraldehyde wherein the predominant route was found to be base-catalyzed enolization of the protonated ketimine. However, some of Hine's interpretations are questionable. These studies will now be discussed.

The major difference in this system, compared to Bender's, is that the equilibrium constant for imine formation with isobutyraldehyde is so large that most of

the aldehyde exists as the imine in the presence of moderate concentrations of methylamine buffer. For this reason, Hine considers this system superior to any previously studied for imine catalysis. However, the high concentration of imine made it necessary to consider many more factors which might influence the overall rate than was necessary for the acetone system. This thoroughness causes the kinetic treatment to be extremely complicated but serves to illustrate the difficulties in determining the desired constants.

Hine measured the enolization by following the loss of deuterium from isobutyraldehyde-2-d. When this was done under conditions where the concentration of methylamine and imine were held constant, the rate of exchange increased with increasing concentrations of methylammonium ions. As with the acetone system, the dependency on methylammonium ions is best attributed to enolization via the protonated imine rather than via general acid catalysis. In contrast to the acetone system, where only a fraction of the total enolization could be accounted for this way, most of the enolization occurs through the imine under Hine's conditions. However, it still remains to be determined precisely how much exchange proceeds through the protonated imine and how much through other routes. For this purpose, Hine considers every possible mechanism for exchange.

There are three species which are capable of exchange: the aldehyde, the neutral imine, and the protonated imine. For each of these species, enolization could be catalyzed by any of the bases in solution--methylamine, the neutral imine, hydroxide ion, and water. The resulting rate equation contains ten terms. After suitable algebraic substitution and rearrangement we have the following equation:

$$k_{cor} = \frac{k_m}{K} + \frac{k_i[I_m]}{K[MeNH_2]} + \frac{k'_m K_{MH}[MeNH_3^+]}{K_{IH}} + \frac{k'_i K_{MH}[I_M][MeNH_3^+]}{K_{IH}[MeNH_2]}$$

$$+ \frac{k'_h K_w}{K_{IH}} + \frac{k'_w K_{MH}[H_2O][MeNH_3^+]}{K_{IH}[MeNH_2]} + k''_m[MeNH_2] + k''_i[I_m]$$

$$+ \frac{k''_h K_w[MeNH_2]}{K_{MH}[MeNH_3^+]}$$

where each small letter k on the right hand side of the equation is a rate constant for one of the base-catalyzed routes as listed in Table 1. Each capital letter, K, is an equilibrium constant as defined below:

$$K_{MH} = \frac{[H^+][MeNH_2]}{[MeNH_3^+]}$$

K_w = ion product of water

$$K = \frac{(\text{imine})}{(MeNH_2)(\text{aldehyde})}$$

$$K_{IH} = \frac{(\text{protonated imine})}{(H^+)(\text{neutral imine})}$$

TABLE 1.--Catalytic Constants in Rate Equation Describing Enolization of Isobutyraldehyde in Methylamine Buffers.

Rate Constant	Species	Base
k_m	aldehyde	$MeNH_2$
k_i	"	neutral imine
k_h	"	OH^-
k''_m	neutral imine	$MeNH_2$
k''_i	"	neutral imine
k''_h	"	OH^-
k'_m	protonated imine	$MeNH_2$
k'_i	"	neutral imine
k'_h	"	OH^-
k'_w	"	H_2O

The constant $k_{cor} = \frac{k_p}{f_I} - \frac{k_h K_w}{K K_{MH}[MeNH_3^+]}$ where k_p is the pseudo first order rate constant from

$$\frac{-d(\text{imine} - d)}{dt} = k_p(\text{imine} - d)$$

and f_I is a factor which corrects for the loss of deuterium from the imine as a result of deuterium exchange from the aldehyde in rapid equilibrium.

As it stands, the equation is too complex to solve for the desired constants. The only recourse is to discard certain terms as "negligible" based on carefully chosen arguments.

First, there are two terms that never entered the equation at all: the water-catalyzed enolization of the aldehyde and the water-catalyzed enolization of the neutral imine. Previous experiments showed the water-catalyzed enolization of the aldehyde to be very small; from this Hine reasoned that the neutral imine would be even less susceptible to water catalysis and could also be ignored.

The next term to be discarded was the last in the above equation by realizing that if the equation is put in the form

$$k_{cor} = C + C'[MeNH_3^+] + \frac{k_h'' K_w [MeNH_2]}{K_{MH}[MeNH_3^+]}$$

a plot of k_{cor} versus $MeNH_3^+$ at constant $MeNH_2$ and imine will be linear only if the last term is negligible. This was found to be the case.

Since this discarded term represents enolization of the neutral imine by hydroxide ion, Hine reasoned that the other weaker bases should be even less capable of catalyzing the enolization of neutral imine. Therefore, the terms containing k_h'', k_m'', k_i'' can also be discarded.

Finally, if the neutral imine functions as a general base catalyst, k_{cor} should increase with increasing imine. Since this does not occur to a significant extent the terms containing k_i, k_i', and k_i'' can be neglected.

With this weeding process complete, the simplified equation is:

$$k_{cor} = \frac{k_m}{K} + \frac{k_h' K_w}{K_{IH}} + \frac{k_m' K_{MH}[MeNH_3^+]}{K_{IH}}$$

Thus, the slope of k_{cor} versus $(MeNH_3)$ gives $k_m' K_{MH}/K_{IH}$ from which k_m', the constant for the methylamine-catalyzed enolization of the protonated inine, can be calculated. For this, K_{IH} must be known--but it is not. Just as with the acetone system, this constant had to be estimated.

The intercept gives the sum of the two kinetically equivalent routes--the amine-catalyzed enolization of the

aldehyde and the hydroxide ion-catalyzed enolization of the protonated imine.

Hine concluded then, that enolization occurs predominantly via amine-catalyzed enolization of the protonated imine with a rate constant being three hundred times greater than the amine-catalyzed enolization of the aldehyde. Hence, as expected, the protonated imine is more reactive than the aldehyde.

In evaluating this work, the arguments used for discarding the terms in the rate law must be questioned. But aside from this, this reviewer is most puzzled by the lack of any arguments for discarding the term for the water-catalyzed enolization of the protonated imine (k_w). Somehow, it just vanished. Recall that with acetone in methylamine buffer Bender found only water-catalyzed enolization of the Schiff base. Without some explanation for this omission, it is difficult to see how the linearity of k_{cor} with ($MeNH_3$) can be interpreted as it has. If the k_w term is not omitted, the slope becomes the sum of the water-catalyzed and amine-catalyzed routes with no means for distinguishing the individual contribution by each.

Enolization: Enzymatic

Rose and Rieder (36) first discovered that in tritiated water, muscle aldolase catalyzed the exchange

of tritium with the pro-S-hydrogen[2] from the hydroxymethyl of dihydroxyacetone phosphate (DHAP). Since DHAP has two equivalent hydrogens at the hydroxymethyl position, the aldolase can apparently distinguish one from the other. This conclusion is supported by the observation that hexose diphosphate (HDP) which results from the condensation of DHAP with G3P, incorporated no tritium; since the hydrogen at the C-3 position of HDP derives from DHAP, random exchange of DHAP hydrogens would necessarily have produced labeled HDP.

In a later detailed study (37) these workers considered the possibility that the exchange reaction might actually depend on contaminating levels of aldehyde. By this view, the aldehyde forms the condensation product which, upon subsequent reversion to trioses, produces labeled DHAP. They dispelled this possibility by showing that HDP actually inhibits the exchange reaction of DHAP.

They went on to show that the kinetics of exchange and condensation were consistent with the following scheme:

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[2]This stereochemical designation is discussed by K. R. Hanson, J. Am. Chem. Soc. 88, 2731 (1966).

$$\text{DHAP} + \text{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-DHAP} \quad \text{(exchange)}$$

$$\text{G3P} + \text{E-DHAP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E-HDP} \xrightarrow{k_3} \text{E+HDP} \quad \text{(condensation)}$$

That is, exchange and condensation occur at the same site on the enzyme, which implies that they are mechanistically linked. According to this scheme, the exchange from tritiated DHAP (in the absence of G3P) should obey the rate law

$$v^x = \frac{k_{-1} E_o}{1 + \frac{K^x}{D}}$$

where v^x is the rate of exchange, K^x the dissociation constant for E-S complex, and D the concentration of DHAP. As predicted from this rate equation, a plot of $1/v^x$ versus 1/D was linear.

The scheme also predicts that as G3P is increased, the rate of exchange from tritiated DHAP will become equal to the rate of condensation. This should occur because at higher levels of G3P, condensation with E-DHAP competes ever more favorably with dissociation of E-DHAP. The results of the experiment were in accord with these predictions.

Since that time, other enzymes have been shown to catalyze similar exchange reactions (38, 39): KDPG aldolase, α-keto glutaric acid aldolase, and acetoacetic acid decarboxylase.

CHAPTER III

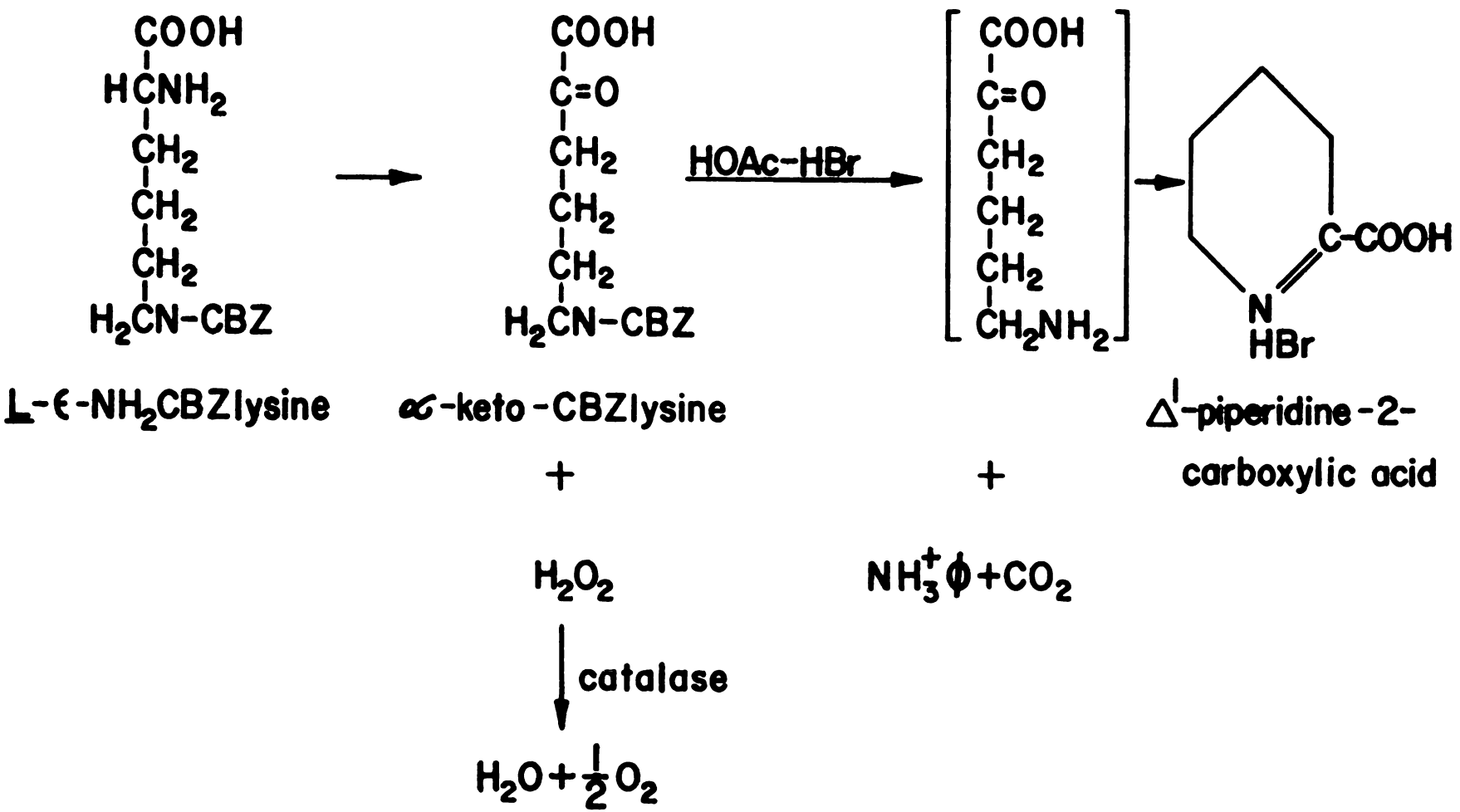
METHODS

Synthesis of Δ^1-Piperidine-2-Carboxylic Acid

The synthesis of Δ^1-piperidine-2-carboxylic acid (Δ^1-PCA) has been accomplished by Meister according to the following procedure (40): First, N-ε-amino-carbobenzoxylysine is converted to α-keto carbobenzoxylysine (α-keto-CBZlysine) by L-amino acid oxidase. Since hydrogen peroxide is a product of the reaction, catalase is included to prevent the decarboxylation of the α-keto acid. The α-keto CBZlysine is isolated from the mixture by acid extraction into ethylacetate followed by evaporation of the solvent and crystallization with petroleum ether. The carbobenzoxy group is removed with acetic acid--HBr reagent from which the transient product ε-amino-α-keto caproic acid is produced. This compound spontaneously cyclyzes to the hydrobromide salt of Δ^1-PCA. The entire sequence is illustrated in Figure 1.

Unfortunately, this author could not realize this synthesis when Meister's procedures were rigorously followed. It has come to his attention that other

Figure 1.--Synthesis of Δ^1-piperidine-2-carboxylic acid.


COOH
HCNH2
CH2
CH2
CH2
H2CN-CBZ
L-ε-NH2CBZlysine
HOAc-HBr
COOH
C=O
CH2
CH2
CH2
H2CN-CBZ
α-keto-CBZlysine
+
H2O2
catalase
H2O+½O2
COOH
C=O
CH2
CH2
CH2
CH2NH2
+
NH3+φ+CO2
C-COOH
N
HBr
Δ1-piperidine-2-
carboxylic acid

laboratories have also met with failures. Macholàn and Svàtek (4) did achieve the synthesis by what they called a "modified" Meister procedure, upon which they did not elaborate.

Because of these difficulties, this author feels it necessary to describe in detail the problems he encountered and the modifications required to successfully synthesize the compound.

At first, the Meister procedure was attempted as directed. 5.0 g α-keto-CBZ lysine were suspended in 200 ml triply distilled water and adjusted to pH 7.0 with 2 N NaOH. After equilibration to 37°C in a water bath, 40 units of L-amino acid oxidase were added. The solution was carefully adjusted to pH 7.0 again, and replaced in the water bath. Oxygen was continuously bubbled through the solution during the course of the reaction. Stirring was effected with a submersible magnetic stirring motor. Foaming was controlled by occasional addition of Corning Antifoam.

The reaction was followed by acid-extraction of the product from 2 ml aliquots with ethylacetate and measuring the characteristic ultraviolet absorption spectrum of the carbobenzoxy (CBZ) group.

The reaction did not proceed as fast as Meister indicated; after 48 hours, the spectral determination indicated that only 2 g α-keto CBZ lysine had been converted.

The reaction was terminated at this point by acidification of the mixture to Congo Red followed by extraction with several portions ethylacetate. The combined extracts were dried overnight (Na_2SO_4). The solvent was then removed in vacuo, which produced a large amount of white crystalline material. This material was redissolved in a small volume of ethylacetate and recrystallized by addition of petroleum ether (bp 40-55°C). After standing at -10°C overnight, the product was collected by suction filtration and washed with petroleum ether. The melting point was 105-106.5°C (reported mp α-keto-CBZlys; 109°C); yield = 1.3 g (26%).

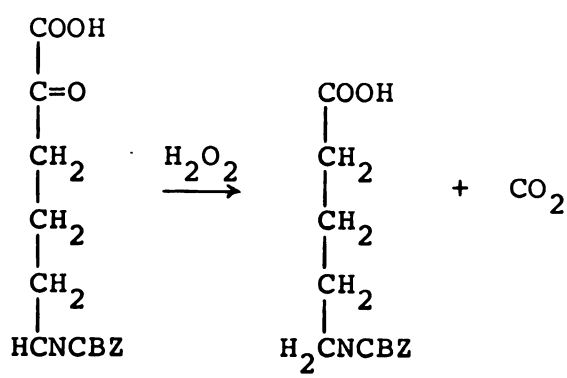
The CBZ group was removed as directed by addition of 1.0 ml HOAc--HBr reagent to 250 mg α-keto-CBZlysine. After an hour when the vigorous reaction had subsided, the white crystalline product was precipitated and washed with ethylether; yield, 80 mg (41%).

However, the product melted at 105°C, far from the reported melting point of 190°C for Δ^1-PCA. In addition, the melting point was quite sharp, indicating that a pure compound had been synthesized. Comparison of the uv-visible absorption spectrum of this material to those published by Macholàn showed definitely that the compound was not Δ^1-PCA. Although the shape of the spectrum could--with some imagination--possibly resemble that of the authentic compound, the extinction coefficient at 256 mμ

was only about 3.0 instead of the reported value 725 mμ. The spectrum did show, however, that the CBZ group had been removed.

The HOAc--HBr treatment was first examined as a source of problems. It has been reported that bromination sometimes occurs as a side reaction (41). However, bromination can successfully be suppressed by addition of phenol to the reaction mixture. When this modification was tried, however, the product had the same characteristics as before. It was therefore concluded that the source of trouble was elsewhere, perhaps in the oxidation of L-ε-amino-CBZlysine itself.

Since an extractable product was formed, oxidation had certainly occurred. However, if for some reason peroxide had not been sufficiently removed by catalase the α-keto derivative could have been quantitatively decarboxylated. In that case, the material isolated would actually be ε-amino-CBZ valeric acid:



To test this possibility, the extractable material was tested for a carbonyl function with 2,4-dinitrophenylhydrazine and semicarbazide. Meister had reported a 2,4-dinitrophenylhydrazone derivative of the α-keto-CBZ-lysine. However, all attempts to form this derivative failed. Likewise, the compound was unreactive to semicarbazide.

As a further test, the titration curves for this compound and the one recovered from HOAc--HBr treatment were determined. If the extractable material is N-ε-aminoCBZvaleric acid, the acid group should have a pK_a between 4 and 5, whereas that for the α-keto product should have a pK_a between 2 and 3. The curve clearly showed only one titratable group with a pK_a between 4 and 5.

The HOAc--HBr treated material showed two titratable groups, one with a pK_a = 4-5, and the other with a pK_a greater than 10. This is consistent with the expected product N-ε-aminovaleric acid.

The above results provide good evidence that N-ε-amino CBZlysine had first been converted to α-keto-CBZlysine then decarboxylated to N-ε-amino-CBZvaleric acid. The decarboxylation problem was eventually solved by using very large amounts of catalase. But in addition, other objectionable features of the synthesis had to be dealt with. First, the CBZlysine is not only insoluble

in water, but floats as a large foam over the solution. This problem is exacerbated by the magnetic stirrer. The most diligent attempts to wet this material down during the course of the reaction were not successful. Second, the pH is not well controlled under these conditions; CBZlysine is not a good buffer near neutrality. Third, when the HOAc--HBr treatment is carried out as directed the solution turns very dark yellow or orange. When ether is added, a small yellow layer settles to the bottom and is very difficult to remove from the product. Fourth, as mentioned earlier, the reaction rate is not as fast as it is reported to be.

With all these objections in mind, the successful synthesis will not be presented. The modifications will be discussed as they arise. First, the magnetic stirrer was replaced by a shaking water bath. Since shaking is not as effective in distributing the bubbled oxygen through the solution the reaction volume was scaled down ten-fold. Shaking was much more effective in keeping the CBZlysine in suspension. The pH was maintained with 0.1 M HEPES buffer, pH 7.5.

500 mg CBZlysine in 20 ml HEPES buffer was placed in a 50 ml Erlynmeyer flask which was then stoppered and vigorously shaken by hand to suspend the CBZlysine as well as possible. After this mixture had reached 37°C on the water bath, the reaction was initiated by the addition of 20 units of L-amino acid oxidase (Worthington) and 10 µl

catalase suspension containing 206,500 U/ml. The level of amino acid oxidase is much higher than the Meister procedure calls for, in order to increase the rate of reaction. More importantly, it was found after repeated attempts, that extraordinary amounts of catalase are needed to prevent decarboxylation. For this reason, 10 µl catalase was added every 15 min throughout the course of the reaction.

Water-saturated oxygen was continuously bubbled through the solution. Foaming was controlled by an occasional drop of 1-octanol.

The reaction was terminated after approximately fourteen hours even though it had not proceeded to completion. After acidification to pH 3.0, the extraction and crystallization was carried out according to Meister's procedure with little complication. Approximately 100 mg product were recovered. Because of the small amount of material, the recrystallized product was collected and washed by centrifugation instead of suction filtration. The product melted at 100-103 (c.f. 106°). This time the product gave a positive semicarbazide test and readily formed a phenylhydrazone derivative, mp 145-155 (c.f. mp 160°C).

The HOAc--HBr treatment was performed as follows: 2 ml HOAC--HBr was pretreated with 50-100 mg phenol, which later prevented formation of the yellow contaminant. 0.5 ml of this reagent was added to 25 mg α-keto-CBZlysine.

After one hour, when CO_2 evolution had ceased, the product was precipitated and washed several times with ether by centrifugation. After perhaps the second ether wash, the product sticks tenaciously to a glass stirring rod. This makes washing impossible, but can be used to advantage by transferring the precipitate on the glass rod to a fresh tube of anhydrous ether. After 15 min of agitiation, the product comes loose from the rod and settles to the bottom of the tube. From this point on, washing can be continued without problems. The product was dried in vacuo overnight (mp 190°C, c.f. 192°C).

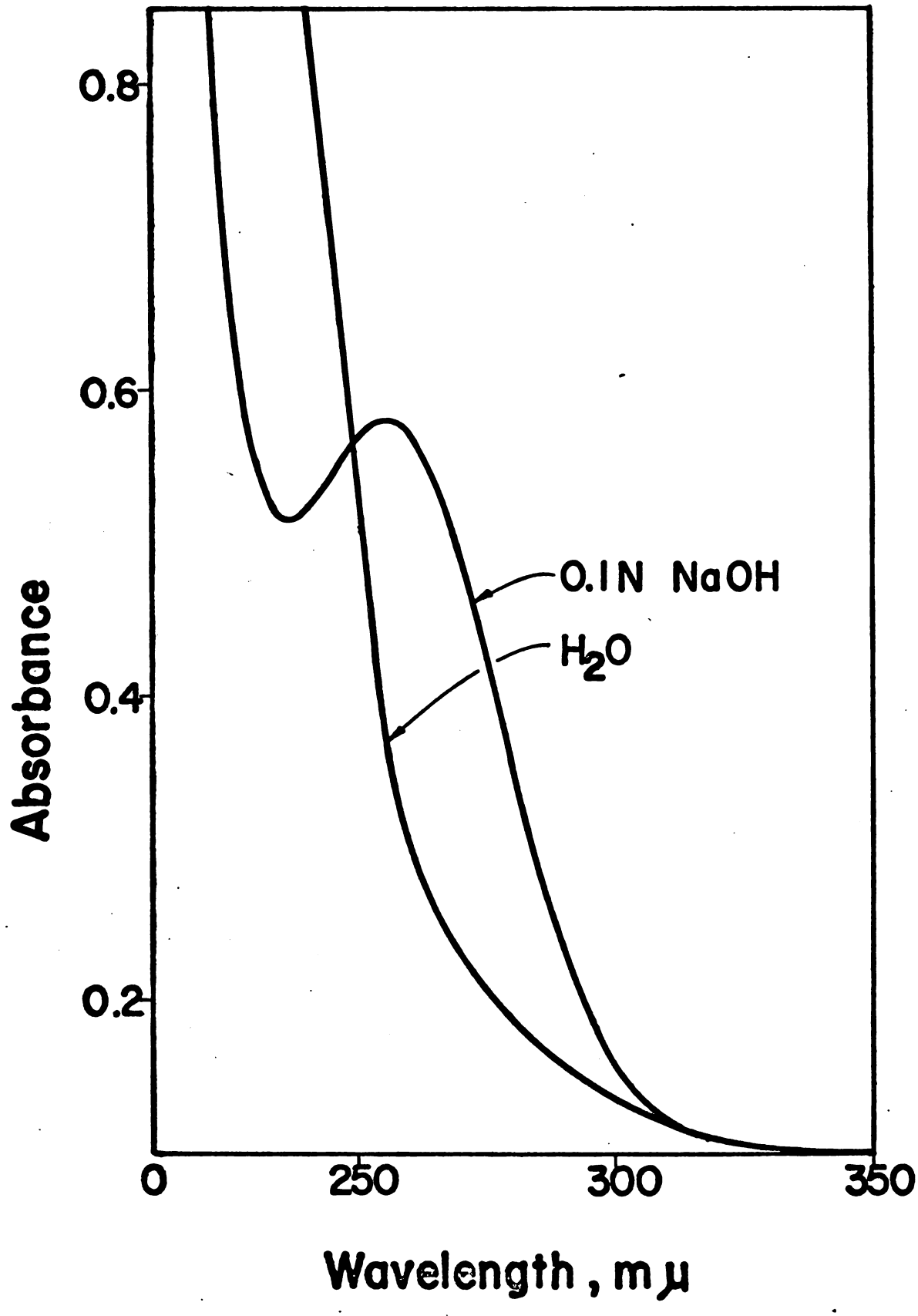
Absorption spectra of the compound in water and 0.1 N NaOH (Figure 2) were identical to those published by Macholàn. In 0.1 N NaOH, the extinction coefficient at 256 mμ (ε = 725) usually agreed with the reported value within 5%.

Buffers

All catalysts except potassium phosphate were recrystallized before use as follows:

n-Butylamine hydrochloride (Eastman) was dissolved in hot alcohol then allowed to cool to room temperature. When the initial precipitation was nearly complete an excess ethyl ether was added which precipitated the remaining material. The mixture was then heated on a steam bath until just enough ether had evaporated to allow dissolution of all the material. At this point, the flask

Figure 2.--Absorption spectra of Δ^1-piperidine-2-carboxylic acid in water and 0.1 N NaOH. The spectra were determined with a Cary Model 15 recording spectrophotometer. Each solution contained 0.1433 mg per ml of the hydrobromide salt of Δ^1-piperidine-2-carboxylic acid.


0.8
0.6
0.4
0.2
Absorbance
0.1N NaOH
H_2O
0
250
300
350
Wavelength , mμ

was quickly stoppered and allowed to cool. Imidazole (Sigma) was recrystallized several times from benzene. n-ε-Aminocaproic acid was recrystallized from EtOH--H_2O--ether. The buffers were adjusted to the desired pH with NaOH or HCl. For most determinations a Beckman pH meter with expanded scale was used. All buffers contained 0.2 M KI and NaCl to maintain the ionic strength at 0.4.

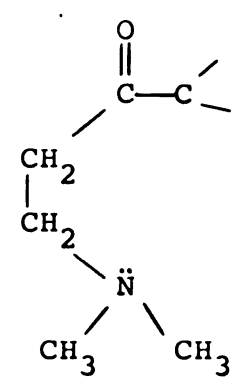
Iodination Procedures

The enolization of ketones (or ketimines) can be followed by rapid halogenation of the enol (or enamine). Under proper conditions, halogenation is zero order with respect to halogen concentration so that the rate of halogenation equals the rate of enolization. In the present case, iodination was used.

In a concentrated solution of KI, I_2 is mostly converted by a rapid equilibrium to I_3^- : $I_2 + I^- \rightleftharpoons I_3^-$. Since the I_3^- species has an intense absorbance maximum at 351 mμ (ε = 26,400), the iodination can be measured spectrophotometrically by following the loss of absorbance at this wavelength.

The iodinations were performed as follows: 1.0 ml buffer was placed in a cuvette and equilibrated in the cuvette compartment of a Gilford Model 2000 spectrophotometer with automatic cuvette changer. A Haake circulating water bath was used to maintain the temperature

at 25°C. After equilibration, iodine in 0.2 M KI was added with a Hamilton microsyringe to a final concentration of approximately 3×10^{-5} M. A blank rate was determined prior to addition of pyruvate. The reaction was initiated by the addition of pyruvate in large excess over iodine, with a Hamilton microsyringe. The rates were linear except near the very end where most of the iodine had been used. This linearity indicated that the reaction was indeed zero order in iodine. As a further check, the reactions were occasionally repeated at different iodine concentrations; in all cases the results were identical. The rate of enolization was calculated from the rate of iodination on the basis of an extinction coefficient 2.6×10^{4} for the triiodide ion (42). Although this method is well established by now, Coward and Bruice (43) have vigorously assaulted the use of halogenation to measure enolization. In particular, some of Bender's work with acetone was questioned. Bruice and Coward were studying the enolization of β-amino ketones of the form



Specifically, they were looking for intramolecular catalysis by the tertiary amino group. They found the reaction of these compounds with iodine to be so fast that the above initial-rate method (in which substrate is in excess over iodine) could not be used. Instead, they followed the first order reaction of substrate with iodine in excess. Since the high concentration of iodine prohibited spectrophotometric methods under these conditions, a pH-stat assay was used to measure the hydrogen ion produced upon iodination.

These workers raised an additional objection to the initial-rate method because such a small fraction of substrate is reacted during the determination. Consequently, they reasoned, a small amount of highly reactive impurity could seriously interfere with the results. While this is true, this objection could be made for many kinetic analyses which utilize initial-rate measurements.

They also found the first order method objectionable because the iodine in such high concentration reacts with the buffer components at a rate comparable to the reaction with substrate. According to them, the reaction of iodine with phosphate buffer is so fast that they questioned the validity of Bender's results with this buffer. However, this author, who has also used phosphate buffer for measuring the enolization of acetone and pyruvate, found the blank rate to be virtually

immeasurable with this buffer in the pH range 5.75-6.50. It is true that reaction with buffer and hydroxide ion does become increasingly bothersome at higher pH, and especially with amine buffers. However, the enolization of acetone and pyruvate is sufficiently fast so that the reaction of iodine with buffer can be subtracted without a severe loss of accuracy.

What is most puzzling to this author is that Coward and Bruice could obtain any meaningful data at all with tertiary amines by iodination procedures. Bender reported that the enolization of acetone in trimethylamine could not be measured by iodination since trimethylamine reacts rapidly and irreversibly with iodine to form an N-iodo complex. (Primary and secondary amines also form this complex but to a lesser degree which does not interfere with the reaction.) Instead, Bender had to use deuterium exchange to measure the enolization with this buffer.

pH-stat Iodination Procedures

Iodination of pyruvate and Δ^1-piperidine-2-carboxylic acid in the absence of buffer was performed on a Sargent Recording pH-stat in order to maintain constant pH. Normally, 0.001 or 0.005 N NaOH was used for this purpose.

Prior to addition of substrate, a solution containing 0.2 M KI, 0.2 M NaCl and 3×10^{-5} M I_2 in 50 ml was equilibrated to 25°C on the pH-stat and adjusted to the desired pH. Although the pH-stat was used to maintain pH, all pH readings were made with a Beckman pH meter with expanded scale and combined glass electrode.

The reaction was initiated by the addition of substrate in 0.5 ml and the iodination followed by withdrawing aliquots at timed intervals and measuring the absorbance at 351 mμ with a Beckman DU spectrophotometer.

Determination of pK_a of n-butylamine

Since the dissociation constant of any acid is sensitive to the ionic strength and, sometimes, the particular ions in solution, the pK_a of n-butylamine had to be determined under iodination conditions (0.2 M KI + NaCl; I = 0.4).

This is done in principle by half neutralizing a solution of n-butylaminehydrochloride with NaOH so that the concentration of conjugate acid equals the concentration of conjugate base. Then, according to the Henderson-Hasselbach equation ($pH = pK_a + \log \frac{n\text{-}BuNH_2}{n\text{-}BuNH_3^{\oplus}}$) the pK_a equals the pH of the solution. Following this procedure, the pH of a solution containing 0.100 M butylaminehydrochloride, 0.2 M KI, 0.1 M NaCl, and 0.05 N NaOH was found to be 10.85. For this determination, a Beckman Model G pH meter was used.

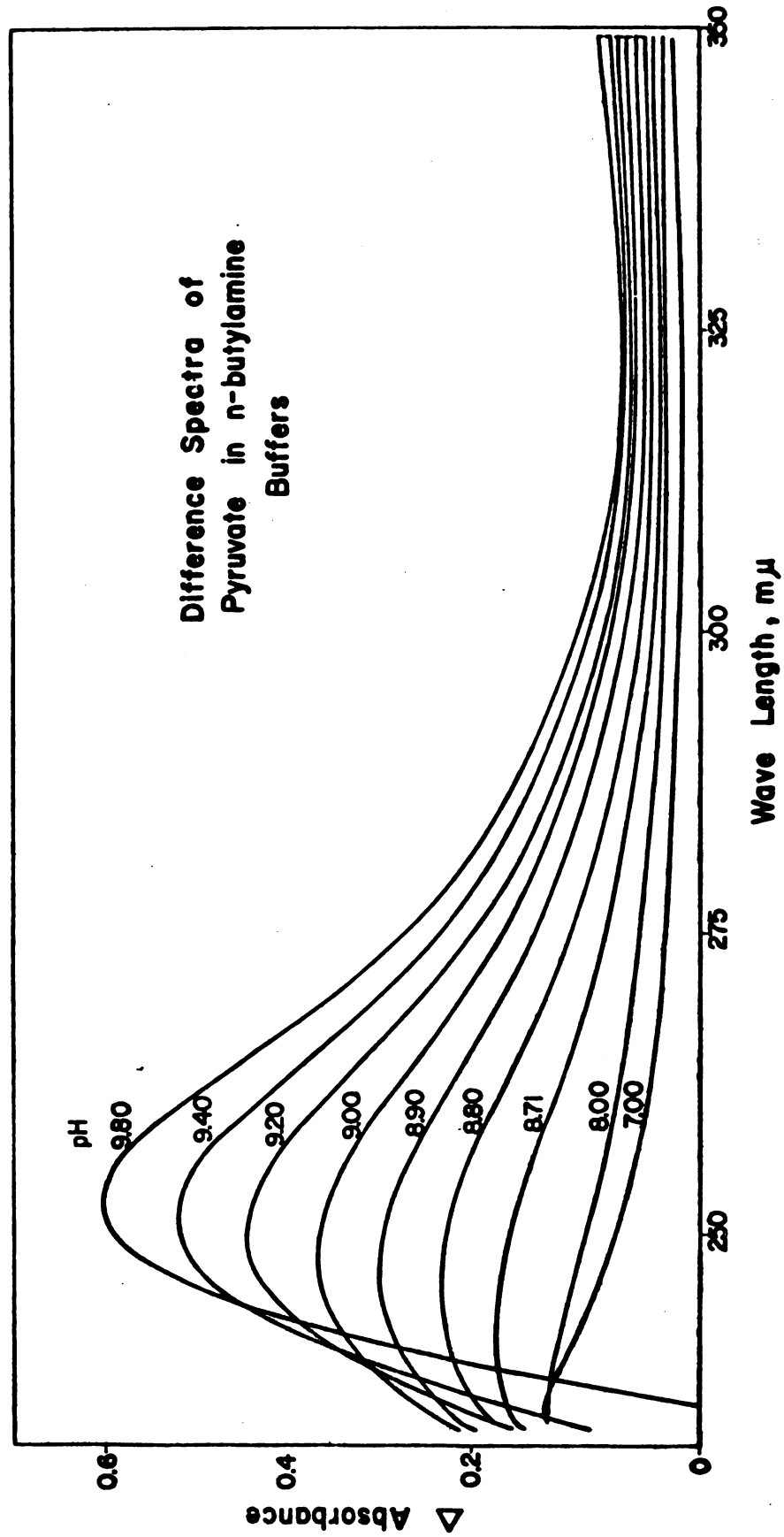
The validity of the technique was assured by comparing the pK_a of n-butylamine in water alone to the literature value (44). The uncorrected value was 10.71 at 25°C, which compared very well to the reported value of 10.66.

However, the pK_a determined by this method has to be corrected for the amount of NaOH needed to achieve pH 10.85 in the absence of amine. This was done with a blank titration of a solution containing 0.2 M NaCl and 0.2 M KI. After appropriate corrections, the pK_a of n-butylamine at 25°C was found to be 10.896.

Determination of the Equilibrium Constant of Schiff Base Formation

Perhaps the best method for determining the equilibrium constant for Schiff base formation (K_s) is the polarigraphic method used by Zuman (45). Zuman determined K_s for a number of aldehydes and ketones (including pyruvate) with glycine, alanine, ammonia, and histamine. Unfortunately, simple amines like n-butylamine were not included. Since polarigraphic apparatus was not available to this author, a spectrophotometric method was devised. When pyruvate is placed in amine buffer, the pyruvyl absorption at 320 mμ is decreased and a new absorption appears at about 250 mμ. Notice that the spectra in Figure 3 are difference spectra. If the absorption of either the Schiff base or pyruvate can be measured

Figure 3.--Difference spectra of pyruvate in n-butylamine buffers. The spectra were determined with a Cary Model 15 spectrophotometer. Two tandom cuvettes were placed in the sample beam, each having a one centimeter light path and a volume capacity of 1.5 ml. One cuvette of the pair contained phosphate buffer and the other contained in one ml, 1.0 M $BuNH_2$ buffer and 5.67×10^{-3} M pyruvate. Likewise, a pair of cuvettes in tandom were placed in the reference beam. One contained n-$BuNH_2$ buffer; the other contained 5.67×10^{-3} M pyruvate in 0.05 M phosphate buffer adjusted to the pH of the amine buffer. In the pH range 6 to 8.0, 0.05 M phosphate was included in the amine buffer to maintain the pH.


Difference Spectra of
Pyruvate in n-butylamine
Buffers
pH
9.80
9.40
9.20
9.00
8.90
8.80
8.71
8.00
7.00
Δ Absorbance
0.6
0.4
0.2
0
250
275
300
325
350
Wave Length, mμ

independently of the other, the equilibrium constant can be readily calculated. Assuming the absorption spectrum of the Schiff base is roughly equivalent to that of Δ^1-piperidine-2-carboxylic acid, it can be seen that the absorption spectra of pyruvate and Schiff base overlap significantly at about 250 mμ, whereas only the pyruvate spectrum shows measurable absorbance at 320 mμ. However, this could be an illusion. The extinction coefficient of pyruvate at 320 mμ is so low ($\varepsilon = 20$) that the "tail" of the more highly absorbant Schiff base peak might overlap significantly with the pyruvate maximum at 320 mμ. For this reason the spectral method, which will now be described, was tested with glycine for which K_s could be compared with the polarigraphically determined value.

The equilibrium expression is

$$K_s = \frac{[S]}{[P][Am:]}$$

where K_s is the equilibrium constant; P is the concentration of pyruvate at equilibrium; Am: is the concentration of the conjugate base of the amine. The following terms are now introduced:

P_o = initial concentration of pyruvate

A_o = absorbance of P_o at 320 mμ.

ε_p = extinction coefficient of pyruvate at 320 mμ.

A = absorbance of P at 320 mμ.

It can be seen that

$$S = P_o - P$$

which is substituted into the equilibrium expression to give

$$K_s = \frac{P_o - P}{(Am:)(P)}$$

Also

$$\frac{P}{P_o} = \frac{\frac{A}{\varepsilon}}{\frac{A_o}{\varepsilon}} = \frac{A}{A_o}$$

So that

$$P = \left(\frac{A}{A_o}\right) P_o$$

Substituting into the previous equilibrium expression:

$$K_s = \frac{(P_o) - \left(\frac{A}{A_o}\right)(P_o)}{(Am:)\left(\frac{A}{A_o}\right)(P_o)}$$

$$= \frac{1 - \left(\frac{A}{A_o}\right)}{(Am:)\left(\frac{A}{A_o}\right)}$$

from which K_s can be determined. Am: is calculated from the known pK_a of the amine.

K_s for the glycine-pyruvate system was determined by measuring the loss of 320 mμ absorbance of a solution containing in 1.0 ml, 1.0 M glycine, and 0.01 M pyruvate adjusted to pH 9.85. The readings were made with a Gilford Recording Spectrophotometer. Equilibrium was essentially attained in less than five minutes. A_o was determined from the absorbance of pyruvate at 320 mμ in 0.05 bicarbonate buffer pH 9.85. Using this method, K_s was found to be 2.54, in excellent agreement with the polarigraphic value of 2.47. This result supports the validity of the spectral method.

In a similar fashion, K_s was determined for the n-$BuNH_2$-pyruvate system at five pH values between 9.55 and 10.65 from equilibrium mixtures containing 0.01 M pyruvate and 0.4 M $BuNH_2$. The results are shown in Table 2. For some reason, K_s decreases with increasing pH; this trend was found to be reproducible. Apparently, another pH-dependent ionization is taking place. It should be mentioned that Zuman did not study the pH-dependence of the equilibrium, but simply measured K_s near the pK_a of the amine.

TABLE 2.--Equilibrium constant of Schiff base formation.

pH	$K_s (M^{-1})$
9.55	14.28
9.75	13.375
10.04	12.182
10.30	10.65
10.65	7.96

Stopped Flow Measurements

All stopped flow determinations were performed with a Durrum Gibson stopped flow apparatus fitted with a Beckman DU optical system. The change in transmittance was recorded with a Tektronix 564 Storage oscilloscope from which photographs of the traces could be made. The temperature was maintained at 25°C with a circulating water bath.

The rate constants for Schiff base formation and hydrolysis were determined from the increase in absorbance at 256 mμ upon mixing equal volumes of 0.01 M pyruvate and 0.4 M n-$BuNH_2$ buffer. Since the amine is in great excess, the forward reaction is pseudo first order in pyruvate; the reverse reaction, of course, is truly first order in Schiff base. Therefore, the rate data can be expressed in terms of a reversible first order process in both directions according to the integrated equation:

$$\ln\left(\frac{A_o - A_{eq}}{A - A_{eq}}\right) = (k_f + k_r)t = k_{obs}t$$

where A_o is the absorbance at time zero, A_{eq} is the absorbance at equilibrium, A is the absorbance at time t, k_f is the pseudo first-order rate constant for the forward reaction, and k_r is the first-order rate constant for the reverse reaction.

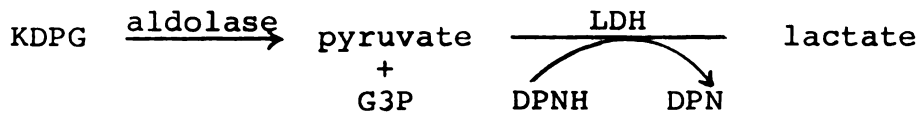
k_{obs} is then determined from a plot of $\ln \frac{A_o - A_{eq}}{A - A_{eq}}$ versus t. k_f and k_r can then be calculated from the two equations $k_{obs} = k_f + k_r$ and $K_{eq} = k_f/k_r$, where K_{eq} is the equilibrium constant for Schiff base formation.

The stopped flow iodinations of Δ^1-piperidine-2-carboxylic acid were performed by mixing in equal volumes a solution containing Δ^1-PCA in imidazole buffer with a solution containing buffer and iodine. As usual, the buffers contained 0.2 M KI and NaCl to maintain the ionic strength at 0.4.

Enzymatic

Crystalline KDPG-aldolase was prepared according to the method of Meloche and Wood (46). Briefly, the purification involves the following steps. First, 200 g Pseudomonas putida cells are disrupted by sonic oscillation and the cell debris removed by centrifugation. Since the aldolase is uncommonly acid stable, the bulk of protein is then removed by acidification to pH 2.0 followed by centrifugation. The supernatant is further fractionated with ammonium sulfate. The final ammonium sulfate step precipitates the aldolase, which is redissolved in a small volume of water. The last step prior to crystallization is fractionation with calcium phosphate gel. Crystallization is effected with ammonium sulfate.

The enzyme is assayed by the method of Meloche and Wood (46) which employs a lactic dehydrogenase coupling system:



The disappearance of DPNH is followed at 340 mμ in a Gilford Recording Spectrophotometer.

CHAPTER IV

RESULTS

Introduction

As discussed in the Introduction, two systems were used to study ketimine-enamine tautomerization: pyruvate in amine buffer and the cyclic ketimine analogue Δ^1-piperidine-2-carboxylic acid.

The tautomerization of Δ^1-piperidine-2-carboxylic acid is simply measured directly by iodination as a function of pH. From these measurements, the rate law is elucidated and the desired constants calculated.

The pyruvate-amine mixtures, however, can only be used for this purpose after certain fundamentals have been established: (1) Schiff base formation must indeed occur, and (2) ketimine-enamine tautomerization must be rate limiting. Once these have been established, the rate law can be elucidated and the constants determined. These constants are then compared to the corresponding constants obtained in buffers which do not form a Schiff base, in order to determine the contribution of the Schiff base towards the overall rate of enolization. This system will be discussed first.

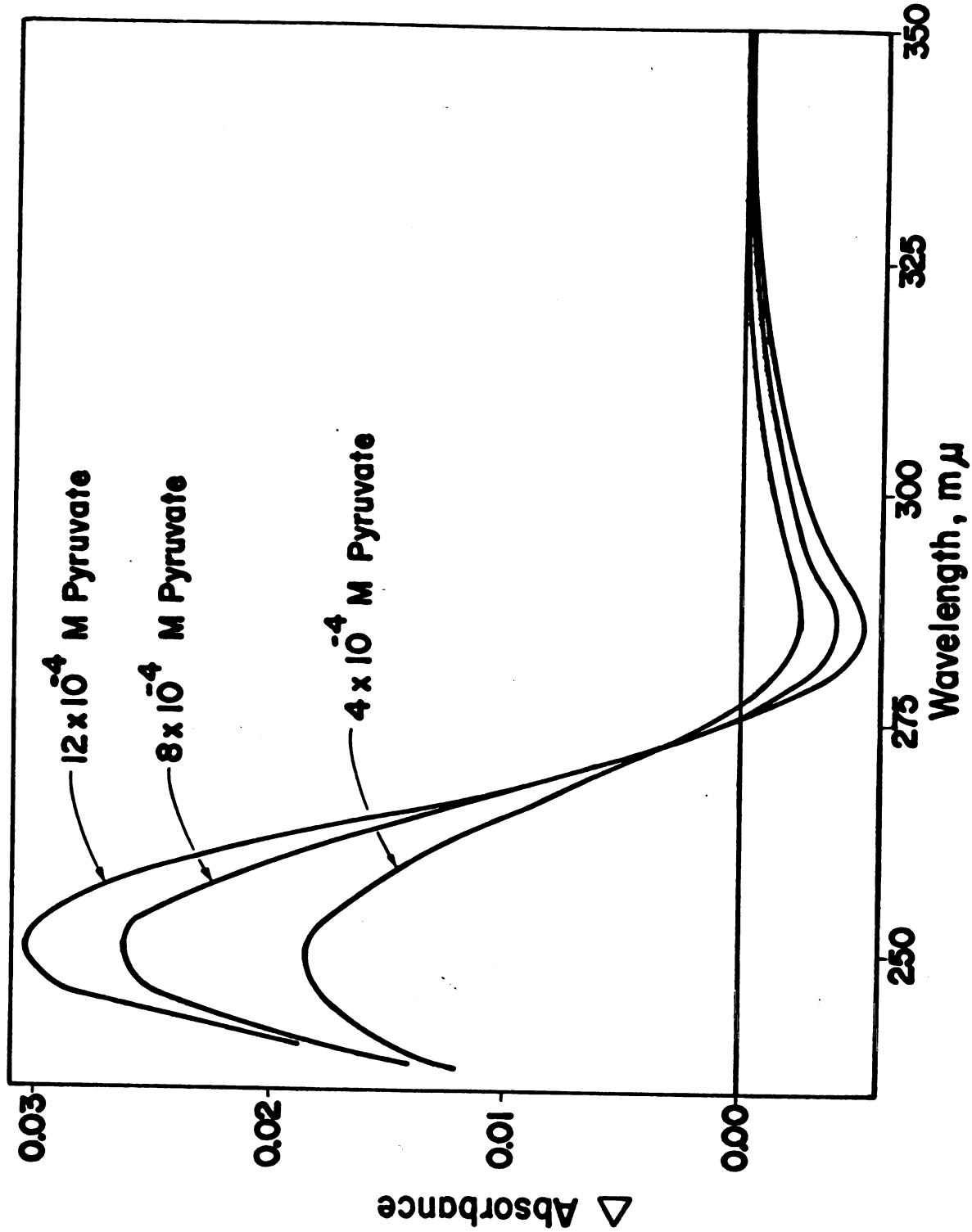
Demonstration of Schiff Base Formation

First, it is necessary to demonstrate that a Schiff base is formed from pyruvate and simple alkyl amines in aqueous solution. This was done by comparing the absorption spectra of mixtures containing pyruvate and amine buffer (Figure 3) to those of the Schiff base Δ^1-piperidine-2-carboxylic acid (Figure 2). As expected, in amine buffer the carbonyl absorbance of pyruvate at 320 mμ decreases while a new absorbance appears below 300 mμ. Furthermore, the pH dependence of the spectra is very similar to that observed for Δ^1-piperidine-2-carboxylic acid.

This conclusion that the spectral changes are characteristic of a Schiff base linkage is also supported by the fact that the spectrally determined equilibrium constant for Schiff base formation from pyruvate and glycine agrees well with the polarigraphically determined value (see Methods, p. 59).

It has been hoped, that the Schiff base formed from pyruvate and amine resembles that formed from pyruvate and the lysine ε-amino group in KDPG aldolase. For this reason, a spectrum of the pyruvate-enzyme complex was determined, as shown in Figure 4. It can be seen (Figures 3 and 4) that the spectrum of the pyruvate-enzyme complex does resemble that of the model-system Schiff base. This does not mean that the enzymatic and

Figure 4.--Difference spectra of pyruvate-KDPG aldolase mixtures. The spectra were determined in a Cary Model 15 spectrophotometer as follows: Two tandom cuvettes were placed in the sample beam, each having a one centimeter light path and a volume capacity of 1.5 ml. One cuvette of the pair contained in 0.5 ml, 0.1 M phosphate buffer and 0.5 M borate buffer, pH 8.5, whereas the other contained in 0.5 ml, 0.5 M borate buffer pH 8.5, 0.1 M phosphate buffer and 5500 units of KDPG aldolase of specific activity 10,000 U/mg, and pyruvate of concentration 4-12 x 10^{-4} M. Likewise, a pair of cuvettes in tandom were placed in the reference beam. One contained in 0.5 ml, 0.5 M borate buffer pH 8.5, 0.1 M phosphate buffer, and 5500 units of KDPG aldolase. The other contained in 0.5 ml, 0.5 M borate buffer pH 8.5, 0.1 M phosphate, and pyruvate.


12 x 10⁻⁴ M Pyruvate
8 x 10⁻⁴ M Pyruvate
4 x 10⁻⁴ M Pyruvate
0.03
0.02
0.01
0.00
Δ Absorbance
250
275
300
325
350
Wavelength, mμ

nonenzymatic Schiff bases are identical in ionic form and environment, but the spectra are at least consistent with the notion that both Schiff bases are similar in many respects.

Kinetics of Schiff Base Formation

Before enolization experiments with pyruvate-amine mixtures can be realized, it is necessary to assure that in amine buffers Schiff base formation is much faster than enolization; if it is not, Schiff base formation could be rate limiting, and the iodination assay for enolization would obviously be a measure of Schiff base formation rather than the ketimine-enamine conversion.

As can be seen from Figures 2 and 3, the rate of Schiff base formation can be measured by the increase in absorbance at 256 mμ. By this method, it was readily observed that Schiff base formation was indeed much faster than the rate of enolization (as measured by iodination) over the pH range 9.2 to 10.6. In fact, the reaction is too fast to permit determination of the rate constant by standard spectrophotometric means.

These simple--and somewhat qualitative--observations provide sufficient evidence that enamine formation is rate limiting. For the experiments planned, it is not necessary to determine the rate constant for Schiff base formation. Nonetheless, it may be of some value to

determine this constant. The rate of Schiff base formation is apparently very fast; how then might it compare to the enzymatic rate of Schiff base formation?

To answer this question, the rate constants for Schiff base formation and hydrolysis of the model reaction in the pH range 9.2 to 10.6 were determined by stopped flow techniques as discussed in the Methods section.

Plots of $\ln(\frac{A_o - A_{eq}}{A - A_{eq}})$ versus t are shown in Figure 5. The rate constants so obtained are summarized in Table 3. Although a full discussion of these results will be deferred to the Discussion section, it may be stated here that the rate constant for Schiff base formation between pyruvate and butylamine is at least two or three orders of magnitude less than the enzymatic rate constant for the enolization of pyruvate. Since the enzymatic rate of Schiff base formation must be as fast as enolization, the enolization constant of the enzyme is a lower limit of the constant for Schiff base formation. Therefore, the enzymatic rate of Schiff base formation is at least two or three orders of magnitude greater than the nonenzymatic rate.

Figure 5.--A typical reaction of pyruvate with butylamine to form Schiff base as measured by stopped flow techniques. The reaction mixture contained, after mixing, 0.2 M $BuNH_2$ buffer and 0.005 M pyruvate. The reaction was followed by the increase in absorbance at 256 mμ. The pH of this particular reaction is 10.56; and the temperature, maintained at 25°C.

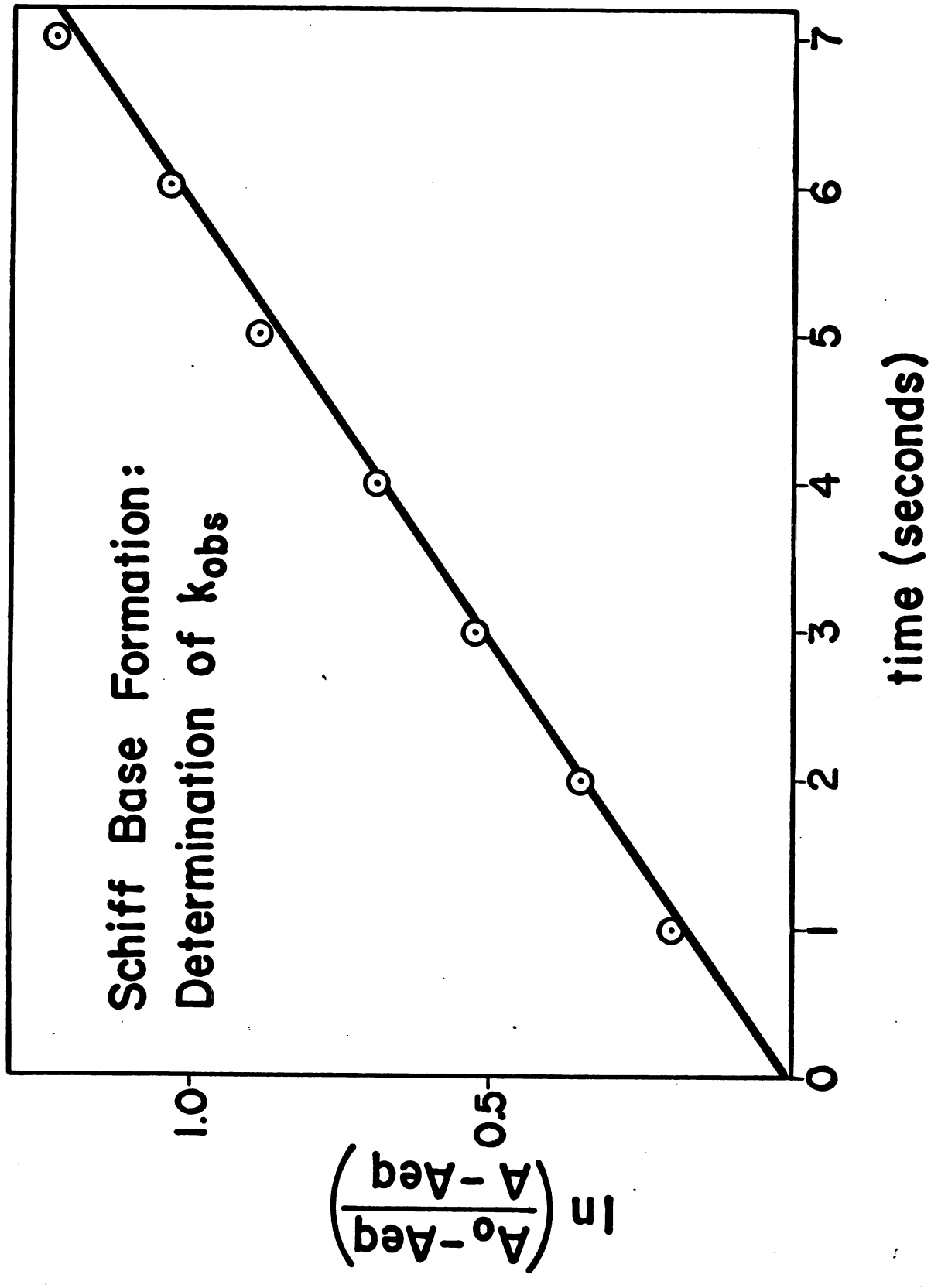

Schiff Base Formation:
Determination of k_{obs}
$\ln \left(\frac{A_o - A_{eq}}{A - A_{eq}} \right)$
1.0
0.5
0
1
2
3
4
5
6
7
time (seconds)

TABLE 3.--Rate Constants for Schiff Base Formation from Pyruvate and Butylamine. [The reaction mixture contained, after mixing in the stopped flow apparatus, 0.2 M $BuNH_2$ buffer and 0.005 M pyruvate. The reaction was followed by the increase in absorbance at 256 mμ.]

pH	k_{obs} (at 0.2 M $BuNH_2$)	k_f $M^{-1}sec^{-1}$	k_r sec^{-1}
10.56	0.1729	0.695	0.034
10.23	0.1264	0.472	0.0315
9.87	0.1039	0.349	0.0342
9.51	0.0939	0.229	0.0458
9.20	0.0940	0.171	0.0597

Enolization of Pyruvate

Since the concentration of Schiff base intermediate is small compared to the concentration of pyruvate, a kinetic analysis similar to that used by Bender is more appropriate than an analysis used by Hine (see Literature Review). Therefore, the enolization of pyruvate in buffers which cannot form a Schiff base was compared to the enolization in primary amine buffers. For this comparison, phosphate and imidazole buffers were used. The results of these experiments are shown in Figures 6-11 and the constants so determined are summarized in Table 4.

Most of the studies with amine were done with n-ε-aminocaproic acid since this amine is not volatile. To assure that the carboxyl group does not contribute to the reaction, the kinetics in this buffer were later corroborated with the kinetics in n-butylamine buffer.

Generally speaking, the kinetics of enolization in phosphate, imidazole, and amine are very similar to those observed for acetone; in all cases, the kinetics could be expressed in terms of the simple rate equation rate = $k_{obs}P = (k_o + k_bB + k_aA)P$. Most importantly, the rate law for amine-catalyzed enolization shows a term corresponding to general-acid catalysis, whose catalytic constant k_a is very much larger than the corresponding constants observed for imidazole or phosphate. As discussed earlier (Literature Review, p. 25) this term is best attributed to water-catalyzed enolization of the

Figures 6-11.--Determination of the constants k_o, k_a, and k_b from the iodination of pyruvate according to the experimental rate equation

$$\text{rate} = k_{obs}P = (k_o + k_bB + k_aA)P$$

For each buffer, two plots are shown. The first is a plot of k_{obs} versus buffer concentration, and the second is a plot of

$$k' = \frac{k_{obs} - k_o}{A + B} \frac{r + 1}{r} \text{ versus } \frac{1}{r}$$

which gives k_a and k_b according to the transformation of the linear rate equation described in the text. The values for k_{obs} were determined from plots of rate versus pyruvate concentration at constant pH and buffer concentration. All reaction mixtures contained 0.2 M KI, I_2, NaCl to maintain the ionic strength at 0.4 and pyruvate in a volume of one ml. The temperature in all cases was maintained at 25°C.

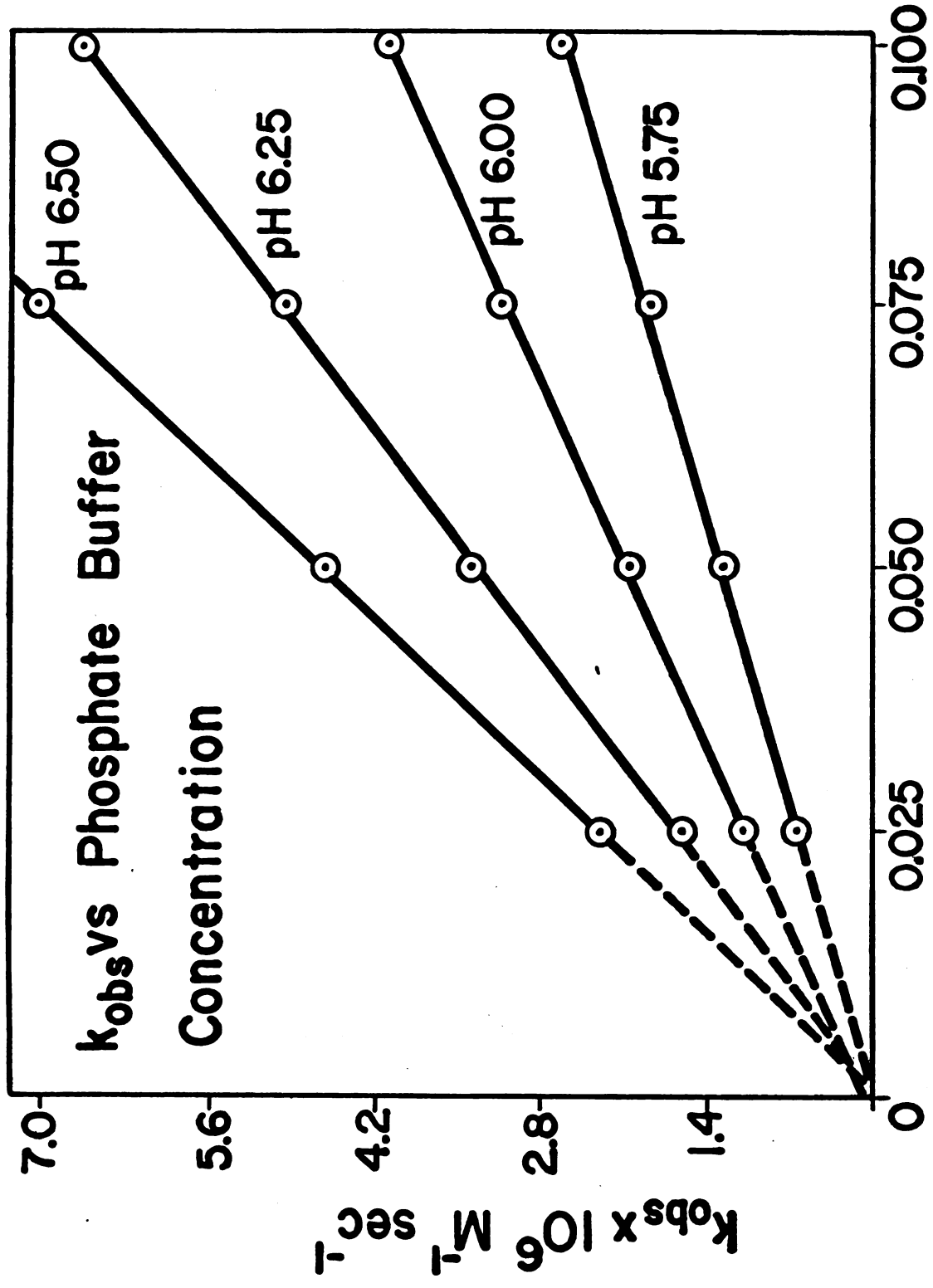

k_{obs} vs Phosphate Buffer Concentration
pH 6.50
pH 6.25
pH 6.00
pH 5.75
$k_{obs} \times 10^6$ M^{-1} sec^{-1}
7.0
5.6
4.2
2.8
1.4
0
0.025
0.050
0.075
0.100
Buffer Concentration (M)

Figure 6

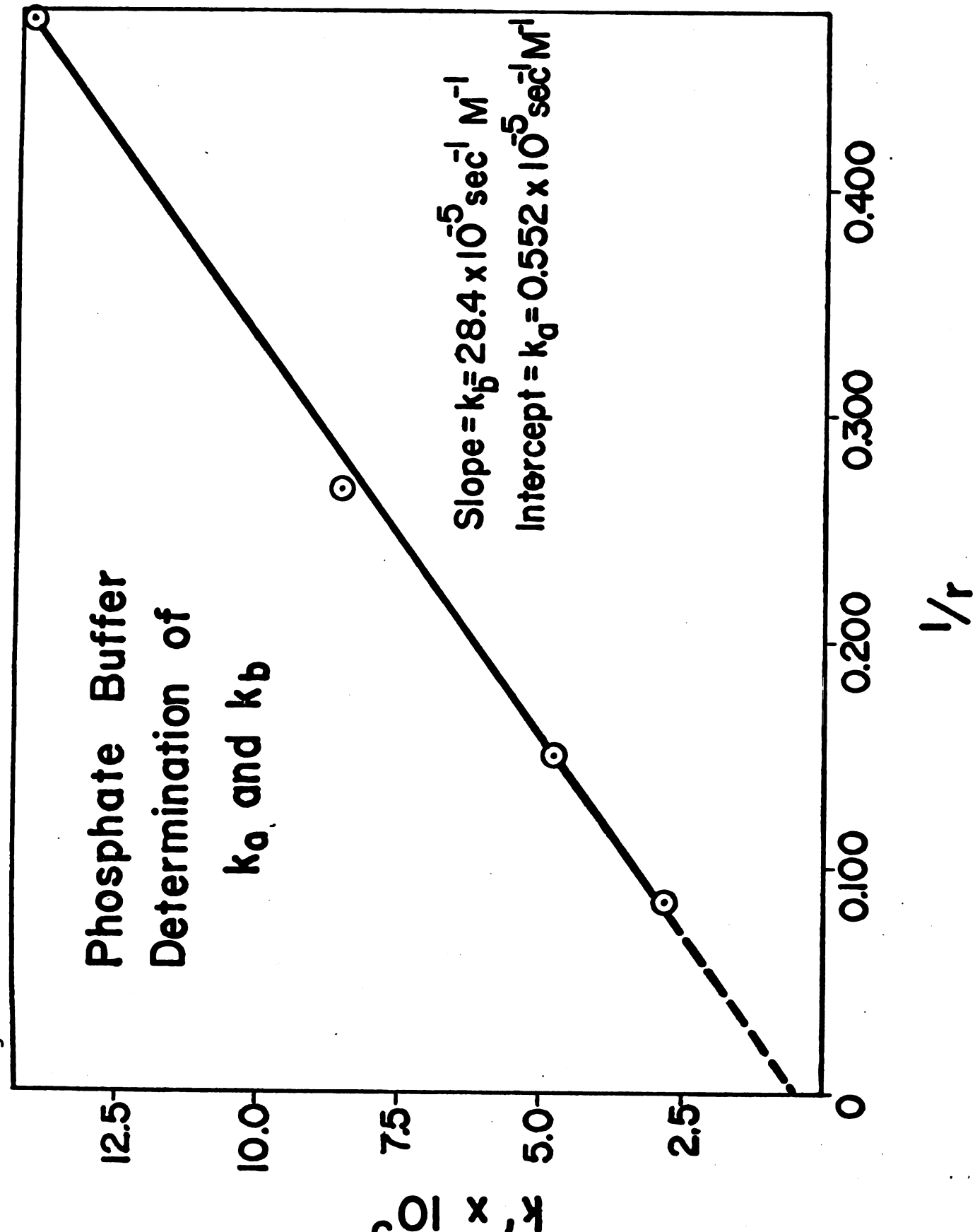

Phosphate Buffer
Determination of
k_a and k_b
Slope = k_b = 28.4 x 10^{-5} sec^{-1} M^{-1}
Intercept = k_a = 0.552 x 10^{-5} sec^{-1} M^{-1}
$k' \times 10^5$
12.5
10.0
7.5
5.0
2.5
0
0.100
0.200
0.300
0.400
1/r

Figure 7

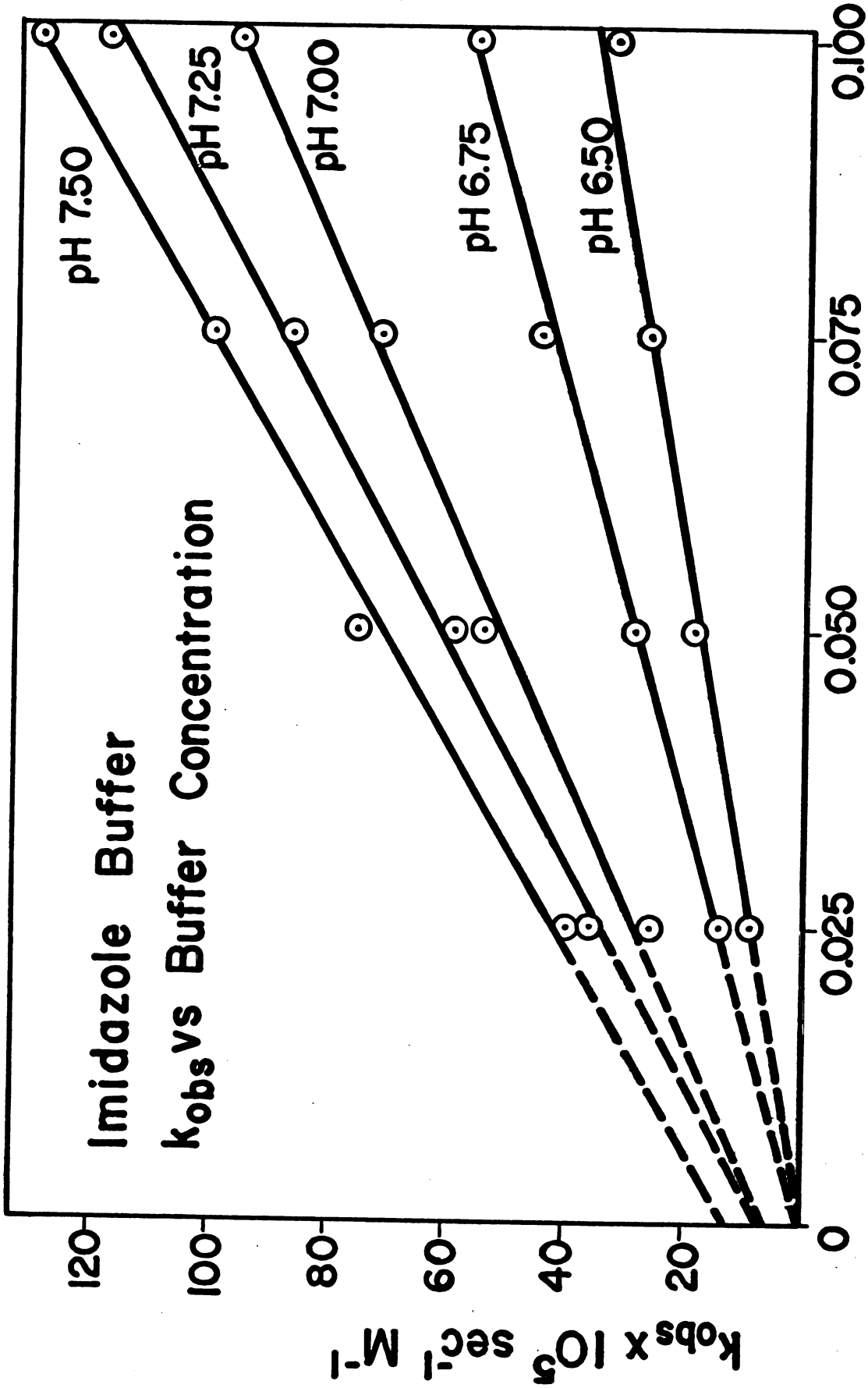

Imidazole Buffer
k_{obs} vs Buffer Concentration
pH 7.50
pH 7.25
pH 7.00
pH 6.75
pH 6.50
$k_{obs} \times 10^3$ sec^{-1} M^{-1}
120
100
80
60
40
20
0
0.025
0.050
0.075
0.100
Buffer Concentration (M)

Figure 8

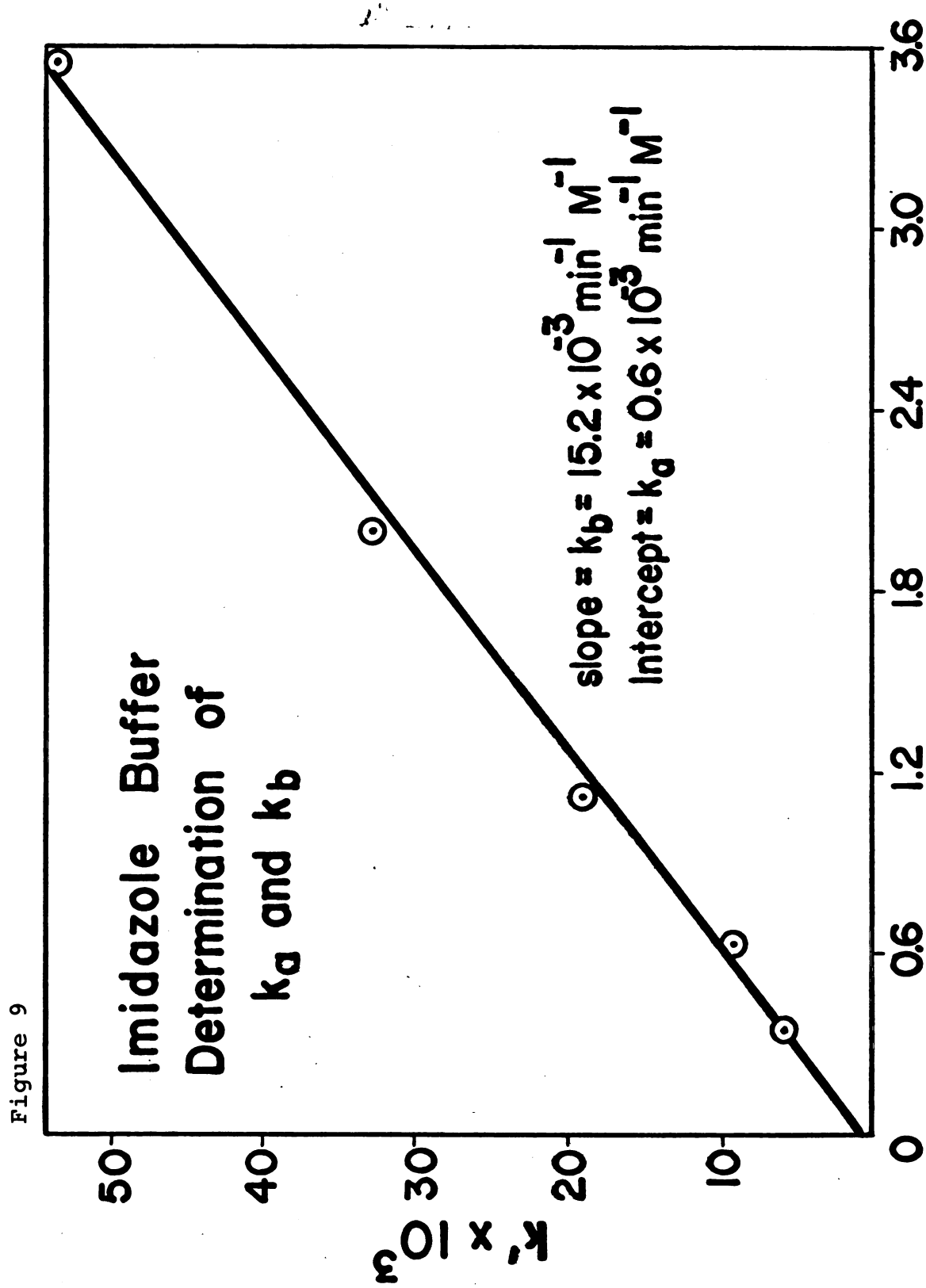

Imidazole Buffer
Determination of
k_a and k_b
slope = k_b = 15.2×10^{-3} min^{-1} M^{-1}
Intercept = k_a = 0.6×10^{-3} min^{-1} M^{-1}
$k' \times 10^3$
1/r
0
0.6
1.2
1.8
2.4
3.0
3.6
10
20
30
40
50

Figure 9

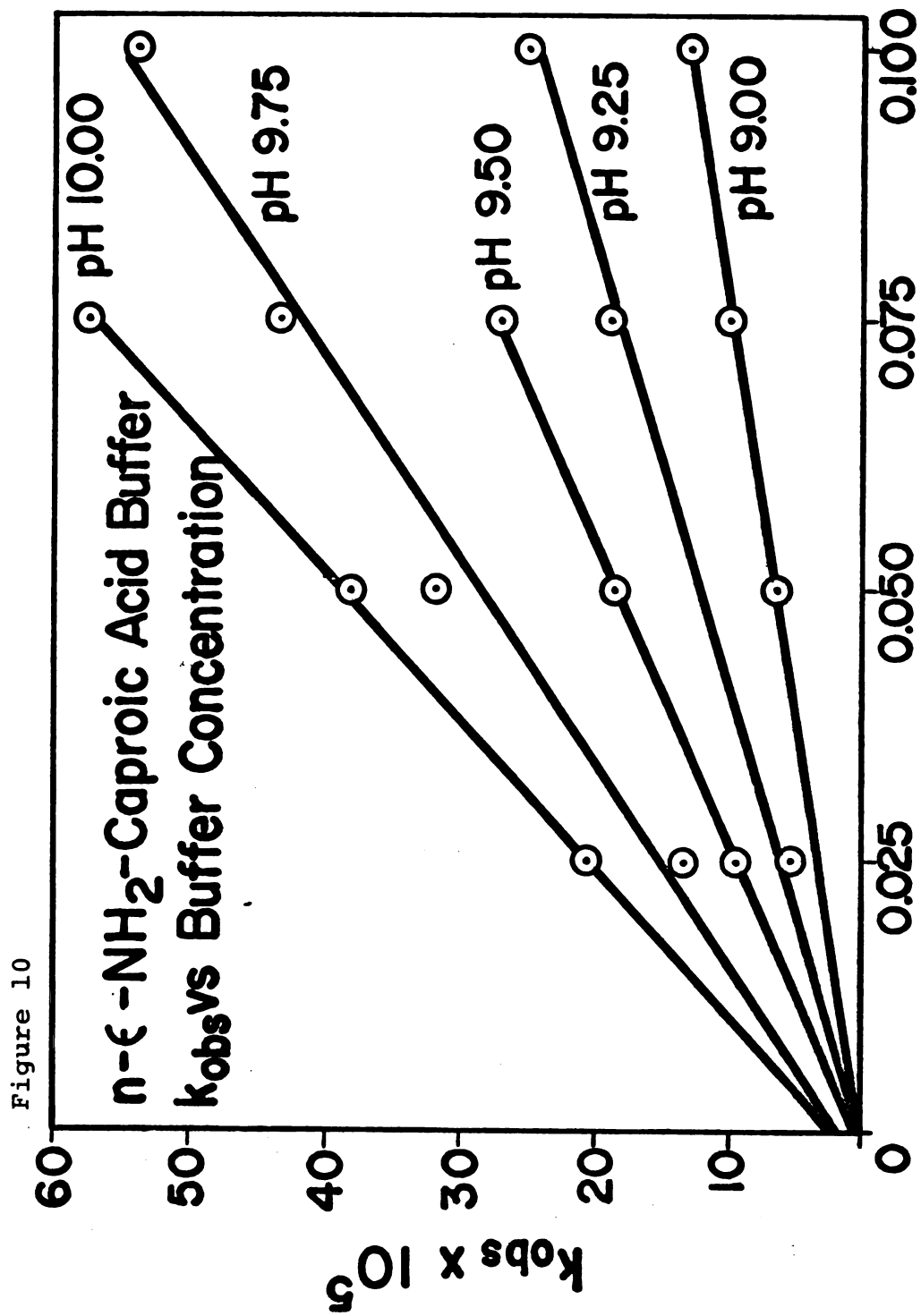

n-ε-NH2-Caproic Acid Buffer
kobs VS Buffer Concentration
pH 10.00
pH 9.75
pH 9.50
pH 9.25
pH 9.00
kobs x 10^5
60
50
40
30
20
10
0
0.025
0.050
0.075
0.100
Buffer Concentration (M)

Figure 10

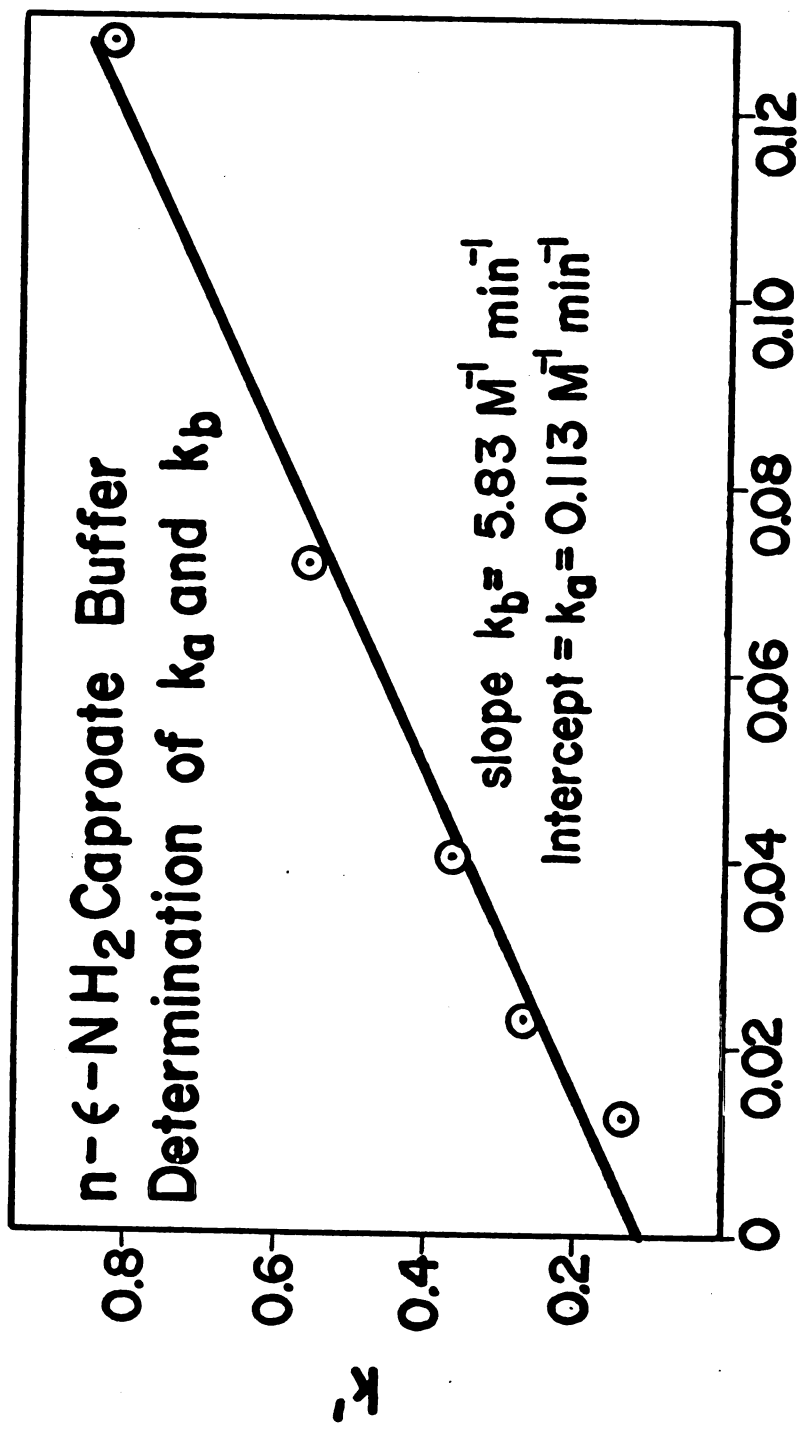
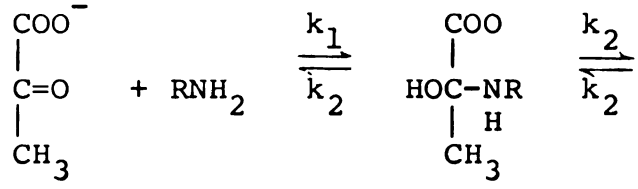

n-ε-NH2 Caproate Buffer
Determination of ka and kb
slope kb= 5.83 M-1 min-1
Intercept = ka= 0.113 M-1 min-1
0.8
0.6
0.4
0.2
0
0.02
0.04
0.06
0.08
0.10
0.12
1/r

Figure 11

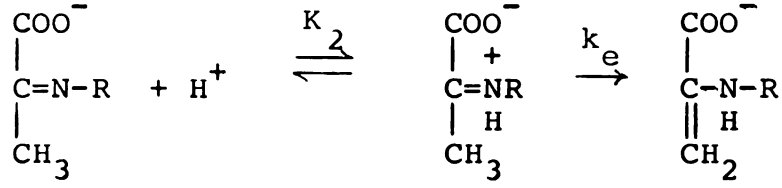
TABLE 4.--Iodination of Pyruvate in Imidazole, Phosphate, and Amine Buffer: Summary of Catalytic Constants k_a and k_b.

Buffer	k_a	k_b
	$M^{-1}\ sec^{-1}$	$M^{-1}\ sec^{-1}$
Phosphate	$< 5.00 \times 10^{-6}$	2.84×10^{-4}
Imidazole	$< 1.00 \times 10^{-5}$	2.53×10^{-4}
N-ε-aminocaproate	1.88×10^{-3}	0.0971

protonated ketimine rather than the general catalysis, for which the following mechanism can be written:



pyruvate carbinolamine



neutral Schiff base protonated Schiff base enamine

where enamine formation is rate limiting. Solving the rate equation for this mechanism in terms of pyruvate and protonated amine gives

$$\text{rate of iodination by ketimine} = \left(\frac{K_a}{K_2}\right)\left(\frac{k_1 k_2}{k_{-1} k_{-2}}\right) k_e [RNH_3^+][P]$$

$$= \left(\frac{K_a}{K_2}\right)(K_1) k_e [RNH_3^+][P]$$

$$\text{so} \qquad k_a = \left(\frac{K_a}{K_2}\right)(K_1) k_e$$

$$\text{and} \qquad k_e = \frac{k_a K_2}{K_a K_1}$$

From this, the rate constant for the conversion of ketimine to enamine, k_e, can be calculated if K_a, K_1, and K_2 are known. K_a, the ionization constant of the amine was determined as described in Methods, p. 55. K_1, the equilibrium constant for Schiff base formation was determined as described in Methods, p. 56. Since, as noted, the equilibrium constant varied somewhat over the pH range studied; an average value of 10 $\underline{M}^{-1}$ was chosen.

As mentioned in the Literature Review, the ionization constant for the Schiff base, K_2, could not be experimentally determined for the acetone or isobutyraldehyde systems. Neither could it be determined for the pyruvate system in this study. However, the stability of the cyclic Schiff base Δ^1-piperidine-2-carboxylic acid to hydrolysis has permitted Macholàn to determine the ionization constant of this compound with good accuracy. The value he obtained was $pK_a = 7.6$. Since this compound is a cyclic analog of the pyruvyl ketimine, this value can properly be applied to those ketimines of pyruvate formed in amine buffers. With the above constants determined, the value of the model system enolization constant, k_e, is calculated to be 0.304 sec^{-1} as compared to the enzymatic rate constant 120sec^{-1}.[3]

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[3]The rate constant for the enzyme-catalyzed enolization was calculated from tritium exchange data previously obtained in this laboratory (58). The value 0.25 μatom/min/International Unit of aldolase at 28°C (measured by the cleavage reaction described in Methods,

Assuming that all of the kinetic interpretations are correct, this result shows quite simply that Schiff base formation between pyruvate and KDPG aldolase is not sufficient to account for the enzymatic rate of enolization. This will be discussed more fully later.

As stated in the Introduction, the purpose of this research is to determine the contribution of the Schiff base to the enzymatic rate. For this purpose, the rate constants for the following three processes must be known: (1) the enzymatic-catalyzed enolization of pyruvate; (2) the nonenzymatic Schiff base-catalyzed enolization of pyruvate; and (3) the "uncatalyzed" enolization of pyruvate, which may be defined as the enolization of pyruvate in water at a pH near the pH-optimum of the enzyme.

The rate constants for the first two processes have already been presented, leaving only that of the uncatalyzed reaction to be determined. Normally, the

---

p. 65) agreed very well with the value reported by Rose, 0.41 μatom/min/International Unit of enzyme (22). After correcting for the isotope effect, Rose estimated the rate of hydrogen exchange to be 1.92 μatoms/min/International Unit of enzyme (unfortunately, Rose did not report the temperature of the reaction). Presumably, Rose used this value to calculate the enolization constant (k_e = 400 sec^{-1}), which appeared in a publication by Westheimer and Tagaki (38) as a personal communication to these authors. Our calculations indicate that this value is correct (we obtain k_e = 423 sec^{-1}) if it is assumed that there is one catalytic site per molecule of enzyme. However, studies in this laboratory have shown that there are three catalytic sites per molecule (59). Therefore, the enolization constant is 423 ÷ 3 = 141 sec^{-1}. At 25°C, this constant is estimated to be 120 sec^{-1}.

uncatalyzed reaction is simply determined from the intercept of a plot of buffer concentration versus rate of constant pH. However, the uncatalyzed enolization was found to be so small compared to the buffer-catalyzed enolization that this method proved useless. The only recourse was to find a method for measuring the enolization in the absence of buffer.

The obvious complication in such an experiment is maintaining the pH. It can be seen that iodination of pyruvate liberates a proton, which causes the pH to drop during the course of the reaction. This problem was readily solved, however, by controlling the pH with a pH-stat, as described in the Methods section.

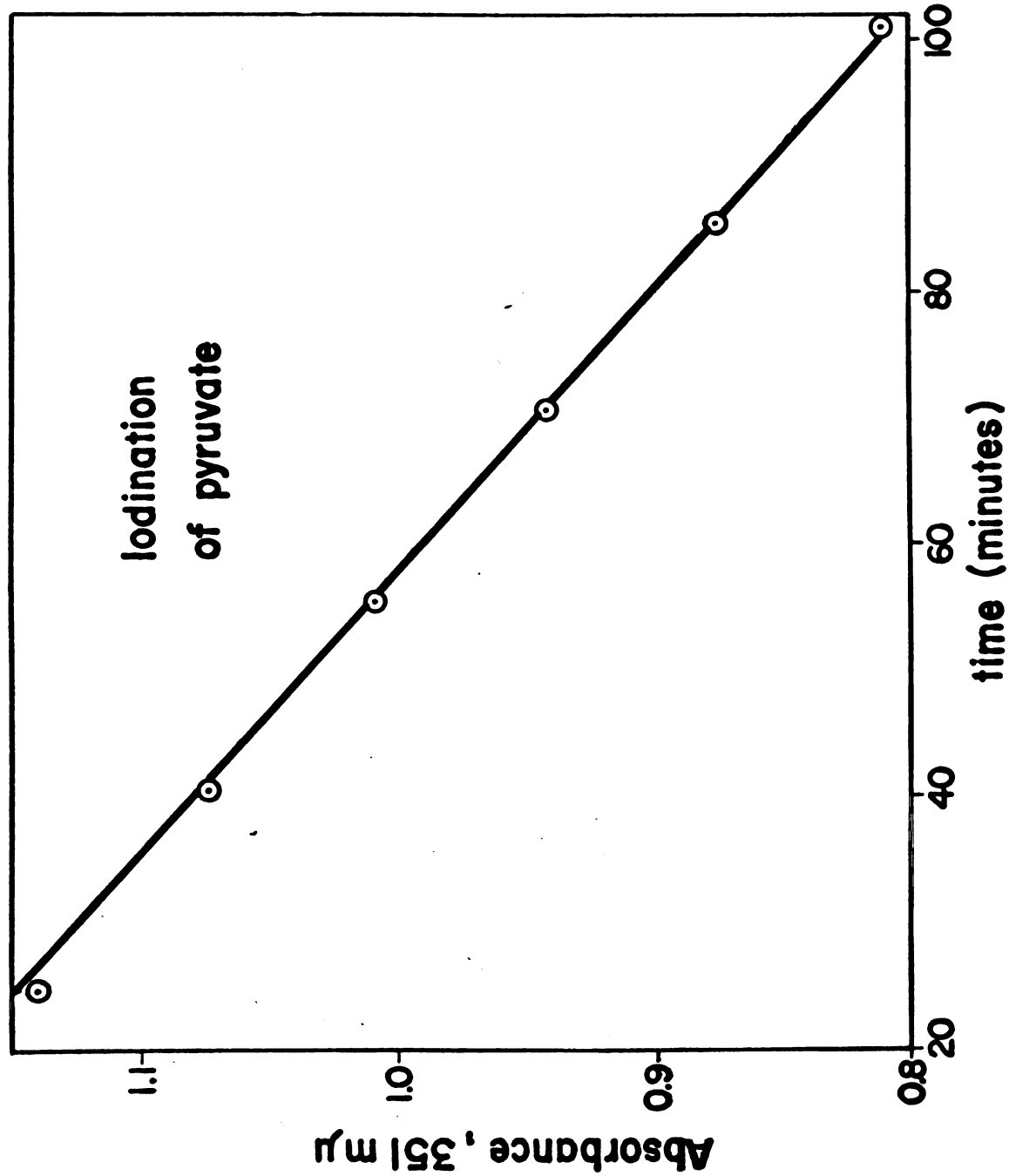
The reaction was measured over the pH range 7.2 to 8.2, which includes part of the pH-optimum range of the aldolase. A typical reaction is shown in Figure 12. The rate constants were determined as follows.

Conceivably, the enolization can be catalyzed significantly by every acid and base species in solution. In that case the rate law would be

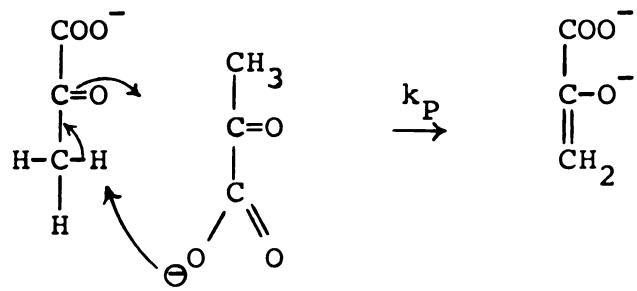
$$\text{rate} = [k_{H_2O}(H_2O) + k_{OH}(OH^-) + k_{H^+}(H^+) + k_P(P)$$

$$+ \ . \ . \ . \ \text{etc.}]P$$

Figure 12.--Typical iodination reaction of pyruvate by the pH-stat method. The reaction mixture contained 0.2 M KI, I_2, 0.2 M NaCl and 10^{-2} M pyruvate in a total volume of 50 ml. The pH of this particular reaction was maintained at 7.5 and the temperature was maintained at 25°C.


Iodination
of pyruvate
Absorbance, 351 mμ
1.1
1.0
0.9
0.8
20
40
60
80
100
time (minutes)

The last term in the equation represents the following:



Although all the constants can be determined, it is much simpler to first assume that some of the terms in the above equation are negligible and plot the data accordingly. If the data fit the simplified equation, there is no need to proceed any further. It appears by inspection that the reaction is predominantly catalyzed by hydroxide ion for which the following rate law can be written:

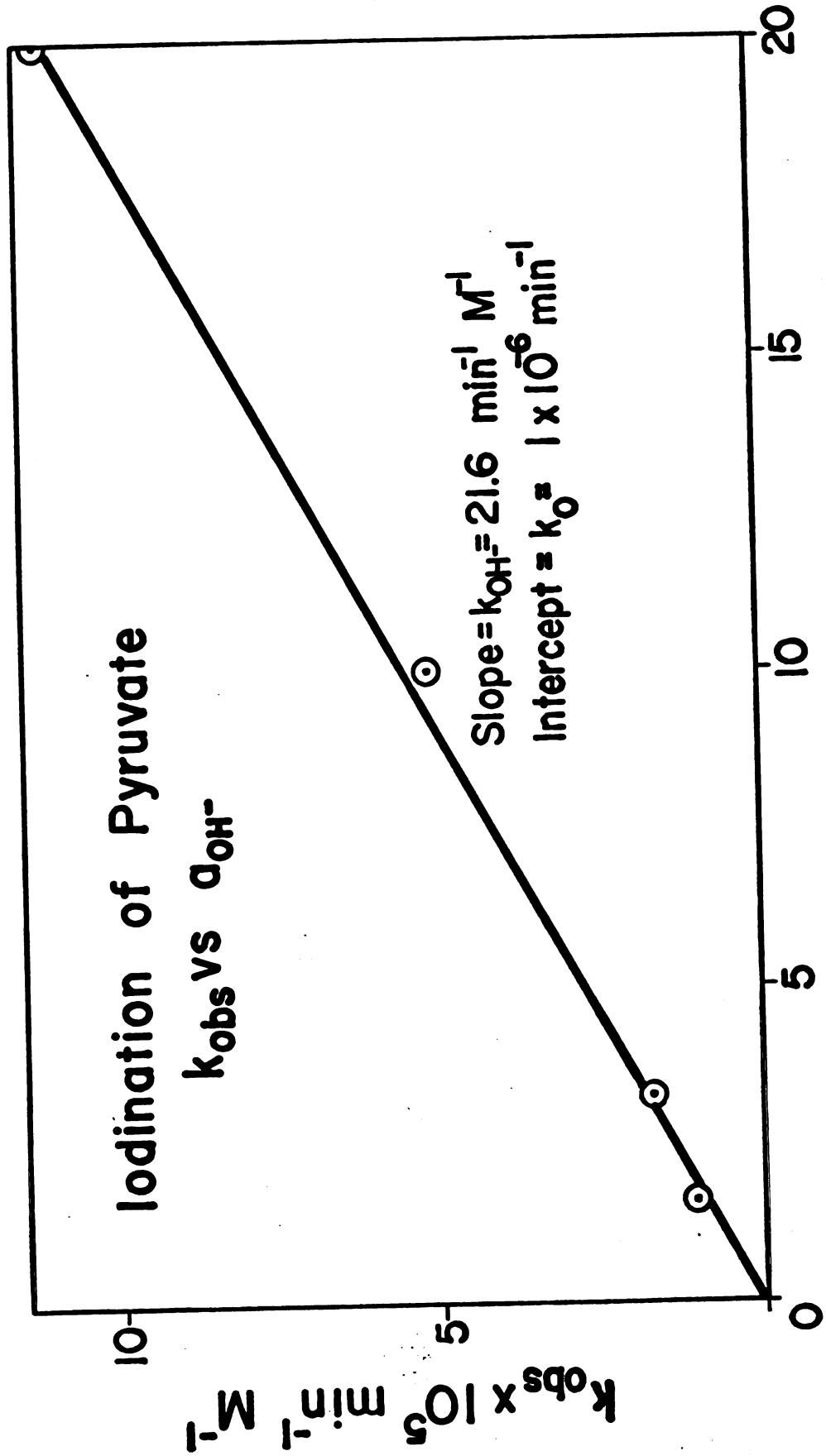
$$\text{rate} = k_{obs}P = k_{OH}(OH^-)(P)$$

Figure 13 shows that a plot of hydroxide ion concentration (actually hydroxide ion activity) versus rate is linear and passes through the origin. This result substantiates the simplified rate law shown above. The rate constant for the hydroxide ion catalyzed enolization as determined from the slope of this plot is $k_{OH} = 0.360 M^{-1} sec^{-1}$. This rate constant enables the calculation of uncatalyzed rate at any pH value within this pH range. At pH 8.0, where the enzyme-catalyzed enolization is maximal, the uncatalyzed rate in terms of 1.0 M pyruvate is 0.36×10^{-6}.

Figure 13.--Determination of the rate equation for the iodination of pyruvate in the absence of buffer. The data for these determinations was obtained by the pH-stat method as described in the text and shown in Figure 12. Figure 13 shows a plot of k_{obs} versus activity of the hydroxyl ion according to the rate law

$$\text{rate} = k_{obs}P = k_{OH^-}(OH^-)(P)$$

Figure 13a shows a log-log plot of the same data.


Iodination of Pyruvate
k_{obs} VS a_{OH^-}
Slope=k_{OH^-}=21.6 min^{-1} M^{-1}
Intercept = k_0 = 1×10^{-6} min^{-1}
$k_{obs} \times 10^5$ min^{-1} M^{-1}
$a_{OH^-} \times 10^7$ M
0
5
10
15
20

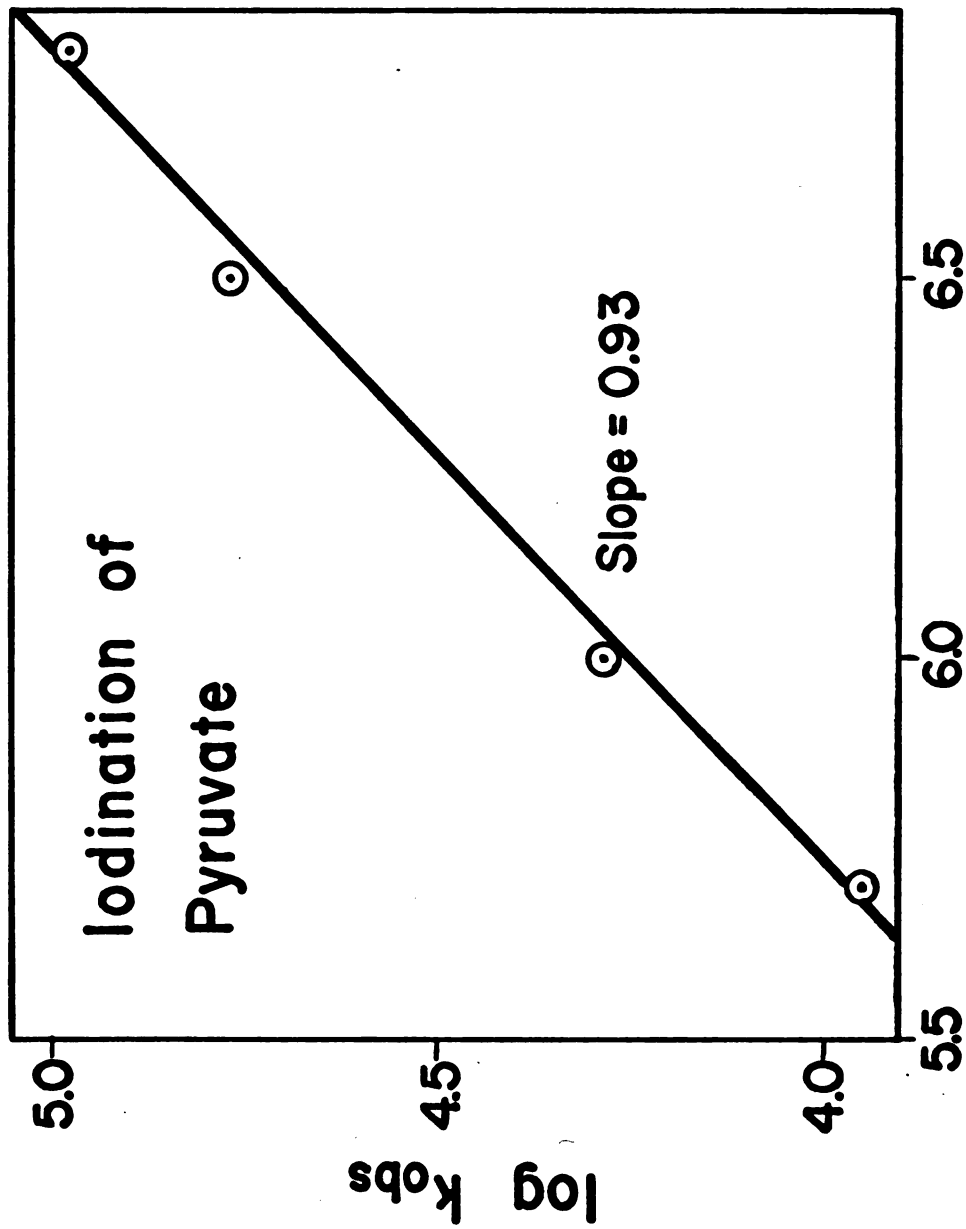

Iodination of
Pyruvate
Slope = 0.93
log k_{obs}
log a_{OH^-}
5.0
4.5
4.0
5.5
6.0
6.5

Figure 13a

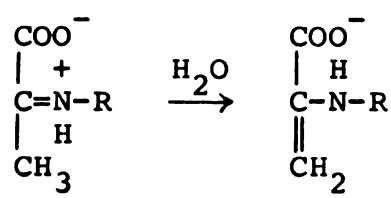
The rate constants for all three processes described earlier can then be summarized below:

1. Enzyme-catalyzed enolization = 120 sec^{-1}
2. Schiff base-catalyzed enolization = 0.304 sec^{-1}
3. "Uncatalyzed" enolization = 0.36 x 10^{-6} sec^{-1}

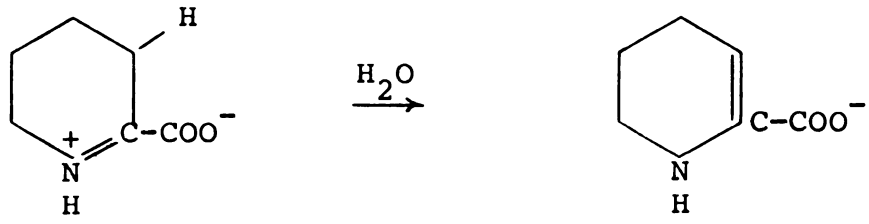
From this it can be seen that enolization of the protonated Schiff base is 10^6 fold greater than the uncatalyzed enolization but 3 x 10^2 fold less than the enzyme catalyzed enolization. Schiff base catalysis, therefore, is highly effective, but only accounts for 0.25% of the enzymatic rate.

Enolization of Δ'-piperidine-2-carboxylic Acid

The shortcomings of the foregoing analyses have already been discussed in the Introduction and Literature Review. To obviate these shortcomings, a direct measure of enamine formation was sought with the stable Schiff base, Δ^1-piperidine-2-carboxylic acid. It was hoped that the rate constant for enamine formation of this compound would agree well with that determined from the enolization of pyruvate in amine buffers. Recall that in amine buffer, an acid-catalyzed term was observed which was interpreted as water-catalyzed enolization of the protonated ketimine:



For comparison, the same constant is desired for Δ^1-piperidine-2-carboxylic acid:



However, preliminary experiments showed immediately that there is a striking difference between the two systems: in imidazole and phosphate buffers, the iodination of Δ^1-PCA is strongly buffer-catalyzed, whereas no buffer-catalyzed enolization of the protonated ketimine was observed with pyruvate in amine buffer. Furthermore, the reaction is too fast in the presence of buffer to allow accurate determinations of the rate constants by ordinary means.

Therefore, the iodination in imidazole buffer was studied by stopped flow techniques as described in Methods. The results at pH 7.25 are shown in Figures 14 and 15. It can be seen that the rate increases linearly with buffer concentration, but the rate of reaction at zero buffer concentration is negligible compared to the buffer-catalyzed rate. Therefore, this method is useless for determining the water-catalyzed enolization.

Figure 14.--Typical iodination reaction of Δ^1-piperidine-2-carboxylic acid measured with the stopped flow apparatus. After mixing, the reaction mixture contained 0.05 M imidazole buffer pH 7.25, 0.2 M KI, 10^{-5} M I_2, NaCl to maintain the ionic strength at 0.4, and 3.7×10^{-4} M Δ^1-piperidine-2-carboxylic acid. Each point on the curve is the calculated absorbance at 351 mμ corresponding to the percent transmittance given by the oscilloscope trace.

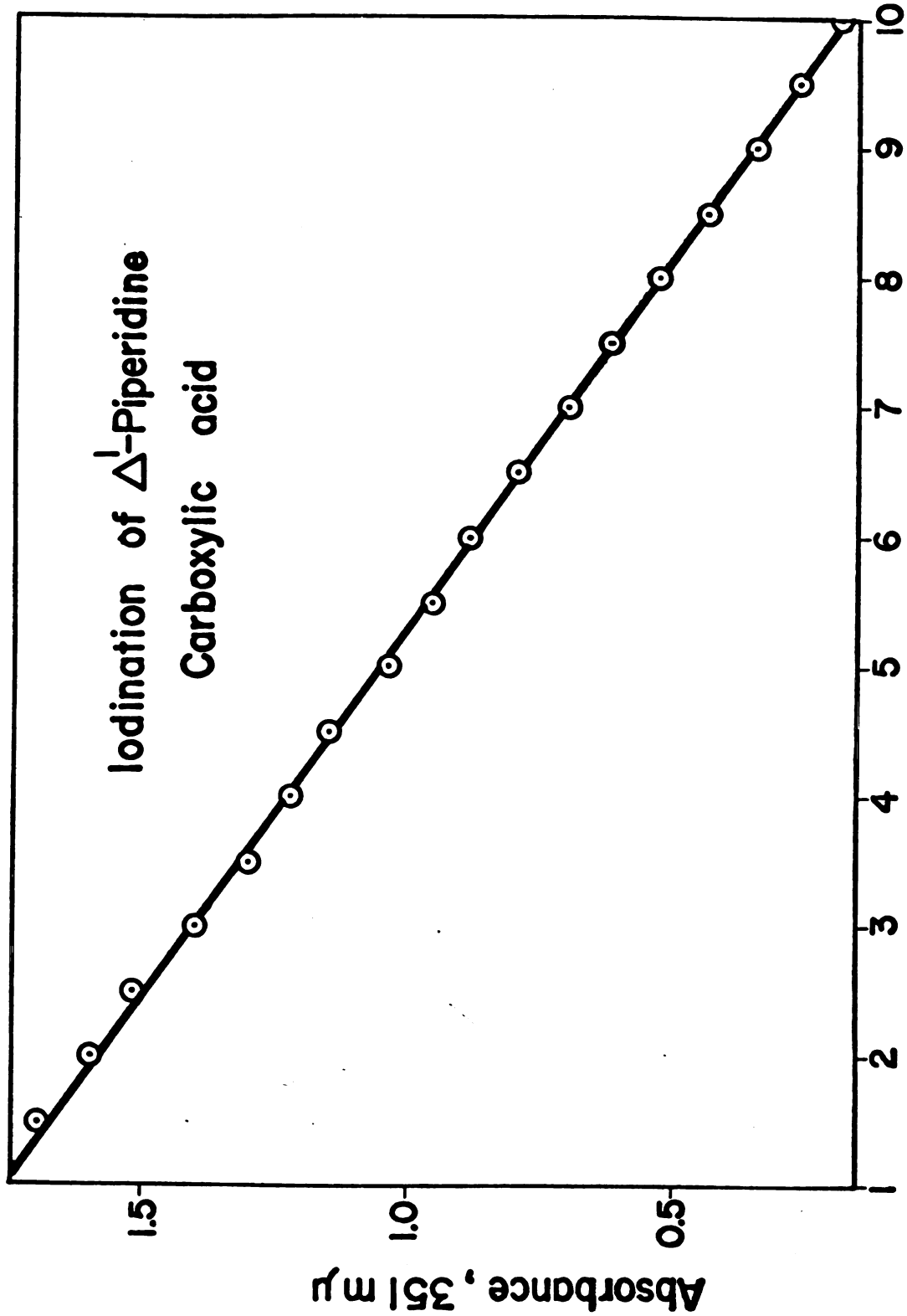
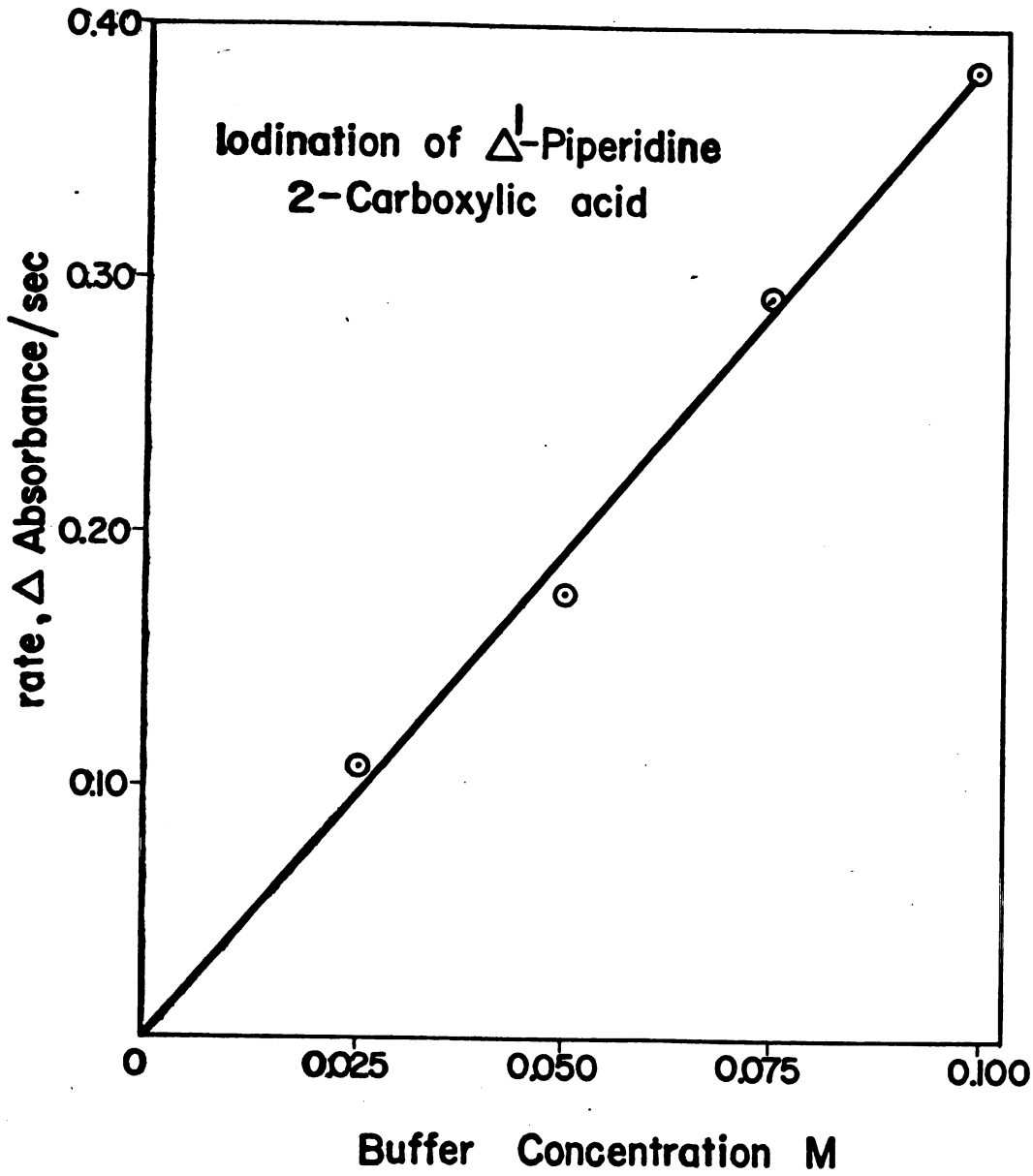

Iodination of Δ¹-Piperidine
Carboxylic acid
Absorbance , 351 mμ
time (seconds)
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
10

Figure 15.--Iodination of Δ^1-piperidine-2-carboxylic acid in imidazole buffer by stopped flow techniques: plot of rate versus buffer concentration.

ne-2-
ped flow
centration.



Fortunately, the reaction is slow enough in the absence of buffer to allow measurements to be made by the pH-stat procedure used for pyruvate.

The iodination was studied over the pH range 7.2 to 8.2, as shown in Figure 16. Table 5 shows the rate of iodination as a function of pH (pH-rate profile) over this range.

The rate data is analyzed in much the same way that it was for pyruvate, only in this case the situation is somewhat more complicated, for the following reason. Since the pK_a for the protonated Schiff base is 7.6, the ratio of protonated to unprotonated form changes dramatically over the pH range studied. And since the enolization constant for the two forms is undoubtedly very different, the complete rate equation must take into account the concentration of each form as a function of pH.

The analysis can be simplified considerably by making the reasonable assumption that enolization of the neutral form is negligible compared to enolization of the protonated form. (If this assumption is not correct, the simplified rate equation will not satisfactorily describe the experimental data.)

Then, if enolization is attributed entirely to the protonated Schiff base, the resulting rate equation is:

$$\text{rate} = [k_{H_2O} + k_{OH^-}(OH^-) + k_s(S)](SH^+)$$

Figure 16.--Typical iodination reaction of Δ^1-piperidine-2-carboxylic acid by the pH-stat method. The reaction mixture contained 0.2 M KI, 0.2 M NaCl, iodine, and 4 x 10^{-4} M Δ^1-piperidine-2-carboxylic acid in a total volume of 50 ml. The pH of this particular reaction was maintained at 7.4; and the temperature, at 25°C.

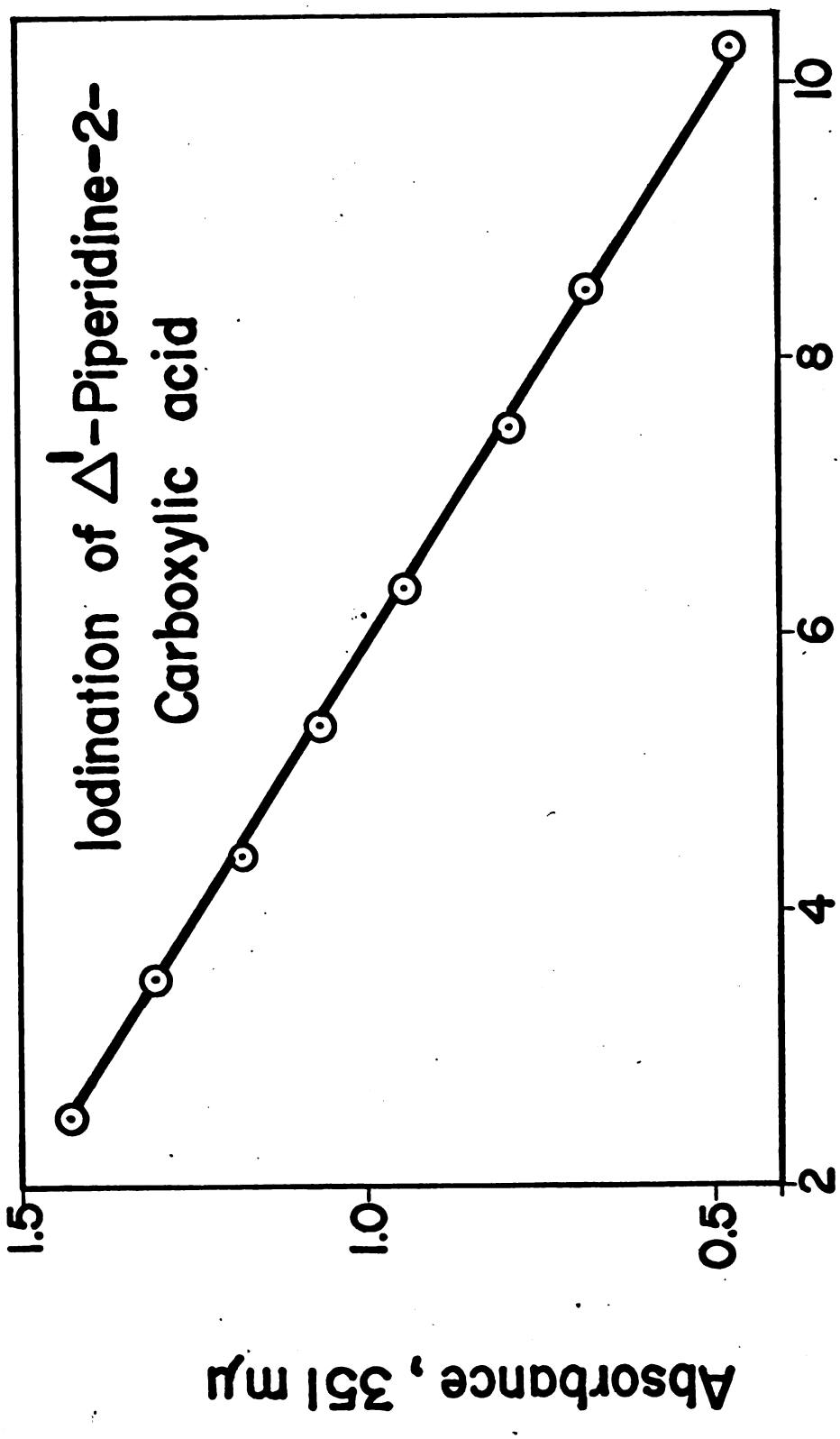
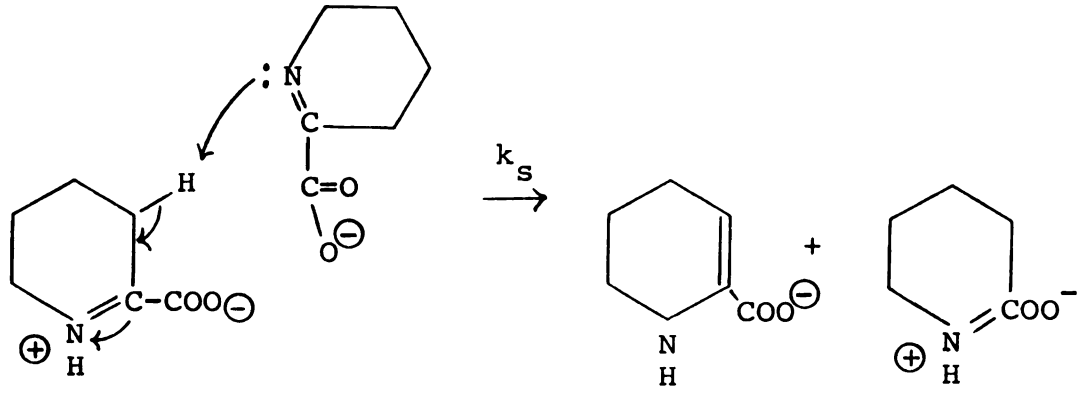

Iodination of Δ¹-Piperidine-2-Carboxylic acid
Absorbance, 351 mμ
time (minutes)
1.5
1.0
0.5
2
4
6
8
10

TABLE 5.--pH-rate Profile of the Iodination of Δ^1-piperidine-2-carboxylic acid by the pH-stat Method.

pH	rate in A_{351} mμ/sec x 10^3
7.15	1.76
7.4	2.13
7.625	2.6
7.90	3.19
8.25	3.76

where the last term represents catalysis by the neutral form of the Schiff base:



By convention, the rate equation is usually expressed in terms of the total concentration of Schiff base, S_o:

$$\text{rate} = \frac{k_{H_2O}\, K'_s(H^+)}{[1 + K'_s(H^+)]} S_o + \frac{k_{OH} K'_s\, K_w}{[1 + K'_s(H^+)]} S_o + \frac{k_s K'_s(H^+)}{[1 + K'_s(H^+)]^2} S_o^2$$

where K'_s is the dissociation constant of the protonated Schiff base and K_w the ion product of water.

As discussed earlier for the enolization of pyruvate under these conditions, it is possible that only one or two of the terms in the rate equation are significant. Assuming first that hydroxide-ion catalysis predominates, the data were plotted according to the rate law

$$k_{obs} = \frac{k_{OH}\ K'_s\ K_w}{1 + K'_s(H^+)}$$

as shown in Figure 17.

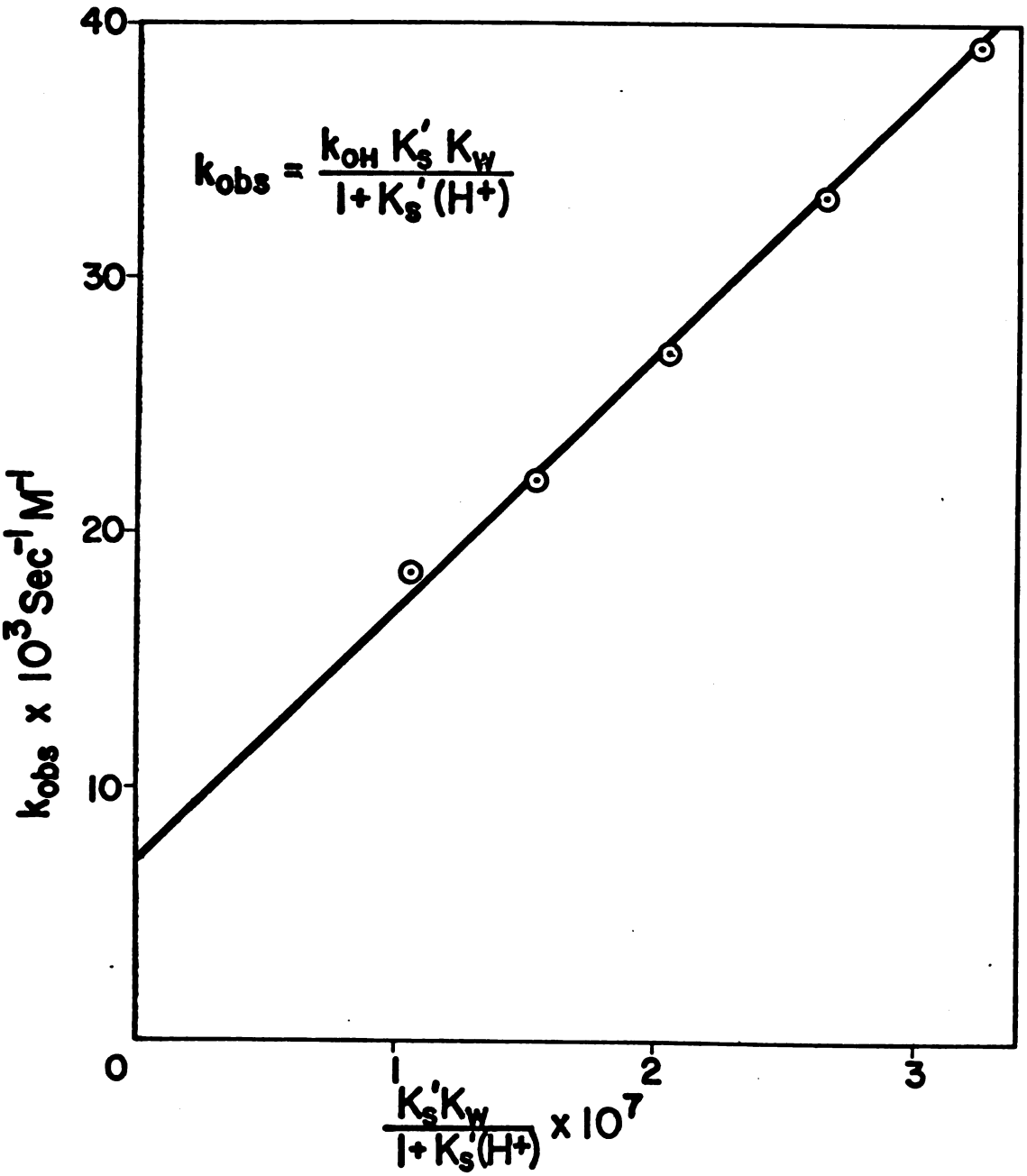
One of two results is expected. If hydroxide-ion catalysis is predominant, the plot of $\frac{K'_s K_w}{1 + K'_s(H^+)}$ versus k_{obs} should be linear and pass through the origin. If, on the other hand, other terms in the rate law are significant, the plot should not be linear.

However, as seen in Figure 17, this plot appears linear and shows a definite positive intercept. At first this is somewhat puzzling since there is no readily apparent way that the complete rate equation could obey such a relationship. Normally, the intercept in such a plot corresponds to a pH-independent reaction; but all three terms in the rate law are pH-dependent.

The answer to this dilemma lies in a rather subtle feature of the last term in the rate expression. A simple calculation shows that while this term is indeed pH-dependent, its value does not change greatly in a pH region about the pK_a of the protonated Schiff base. Therefore, its effect on a plot of k_{obs} versus $\frac{K'_s K_w}{1 + K'_s(H^+)}$ is not great enough to cause a measurable deviation from linearity; instead, this term appears to be pH-independent when measured against the hydroxide ion catalysis.

Figure 17.--Determination of the rate law for the iodination of Δ^1-piperidine-2-carboxylic acid in the absence of buffer. All data were obtained by the pH-stat method described in the text, as shown in Figure 16 and Table 5. The data so obtained are plotted here according to the simplified rate law

$$\text{rate} = k_{OH^-}(SH^+)(OH^-)$$


$k_{obs} = \frac{k_{OH} K_s' K_w}{1 + K_s'(H^+)}$
40
30
20
10
0
1
2
3
$k_{obs} \times 10^3 Sec^{-1} M^{-1}$
$\frac{K_s' K_w}{1 + K_s'(H^+)} \times 10^7$

The catalytic constant for this term, k_s, can then be determined from a plot of rate/S_o versus S_o at constant pH according to the expression:

$$\frac{\text{rate}}{S_o} = \frac{k_{H_2O}\, K'_s(H^+)}{[1 + K'_s(H^+)]} + \frac{k_{OH}K'_sK_w}{[1 + K'_s(H^+)]} + \frac{(S_o)k_sK'_s(H^+)}{[1 + K'_s(H^+)]^2}$$

This plot is shown in Figure 18. Once k_s is determined, the other two constants can be determined from a plot of

$$\frac{1}{S_o}\frac{(\text{rate})[1 + K'_s(H^+)]}{[H^+]} - \frac{k_sK'_sS_o}{[1 + K'_s(H^+)]} \text{ versus } \frac{1}{H^+}$$

according to the following rearrangement of the rate expression as shown in Figure 19:

$$\frac{1}{S_o}\frac{\text{rate}[1 + K'_s(H^+)]}{[H^+]} - \frac{k_sK'_sS_o}{[1 + K'_s(H^+)]} = \frac{k_{OH}K'_sK_w}{H^+} + k_{H_2O}k_s$$

where k_{OH} can be determined from the slope and k_{H_2O} from the intercept. k_{OH} was found to be 1.14×10^3 $M^{-1}sec^{-1}$ and k_{H_2O} less than 6×10^{-5} sec^{-1}.

These results are quite different than those obtained from the pyruvate amine mixtures. This will be discussed in detail in the Discussion.

For convenience, all the catalytic constants determined in this research are tabulated in Table 6. In a number of cases, constants determined from intercepts could not be distinguished from zero with

Figure 18.--pH-stat iodination of Δ^1-piperidine-2-carboxylic acid. Determination of the constant k_s by a plot of rate/S_o versus S_o, according to the rate equation

$$\frac{\text{rate}}{S_o} = \frac{k_{H_2O}K'_s(H^+)}{[1 + K'_s(H^+)]} + \frac{k_{OH}K'_sK_w}{[1 + K'_s(H^+)]} + \frac{k_sK'_s(H^+)S_o}{[1 + K'_s(H^+)]^2}$$

The slope is the quantity

$$\frac{k_sK'_s(H^+)}{[1 + K'_s(H^+)]}$$

The intercept is the sum of the first two terms in the rate expression. The reaction mixture contained in addition to substrate, 0.2 M KI, I_2, and 0.2 M NaCl in a total volume of 50 ml. The pH was maintained at 7.6; and the temperature, at 25°C.

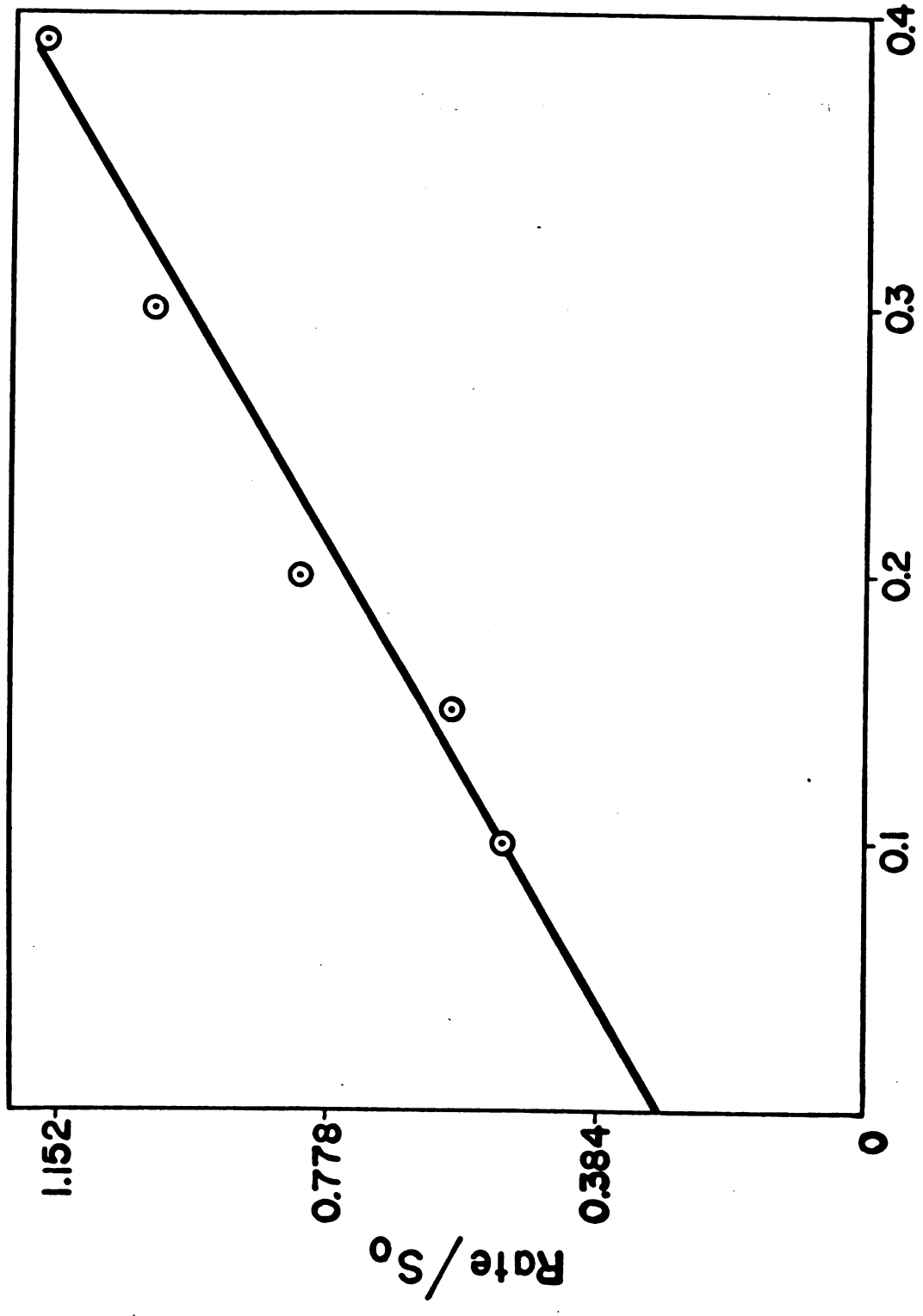

Rate / S_o
1.152
0.778
0.384
0
0.1
0.2
0.3
0.4
$S_o \times 10^3$ M

Figure 19.--Iodination of Δ^1-piperidine-2-carboxylic acid by the pH-stat method; determination of the constants k_{OH^-} and k_o from a plot of

$$\frac{1}{S_o}\,\frac{\text{rate}[1 + K'_s(H^+)]}{(H^+)} - \frac{k_s K'_s S_o}{[1 + K_s(H^+)]} \text{ versus } \frac{1}{H^+}$$

according to the rate equation shown in the figure. The slope is given by $k_{OH^-}K'_sK_w$ and the intercept by $k_{H_2O}K'_s$. The data for this plot have been presented in Figure 18 and Table 5.

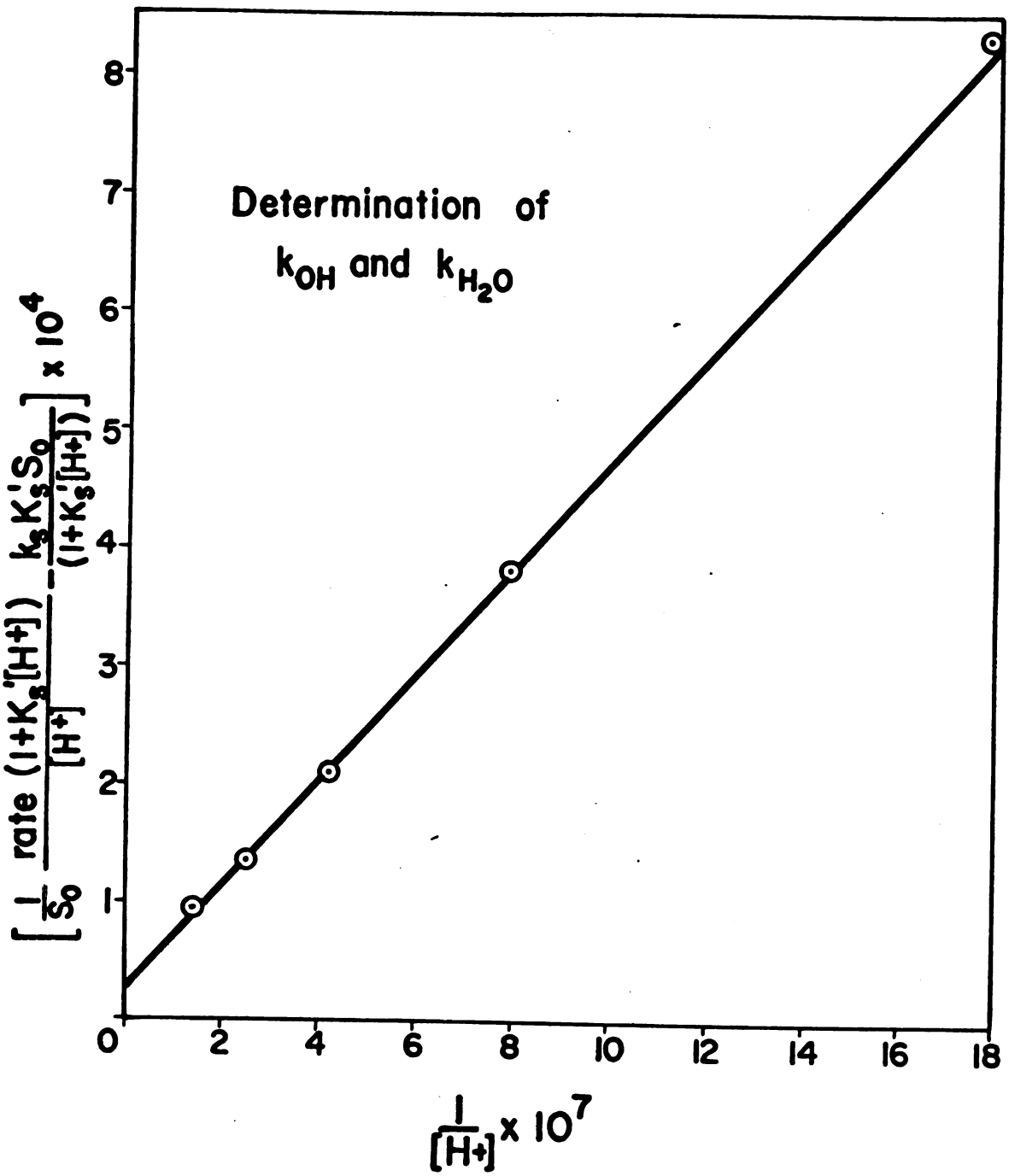
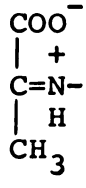

Determination of
k_{OH} and k_{H_2O}
$\left[\frac{1}{S_0}\frac{\text{rate}\,(1+K_s'[H^+])}{[H^+]} - \frac{k_s K_s' S_0}{(1+K_s'[H^+])}\right] \times 10^4$
$\frac{1}{[H^+]} \times 10^7$
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18

TABLE 6.--Complete Rate Constant Table.

Substrate	Catalyst	Constant
Pyruvate	H_2O	$k_{H_2O} = <1 \times 10^{-6}\ min^{-1}$
	OH^-	$k_{OH^-} = 0.360\ M^{-1}sec^{-1}$
	phosphate	$k_b = 2.84 \times 10^{-4}\ M^{-1}sec^{-1}$
	phosphate	$k_a = <5.52 \times 10^{-6}\ M^{-1}sec^{-1}$
	imidazole	$k_b = 2.53 \times 10^{-4}\ M^{-1}sec^{-1}$
	imidazole	$k_a = <1 \times 10^{-5}\ M^{-1}sec^{-1}$
	N-ε-amino-caproate	$k_b = 0.0971\ M^{-1}sec^{-1}$
	N-ε-amino-caproate	$k_a = 1.88 \times 10^{-3}\ M^{-1}sec^{-1}$
pyr-ketimine	H_2O	$k_e = 0.304\ sec^{-1}$
		
Δ^1-Piperidine-2-Carboxylic Acid	H_2O	$k_{H_2O} = <6 \times 10^{-5}\ sec^{-1}$
	OH^-	$k_{OH^-} = 1.14 \times 10^{3}\ M^{-1}sec^{-1}$
	Δ^1-PCA	$k_s = 0.125\ M^{-1}sec^{-1}$
	imidazole	$k_{obs} = 0.182\ M^{-1}sec^{-1}$*
		$k_{I_m} = 0.41\ M^{-1}sec^{-1}$**
Aldolase-pyruvate Schiff base		$k_{enz} = 120\ sec^{-1}$

*pH 7.25

**In terms of 1 M SH^+ and 1 M Im:

confidence; therefore, an upper limit was placed on these constants as indicated by the sign, <, preceding the value of the constant.

CHAPTER V

DISCUSSION

The rate of tautomerization of simple aliphatic imines has now been studied in four systems: acetone in amine buffer (34); isobutyraldehyde in amine buffer (47); pyruvate in amine buffer; and the cyclic ketimine Δ^1-piperidine-2-carboxylic acid in the presence and absence of buffer (present research).

Unfortunately, the results obtained with these systems differ from one another in many important respects. To be sure, certain quantitative differences could be expected. For instance, it would not be surprising to find that the enolization constants for a cyclic ketimine and the imines of an aldehyde, ketone, and α-keto acid differ by perhaps two orders of magnitude. What is disturbing is the qualitative differences observed among these systems. Specifically, the ketimine-enamine conversion determined from acetone in methylamine buffer and pyruvate in amine buffer was found to be catalyzed only by water and not by general-bases. On the other hand, the corresponding tautomerization of the imine of

isobutyraldehyde and Δ^1-piperidine-2-carboxylic acid proceeds predominantly through a general-base-catalyzed route. Adding to the confusion, Bender found that general-base catalysis of the tautomerization of the acetone imine was evident in the presence of certain amines, such as glycine, ethylenediamine, p-toluidine, but not in the presence of other amines such as methylamine, ethanolamine, and hydroxymethylaminomethane.

Any attempt to rationalize these results must proceed along two lines: (1) some, if not all, kinetic analyses and interpretations are incorrect, and (2) in the case of Δ^1-piperidine-2-carboxylic acid the effect of the ring is so great that the properties of this imine are not at all comparable to those of the acyclic imines. The kinetic analyses will be discussed first.

In this author's opinion, the interpretations of the rate data obtained for the acetone and pyruvate systems are reasonable; the most likely source of the acid-catalyzed enolization appears to be water-catalyzed enolization of the protonated ketimine. Whether the rate data are correct is another matter. There is always the possibility that a small unknown systematic error is causing terms to appear in the rate law that do not arise from the reaction supposedly being measured. The acetone and pyruvate systems are most susceptible to this kind of error since the term for acid catalysis is so much smaller

than the one for base catalysis. However, there is no readily apparent source of this error in these cases.

The isobutyraldehyde system should be relatively insensitive to this type of error since most of the enolization proceeds through the protonated imine. However, as discussed in the Literature Review, some of the interpretations of the rate data are questionable. For instance, the conclusion that most of the enolization proceeds through general base-catalyzed enolization of the protonated imine seems to depend on an a priori assumption that water-catalyzed enolization of the protonated imine is negligible. Recall that in the acetone system, only water-catalyzed enolization was observed with many of the amines used.

The kinetics of the Δ^1-piperidine-2-carboxylic acid system are unquestionably the least ambiguous of all four systems. The experimental rate equation shows quite clearly that in the absence of buffer, enolization is dependent on hydroxyl ion concentration and a protonated form of the substrate having a $pK_a = 7.6$, which is precisely the pK_a value Macholan (4) obtained, by titration, for the protonated ketimine of this compound. This provides strong evidence that the observed enolization results from the protonated ketimine. It follows, then, that the large stimulation of the rate by buffer or hydroxide ion can only be attributed to base-catalyzed enolization of the protonated ketimine.

It is worth re-emphasizing the advantages the Δ^1-piperidine-2-carboxylic acid system has over the other three studied. First, the compound exists almost entirely as the Schiff base. This obviates all the problems that arise from general acid-base-catalyzed enolization of the free ketone or aldehyde. Second, the pK_a of the protonated imine can be experimentally determined for this compound, whereas this important constant had to be approximated in the other systems. Third, the enolization could be measured on both sides of the pK_a. Such an analysis provides the soundest kinetic treatment when ionizable substrates are involved. In the other systems, enolization could only be studied on the alkaline side of the pK_a value.

However, as mentioned earlier, the disadvantage of this system is the unknown effect the ring might have on enolization. But it is possible to get a rough idea of the magnitude and direction of this effect from studies of the enolization of cyclic ketones, and the isomerization of cyclic olefins where the starting double bond is exocyclic to the ring.

The results of these studies are somewhat surprising. When Schriesheim *et al.* (48), and Schechter *et al.* (49), compared the rate of enolization of cyclic ketones, they found that the order of reactivity was cyclohexanone > cyclopentanone > cyclobutanone. However,

the order of reactivity was reversed in the base-catalyzed route. Furthermore, the base-catalyzed enolization of the cyclic ketones was much faster than the base-catalyzed enolization of corresponding acyclic ketones. Schriesheim et al. (48) observed a similar order of reactivity for the isomerization of cyclic olefins. Two explanations were offered by the two groups of investigators. Schechter et al. (49) suggested that two principles governed the reactivity of these compounds: (1) the transition state for an acid-catalyzed enolization most resembles the enol, whereas the transition state for a base-catalyzed reaction most resembles the ketone; (2) the acidity of a proton alpha to a carbonyl increases as the size of the ring decreases. Both of these principles originated in the work of other investigators (50-52).

The order of reactivity can then be explained as follows: In the acid-catalyzed route, the ring is forced to accommodate a substantial amount of double bond character; this becomes ever more difficult as the size of the ring decreases. Therefore, larger cyclic ketones should undergo enolization more readily than do smaller cyclic ketones. On the other hand, since the transition state for base-catalyzed enolization most resembles the ketone, the ring does not suffer a great increase in strain in the transition state. Therefore, the most important factor governing base-catalyzed enolization is the acidity of the alpha proton, which increases as the ring size decreases.

As a result, the base-catalyzed enolization of smaller rings should be more favorable than that of larger rings.

An alternative explanation for the order of reactivity in base-catalyzed enolization was offered by Schriesheim et al. (48). These workers showed with molecular models that as the ring size decreases, the orientation of the carbon-hydrogen bond in relation to the plane of the carbonyl approaches the perpendicular. Since this perpendicular relation is known to be most favorable for enolization, the rate of enolization should increase with decreasing ring size.

Whatever the explanation, the important experimental findings of these studies which pertain to the present research is that base-catalyzed enolization of a cyclic six-member ketone is significantly larger than the corresponding base-catalyzed enolization of an acyclic ketone by a factor of perhaps ten.

This is in sharp contrast to results obtained in this study which indicate that the water-catalyzed enolization of the protonated ketimine of pyruvate is 2×10^4 times greater than the water-catalyzed enolization of the protonated ketimine of Δ^1-piperidine-2-carboxylic acid.

Now that all these systems have been discussed in detail, it is appropriate to attempt to reach some conclusions about the results of the present research. In

order to do this, the following dilemma must be considered: the system with pyruvate in amine buffer provides precisely the ketimine which is to be studied, but the kinetics may be ambiguous. On the other hand, Δ^1-piperidine-2-carboxylic acid system provides a ketimine that is somewhat different than the one intended for study, but the kinetics are unambiguous. Which system, then, is a more reliable measure of the ketimine-enamine tautomerization of an acyclic ketimine of pyruvate?

In the opinion of this author, the piperidine system is the more reliable. As discussed earlier, the only objection to this system is the possibility that the enolization of a cyclic ketimine might be greatly different than the enolization of an acyclic ketimine. If this explanation is used to account for the differences of the two systems, it would be necessary to conclude that the tautomerization of a cyclic six-membered ketimine is 2×10^4 less than that of an acyclic ketimine. This conclusion could not differ more, in direction and magnitude, from the results obtained with cyclic ketones, where the base-catalyzed enolization of cyclohexanone was found to be greater than that of the acyclic ketone by a factor of approximately ten.

To make the comparison complete, we must consider differences in the rate at which methyl and methylene hydrogens (alpha to a carbonyl) undergo exchange. Rappe

and Sachs (53) showed that the exchange rate of methylene hydrogens is 0.6 to 0.23 that of methyl hydrogens. This effect should nearly compensate for the stimulation effect of the ring.

Therefore, it seems most reasonable to conclude, tentatively at least, that the results obtained with the pyruvate and acetone systems are questionable, while those obtained with the Δ^1-piperidine system are probably a good measure of the tautomerization of acyclic ketimines.

Using Δ^1-piperidine-2-carboxylic acid as a model for the aldolase reaction, the role of the Schiff base in the enzymatic process can now be discussed.

These studies have established the contribution of Schiff base catalysis alone to the overall enzymatic rate; clearly, Schiff base formation alone can only account for a fraction of the catalysis, as shown in Table 7 which compares the enzymatic rate constant with the pseudo first order rate constants for pyruvate and Δ^1-piperidine-2-carboxylic acid at pH 8.0. The enolization of Δ^1-piperidine-2-carboxylic acid is approximately 10^3 times greater than pyruvate but 2.5×10^5 times less than the enzymatic enolization. However, the enolization of pyruvate and Δ^1-piperidine-2-carboxylic acid is strongly catalyzed by general bases. Table 8 compares the enzymatic enolization with the imidazole-catalyzed enolization of pyruvate and protonated ketimine of Δ^1-PCA. Once again, the enolization

TABLE 7.--Comparison of the Enzymatic Rate Constant of Enolization with the Corresponding Pseudo First Order Rate Constants for Pyruvate and Δ^1-PCA at pH 8.0.

Compound	Rate Constant of Enolization*
	sec^{-1}
Enzyme	120
Δ^1-piperidine-2-carboxylic acid	5×10^{-4}
Pyruvate	3.6×10^{-7}

*There is some difficulty comparing the rate constant of the enzymatic enolization to the rate constants for Δ^1-PCA and pyruvate. For both of these nonenzymatic reactions, the rate constants have been expressed in terms of 1.0 M pyruvate or Δ^1-PCA and 10^{-6} M hydroxide (pH 8.0). Therefore, the nonenzymatic rate constants are clearly pseudo first order constants. The order of the enzymatic process is not so clear, since it is not known whether water, hydroxide ion, or an amino acid residue catalyzes the tautomerization of the pyruvate-enzyme Schiff base. Therefore, the enzymatic rate constant could only be calculated from the rate of tautomerization of 1.0 M pyruvate-enzyme complex. Consequently, the enzymatic constant can only be expressed as a true first order constant, although the reaction may be of higher order. But as long as the physical significance of these constants has been stated, the comparisons can be made, and evaluated accordingly.

TABLE 8.--Comparison of the Enzymatic Enolization with Imidazole-Catalyzed Enolization of Pyruvate and Protonated Ketimine of Δ^1-PCA.

Compound	Rate Constant
Enzyme	120 sec^{-1}
Δ^1-piperidine-2-carboxylic acid	0.41 $sec^{-1}M^{-1}$
Pyruvate	0.253 x 10^{-3} $sec^{-1}M^{-1}$

of Δ^1-piperidine-2-carboxylic acid is approximately 2×10^3 times greater than pyruvate, but under these conditions it is only 3×10^2 times less than the enzymatic enolization.

From this information, it is possible to construct a hypothetical model for the enzyme-catalyzed reaction by proposing that catalysis is effected by an amino acid side chain functioning as a general base to catalyze the enolization of the protonated ketimine of pyruvate. If the reasonable assumption is made that the base is held in close proximity to the methyl hydrogens, the effective base concentration relative to the methyl protons could reasonably be assigned a value of ten (if not higher). So, if the imidazole group of histidine were the base, the rate constant for this enolization would be ten times the value of the rate constant determined with imidazole buffer, i.e., 4.1 sec^{-1}. This value is now one hundred times less than the enzyme-catalyzed rate. But if the base is stronger than imidazole, such as the ε-amino group of lysine, thiolate anion of cysteine, or phenolate anion of tyrosine, the rate constant would be as large as that observed for the enzyme-catalyzed reaction. This conclusion derives from a rough interpolation of the rate constants for the imidazole-catalyzed enolization and the hydroxide ion-catalyzed enolization. Of course, this model must remain

speculative until it can be shown that an amino acid residue does function as a general base in the catalytic reaction. However, there is some circumstantial evidence for the involvement of a general base in aldolase reactions (aside from the usual plethora of "active site" studies involving the systematic destruction of every possible amino acid side chain). Muscle aldolase catalyzes the enolization of dihydroxyacetonephosphate by a Schiff base mechanism analogous to that of the KDPG aldolase. However, only one of the hydroxymethyl hydrogens is exchanged. If Schiff base formation alone provided sufficient activation of the enolization reaction, it would be difficult to explain the stereospecificity of the exchange reaction, since both hydroxymethyl hydrogens should undergo spontaneous exchange. The simplest explanation for the stereoselectivity is that binding of the substrate places only one of these hydrogens in proximity with a general-base catalyst in the site. Moreover, it should not be overlooked that dihydroxyacetonephosphate has four potentially exchangeable hydrogens when those at the carbon 1 position are included. Since these hydrogens do not exchange at all, it seems that Schiff base catalysis alone is not sufficient.

Although this research has been concerned with Schiff base-catalyzed enolizations, it is worth discussing the problem of Schiff base formation itself. Both the

kinetic and equilibrium constants obtained for Schiff base formation show clearly that this part of the enzyme-catalyzed reaction must itself be enzyme-catalyzed. For example, the equilibrium constant of Schiff base formation from pyruvate and butylamine was found to be 10 M^{-1}; by comparison, the binding constant of pyruvate to KDPG aldolase is 2×10^4 M^{-1} (determined from the K_m). But this is not at all surprising. Most likely, pyruvate first forms an energetically favorable noncovalent complex with the enzyme prior to Schiff base formation, so that the overall equilibrium constant of Schiff base formation would be the product of the equilibrium constant for noncovalent binding and the intrinsic equilibrium constant of Schiff base formation. Other factors may play a role in enhancing enzymatic Schiff base formation, such as maintenance of an uncharged lysine at neutral pH. From the extensive investigations of Schiff base formation, even more factors might be imagined such as acid-catalyzed dehydration of the carbinolamine at the active site.

From this very general description, it is obvious that the enzyme-catalyzed formation of Schiff base is every bit as mysterious as the enolization, if not more so. And, apparently, it would be even more difficult to construct a nonenzymatic model of this reaction than for the enolization reaction.

However, the properties of Δ^1-piperidine-2-carboxylic acid may well have some bearing on Schiff base

formation as well as enolization. Consider the remarkable stability of this compound to hydrolysis, as illustrated in the following example.

The equilibrium constant for Schiff base formation from pyruvate and butylamine is 10 M^{-1}. Then at pH 7.6 where the concentration of protonated imine equals that of neutral imine we can write

$$2K_s = \frac{(S_T)}{(P)(RNH_2)}$$

where S_T is the total concentration of imine. In a solution containing 1.0 M pyruvate and 1.0 M amine, the concentration of neutral amine will be approximately 10^{-3} M. From this, the ratio S_T/P is readily calculated to be 2×10^{-2}. In other words, the equilibrium ratio of Schiff base to pyruvate is only one to fifty. However, the equilibrium ratio of Δ^1-piperidine-2-carboxylic acid to the open chain form at this pH lies almost exclusively (within experimental measurement) in the direction of the Schiff base. Another simple calculation reflects the possible implications of this phenomenon.

Let us assume, just to make the point, that the equilibrium constant for the ring closure can be calculated by the same equation used for the pyruvate amine mixture. The results of this calculation are startling: the value obtained for the equilibrium constant is 1×10^4; the value of the dissociation constant is the reciprocal,

1×10^{-4}, which is comparable to the K_m of pyruvate for KDPG aldolase!

The question to be answered is how this intramolecular formation of Schiff base can be so much more favorable than the intermolecular reaction between pyruvate and amine. The first explanation that comes to mind is that the "effective concentration" of reacting groups is much higher in the intramolecular reaction. However, a simple calculation shows that if the intrinsic equilibrium constant for Schiff base formation in the intramolecular reaction is the same as that for the intermolecular reaction (10 $\underline{M}^{-1}$), the "effective concentration" of amine would have to be an impossible 1000 $\underline{M}$ to account for the stability of the cyclic Schiff base at pH 7.6.

This phenomenon is actually fairly common for intramolecular reactions. Since this subject has been discussed in detail by Jencks (54) and others referenced within, a detailed discussion of possible explanations for the behavior of intramolecular reactions will not be presented here. Suffice it to say that factors other than local concentration effects are involved. Three such factors which have been suggested are rotamer orientation, changes in solvation, and orbital overlap. Two of the more impressive studies of intramolecular reactions are worth mentioning. Bruice and Pandit (55, 56) found that the rate of nucleophilic attack of carboxylate on an ester increases dramatically as the groups are forced

together by the geometrical constraints of the molecule. Similarly, Storm and Koshland (57) found enormous accelerations in the rate of lactone formation when the reacting groups were forced into close proximity with the proper orientation.

Whatever the explanation for the rate accelerations, both studies show clearly that maximal rate accelerations only occur when severe limitations are imposed on the number of possible orientations the reacting groups can assume in relation to one another. Presumably, then, this precise orientation is effected in an enzyme catalyzed reaction.

But the stability of Δ^1-piperidine-2-carboxylic acid indicates that this may not always be necessary. The only apparent restriction of the relative positions of the reacting groups in the open chain form is that they cannot be separated by more than about ten angstroms. Therefore, the highly favorable ring closure reaction must be explained in other ways.

This may have important consequences regarding not only enzymatic Schiff base formation, but also substrate binding and enzyme catalysis in general. Specifically, substrate binding and catalysis may be effected by a more subtle mechanism than directly jamming the reacting or binding groups together in a unique orientation. Perhaps the initial binding of substrate is weak, but the presence of substrate causes the entire active site to change to an

energetically more favorable configuration that includes the substrate in much the same way that the rather distant amino and carbonyl groups of the open chain form of Δ^1-piperidine-2-carboxylic acid are combined to form the more stable Schiff base. This view, of course, is essentially the Koshland induced-fit model.

It seems, then, that Δ^1-piperidine-2-carboxylic acid may well be a good model for many enzymatic processes.

Finally, the possible role of the Schiff base in substrate binding should be mentioned. Throughout this work the catalytic role of the Schiff base has been emphasized, but it is difficult to overlook the possibility that the enzyme might use this covalent bond to assist the binding of pyruvate. Moreover, the covalent bond is an ideal way to bind <u>intermediates</u>. Consider the following scheme for the enzymatic aldolization of pyruvate and G3P:

$$E + P \rightleftharpoons E\cdot P \rightleftharpoons EP \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} EP^- \xrightarrow{+G3P} \text{product}$$

noncovalent interaction — Schiff base — enol(ate)

Since this is an ordered mechanism, the enzyme must efficiently bind the enol (enolate) form of pyruvate as well as pyruvate itself; otherwise, the enol would readily dissociate from the enzyme. In order to

effectively bind pyruvate in both keto and enol forms, the enzyme might have to undergo a conformational change upon enolization. However, this is not necessary with a Schiff base mechanism since the enol form of pyruvate is a covalently bound enamine which cannot dissociate directly from the enzyme. This argument, of course, may be teleology at its worst, but still it is an attractive possibility.

REFERENCES

1. Koshland, D. E., Jr., and Neet, K. E. Ann. Rev. Biochem., 37, 359 (1968).

2. Ingram, J. M., and Wood, W. A. J. Biol. Chem., 240, 4146 (1965).

3. Rose, I. A., and O'Connell, E. L. Arch. Biochem. and Biophys., 118, 758 (1965).

4. Macholàn, L., and Svàtek, E. Coll. Czech. Chem. Comm., 25, 2564 (1960).

5. Bruice, T. C., and Benkovic, S. In Bioorganic Mechanisms. New York: W. A. Benjamin, Inc., 1966, p. 181.

6. Snell, E. E., Fasella, P. M., Braunstein, A., and Fanelli, A. R. Chemical and Biological Aspects of Pyridoxal Catalysis. New York: Pergamon Press, 1963.

7. Bruice, T. C., and Benkovic, S. In Bioorganic Mechanisms. New York: W. A. Benjamin, Inc., 1966.

8. Jencks, W. P. In S. G. Cohen, A. Streitweish, Jr., and R. W. Taft (eds.). Progress in Physical Organic Chemistry, Vol. 2. New York: Interscience Publishers, Inc., 1964, p. 63.

9. Acree, S. F., and Johnson, J. M. Am. Chem. J., 38, 308 (1907).

10. Barret, E., and Lapworth, A. J. Chem. Soc., 93, 85 (1908).

11. Bodforss, S. Z. Physik. Chem., 109, 223 (1924).

12. Olander, A. Z. Physik. Chem., 129, 1 (1927).

13. Bartlett, P. D., and Conant, J. B. J. Am. Chem. Soc., 2881 (1932).

14. Jencks, W. P. J. Am. Chem. Soc., 81, 475 (1959).

15. Westheimer, F. H., and Cohen, H. J. Am. Chem. Soc., 60, 90 (1938).

16. French, C. C. J. Am. Chem. Soc., 51, 3215 (1929).

17. Miller, J. G., and Kilpatrick, M. J. Am. Chem. Soc., 53, 3217 (1951).

18. Speck, J. C., Jr., and Forist, A. A. J. Am. Chem. Soc., 79, 4659 (1957).

19. Pontremoli, S. Proc. Natl. Acad. Sci., 47, 1942 (1961).

20. Grazi, E., Cheng, T., and Horecker, B. L. Bioch. and Biophys. Res. Comm., 7, 250 (1962).

21. Speck, J. C., Jr. J. Am. Chem. Soc., 85, 1012 (1963).

22. Rose, I. A., and O'Connell, E. L. Arch. Bioch. Biophys., 118, 758 (1967).

23. Cash, D. J., and Wilson, I. B. J. Biol. Chem., 241, 2490 (1966).

24. Pedersen, K. J. J. Am. Chem. Soc., 60, 595 (1938).

25. Pedersen, K. J. Acta. Chem. Scand., 8, 710 (1954).

26. Hay, R. W. Aust. J. Chem., 18, 337-51 (1966).

27. Guthrie, J. P., and Westheimer, F. H. Fed. Proc., 26, 562 (1967).

28. Jencks, W. P. In Catalysis in Chemistry and Enzymology. New York: McGraw Hill, 1969, p. 18.

29. Hamilton, G. H., and Westheimer, F. H. J. Am. Chem. Soc., 81, 6332 (1959).

30. Fridovich, I., and Westheimer, F. H. J. Am. Chem. Soc., 84, 3209 (1962).

31. Warren, S., Zerner, B., and Westheimer, F. H. Biochemistry, 5, 817 (1966).

32. Kobes, R. D., and Dekker, E. E. Bioch. and Biophys. Res. Comm., 27, 607 (1967).

33. Ingram, J. M., and Wood, W. A. J. Biol. Chem., 241, 3256 (1966).

34. Bender, M. L., and Williams, A. J. Am. Chem. Soc., 88, 2502 (1966).

35. Hine, J., Menon, B. C., Jenson, J. H., and Mulders, J. J. Am. Chem. Soc., 88, 3367 (1966).

36. Rose, I. A., and Rieder, S. V. J. Am. Chem. Soc., 77, 5764 (1955).

37. Rose, I. A., and Rieder, S. V. J. Biol. Chem., 231, 315 (1958).

38. Tagaki, W., and Westheimer, F. H. Biochemistry, 7, 901 (1968).

39. Kobes, R. D. Thesis. University of Michigan, 1967.

40. Meister, A. J. Biol. Chem., 206, 577 (1954).

41. Weisblat, D. I., Magerlein, B. J., and Meyers, D. R. J. Am. Chem. Soc., 75, 3630 (1953).

42. Harper, E. T., and Bender, M. L. J. Am. Chem. Soc., 87, 5625 (1965).

43. Coward, J. K., and Bruice, T. C. J. Am. Chem. Soc., 91, 5339 (1969).

44. Hall, N. F., and Sprinkle, M. R. J. Am. Chem. Soc., 54, 3469 (1932).

45. Zuman, P. Coll. Czech, Chem. Comm., 15, 839 (1950).

46. Meloche, H. P., and Wood, W. A. J. Biol. Chem., 339, 3515 (1964).

47. Hine, J., Menon, B. C., Jenson, J. H., and Mulders, J. J. Am. Chem. Soc., 88, 3367 (1966).

48. Schriesheim, A., Muller, R. J., and Rowe, C. A., Jr. J. Am. Chem. Soc., 84, 3164 (1962).

49. Shechter, H., Collis, M. J., Dessy, R., Okuzumi, Y., and Chen, A. J. Am. Chem. Soc., 84, 2905 (1967).

50. Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., and Schaad, L. J. J. Am. Chem. Soc., 80, 5855 (1958).

51. Tamres, M., and Searles, S. J. Am. Chem. Soc., 81, 2100 (1959).

52. Campbell, H. J., and Edwards, J. T. Can. J. Chem., 38, 2109 (1960).

53. Rappe, C., and Sachs, W. H. J. Org. Chem., 32, 4127 (1967).

54. Jencks, W. P. In Catalysis in Chemistry and Enzymology. New York: McGraw Hill, 1969.

55. Bruice, T. C., and Pandit, U. K. J. Am. Chem. Soc., 82, 5328 (1960).

56. Bruice, T. C., and Pandit, U. K. Proc. Natl. Acad. Sci., 46, 402 (1960).

57. Storm, D. R., and Koshland, D. E., Jr. Proc. Natl. Acad. Sci., 66, 445 (1970).

58. Barran, L. R. Thesis. Michigan State University, 1969.

59. Hammerstedt, R. H., Möhler, H., Decker, K. A., and Wood, W. A. Manuscript received for publication.