Simulation of the active site of vitamin B6-dependent enzymes

Tom Lyons Fisher

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Simulation of the active site of vitamin B\textsubscript{6}-dependent enzymes

by

Tom Lyons Fisher

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

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Head of Major Department

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## Synthesis of the 6-membered imine ring analog and its derivatives

### DISCUSSION

- **Oxidation methods**
- **5-Deoxy analogs**
- **Intermediates of the attempted syntheses of the 5-membered imine ring analog**
- **The 6-membered imine ring analog and related compounds**
- **The 7-membered imine ring analog**

### CONCLUSION

### ACKNOWLEDGMENT

### BIBLIOGRAPHY
DEDICATION

To my Mother and Father, both of whom deserve a Ph.D. more than I; and to Adrienne, who made life bearable.
INTRODUCTION

At the present time vitamin B₆ research is at a very interesting stage of its evolution: the knowledge of the coenzyme is sufficiently extensive to use as a probe for the elucidation of the mechanisms of vitamin B₆-dependent enzymes. This extensive knowledge is the result of almost forty years of investigation in which the great importance and elegant simplicity of this vitamin's function have gradually become clear.

It is known that vitamin B₆ occupies a central role in intermediary metabolism. Its function is to mediate transformations involving amino acids. It catalyses the transfer of nitrogen (transamination), promotes the alteration of other functional groups (decarboxylation, eliminations) and alters the stereochemistry (racemization) of amino acids.

Vitamin B₆ can be removed from enzymes, and it and its analogs can be shown to catalyze, in model systems, all of the reactions of which the holoenzymes are capable, albeit at a significantly slower rate and with lower yields (which implies that the mechanisms are not identical).

A knowledge of the metabolic role and the model system behavior of B₆ led to the Braunstein-Snell hypothesis, a detailed description of the mechanism of coenzymic catalysis which is consistent with all of the experimental data.
In view of the definitive mechanistic knowledge of the model systems, and the demonstrable similarity of them to the enzymic systems, it should be fruitful to draw analogies between these two systems in order to simplify the elucidation of the enzymic mechanisms. How valuable these analogies turn out to be is dependent upon the ability of the model system analyst to develop even better approximations of the enzymic situation, primarily through the use of increasingly sophisticated $B_6$ analogs.
HISTORY

Discovery of pyridoxol

Vitamin B₆ research began in 1934 with the recognition of a nutritional factor of the vitamin B complex, distinct from vitamins B₁ and B₂, which prevented a certain dermatitis in rats (György, 1934). Reasonably pure crystals of the vitamin were isolated from rice bran 4 years later (Keresztesy and Stevens, 1938a).

Proof of structure of pyridoxol

Availability of the pure compound quickly led to the elucidation of the structure (Figure 1). Briefly, the evidence for the structure, a combination of results from two laboratories (Keresztesy and Stevens, 1938b; Kuhn and Wendt, 1939; Kuhn et al., 1939a,b), was: The free base possessed an empirical formula C₁₇H₂₁NO₃. Titration showed that this was also the molecular formula. The ultraviolet spectrum revealed an aromatic substance with two ionizable groups; a red-brown ferric chloride test result suggested a 3-hydroxypridine.

Mild oxidation of the methyl ether of the vitamin gave a lactone and a dibasic acid. The stoichiometry of the oxidation and the lactone formation indicated vic-hydroxymethyl groups. Neither of the groups was in the α-position as shown by the ferric chloride test. Thus all groups had been positioned except the methyl. A mixture melting point determination of the oxidation product and 3-methoxy-2-methyl-4,5-
dihydroxymethyl pyridinecarboxylic acid assigned the methyl group to the 2-position.

**Synthesis of pyridoxol**

The complete synthesis of vitamin B₆ was described by Harris and Folkers (1939) (Figure 1). An interesting feature of the synthesis is the necessity of reducing the nitro and nitrile groups sequentially even though the reaction conditions are similar. The synthesis is, of course, of historical interest only. It has been supplanted by a number of more sophisticated routes, an example of which is shown in Figure 2 (Harris et al., 1962).

**Other compounds with vitamin B₆ activity**

Although the structure of pyridoxol was firmly established by 1942, it was clear from the work of Snell et al. (1942) that it was not the sole naturally-occurring substance which possessed vitamin B₆ activity. Indeed, "pseudopyridoxine" seemed to be significantly more potent than pyridoxol when tested on lactic acid bacteria.

Snell observed that pyridoxol could be made more active by subjecting it to conditions expected to cause amination or oxidation (Snell, 1944). He correctly inferred that an interconvertible amine and aldehyde were involved.

Using these suggestions, and the key intermediate 4-methoxypyridoxol (19, Figure 3) (Harris, 1940), the research
Figure 1. First complete synthesis of pyridoxol hydrochloride (Harris and Folkes, 1939)
Figure 2. A modern pyridoxol synthesis (Harris et al., 1962)
Figure 3. Synthesis of pyridoxal, pyridoxamine and isopyridoxamine (Harris et al., 1944a)
1) $\text{HONO}$

2) ethyl acetat

$\text{H}_2/\text{Pt}$

$\text{NaOCH}_3$

$\text{NH}_3$

$\text{KMN}_4$

$\text{NH}_2\text{OH}$

$\text{NH}_3$

$\text{NH}_3$

$\text{H}^+ \text{Cl}^-$
staff at Merck and Company synthesized the 4-aldehyde (pyridoxal, \(^1\)) and the 4- and 5-amine derivatives (pyridoxamine, \(^2\), and isopyridoxamine, \(^3\), respectively) (Harris et al., 1944a). Biological assay showed that only the 4-compounds were active. Somewhat later the 5-aldehyde (isopyridoxal) was synthesized (Harris et al., 1944b) and found inactive (Snell and Rannefeld, 1945), as expected. Thus pyridoxal and pyridoxamine must also be considered vitamin B\(_6\). Yet the catalog of compounds possessing vitamin B\(_6\) activity was not complete.

It was known that vitamin B\(_6\) was required by tyrosine decarboxylase (Gunsalus and Bellamy, 1944). But when pyridoxal was added to a dried cell enzyme preparation only minor re-activation occurred. A very substantial synergistic effect was observed, however, when both pyridoxal and adenosine triphosphate were added. Crude synthesis of an active phosphorylated derivative of pyridoxal, using thionyl chloride and silver dihydrogen phosphate, suggested that the actual coenzyme was pyridoxal-5'-phosphate ("codecarboxylase", \(^4\), Figure 4a) (Gunsalus et al., 1944).

The structure of codecarboxylase was established (Heyl et al., 1951) by a synthetic method which was not a feasible preparative route. A later method circumvented the generally unsatisfactory direct phosphorylation of pyridoxal or oxidation of pyridoxol phosphate by phosphorylating pyridoxamine. A
Figure 4a. Synthesis of pyridoxal phosphate via pyridoxamine phosphate (Wilson and Harris, 1951)

Figure 4b. A modern method of pyridoxal phosphate synthesis (Iwanami et al., 1968)
\[ \text{Cl}^+\text{H}_3\text{N}^+ \xrightarrow{\text{H}_2\text{PO}_4 + \text{P}_2\text{O}_5} \text{H}_2\text{N}^+ \xrightarrow{\text{O}_2 \text{charcoal}} \text{O} - \text{P} - \text{OH} \xrightarrow{1\text{N HCl}} \text{O} - \text{P} - \text{OH} \]
unique oxidation, in which pyridoxamine phosphate (24) and atmospheric oxygen were co-adsorbed on charcoal, was used to obtain pyridoxal phosphate (Wilson and Harris, 1951, Figure 4a). An excellent modern method for the preparation of pyridoxal phosphate utilizes para-toluidine for protection of the aldehyde during phosphorylation (Iwanami et al., 1968, Figure 4b).

At the present time it is believed that vitamin B\textsubscript{6} activity is confined to pyridoxol, pyridoxal, pyridoxamine, and their 5'-phosphates. A concise review of the proofs of structure is available (Wibaut, 1953).

It is interesting to note that the biosynthesis of B\textsubscript{6} has remained entirely obscure until quite recently (Hill and Spenser, 1970) due to the difficulty of identifying the small quantities of precursors present in normal organisms.

**Metabolic importance of vitamin B\textsubscript{6}**

Concurrently with the determination of the various vitamin B\textsubscript{6} structures, examination of the biological roles of these compounds was taking place. As was pointed out previously, the first enzyme which was found to require pyridoxal phosphate was tyrosine decarboxylase from Streptococcus faecalis (Gunsalus et al., 1944). By the time the first transaminases were shown to be pyridoxal phosphate-requiring (Lichstein et al., 1945), a large number of decarboxylases were known.
Gradually biochemists were discovering the diversity and importance of this coenzyme (Snell, 1958). There was steadily increasing incentive to ascertain the mechanism of action of vitamin B₆—specifically pyridoxal- and pyridoxamine-phosphate.

**Model systems**

The obvious approach to the problem was to study the least complex system possible in the hopes of gleaning data which could then be applied to more complex systems—in other words, to study model systems. Although there is always an inherent danger of reducing the complexity of biological systems to such an extent that the results are no longer relevant, there was reason to believe that this would not be the case with vitamin B₆ models because Snell (1945) was able to demonstrate reversible transamination with pyridoxal and pyridoxamine. Eventually, highly refined model systems and analytical methods were developed for the study of transamination, in particular (Metzler and Snell, 1952). One significant finding resulting from early work on model systems was a rate enhancement caused by some metallic ions (Metzler and Snell, 1952) due to their ability to form complexes—some of which were isolable (Baddiley, 1952)—with pyridoxal-amino acid imines.

**The Braunstein-Snell hypothesis**

The results from enzymic and model systems enabled Braunstein and Shemyakin (1953) in the Soviet Union
and Metzler, Ikawa, and Snell (1954a) in the United States to independently propose a detailed mechanism of catalysis by pyridoxal and pyridoxamine. This proposal, with slight modifications, is now regarded as the fundamental tenet of vitamin B₆ research. The Braunstein-Snell hypothesis is thoroughly discussed in a number of works (Guirard and Snell, 1964; Bruice and Benkovic, 1966, Ch. 8; Jencks, 1969, p 133). A modified mechanism, which, as will be pointed out later, is more appropriate for the enzymic model, is presented here (Figure 5).

At approximately physiological pH the coenzyme has the zwitterionic structure. The amine group on the amino acid is protonated. It is clear that a protonated amine is not particularly nucleophilic, so it has been postulated that the phenolate anion may aid in neutralizing the positive charge in the manner shown. In any event, the nitrogen does act as a nucleophile as it attacks the carbonyl carbon to give the unstable intermediate. undergoes a prototropic shift from nitrogen to oxygen to produce the carbinolamine. The nitrogen probably regains the proton lost during the nucleophilic attack at this time, so the net effect is a shift of a proton from one oxygen to another. Dehydration of the carbinolamine, yields an aldimine (Schiff's base) which is hydrogen-bonded. At this point the coenzyme is poised to fulfill its function as an electron sink, and at this point, too, the reaction pathways begin to diverge. Any of the bonds
Figure 5. Simplified mechanism of vitamin B₆ catalysis
to the 2-C of the amino acid except the C-N bond may now be labilized; if decarboxylation is going to occur, the 1,2-C bond is broken to give \(31\), if side-chain elimination is going to take place, the 2,3-C bond is broken to give \(32\), and if 2,3- or 3,4-elimination, 3-decarboxylation, racemization, or transamination is going to occur, the 2-C-H bond is broken to give \(33\). Whichever bond is destroyed, the coenzyme assumes the semiquinoid structure. Protonation at 2-C and reversal of the previous steps will now yield the decarboxylated, eliminated, or racemized product. Protonation at the former carbonyl carbon and subsequent decomposition of the ketimine, \(34\), results in a 2-ketoacid and a pyridoxamine-type compound—products of transamination—or 3-decarboxylation, 2,3-elimination, or 3,4-elimination.

What experimental evidence supports this mechanism? Perhaps the most convincing finding is the predictable products resulting from both model systems (Werle and Koch, 1949; Metzler and Snell, 1952; Olivard et al., 1952; Metzler et al., 1954b) and enzymes (Baddiley and Gale, 1945; Meister and Tice, 1950; Cammarata and Cohen, 1950; Feldman and Gunsalus, 1950).

Model system studies of non-pyridyl systems (Ikawa and Snell, 1954) and the intrinsic reasonableness of the mechanism as based on inferences from well-studied organic systems (Bruice and Benkovic, 1966, Ch. 8) lend considerable credence to the hypothesis.
Quantum-mechanical calculations confirm the salient features of the mechanism (Pullman, 1963).
OVERVIEW

Instrumental analysis of model systems

Instrumental analysis of model systems has until recently been confined to spectrophotometric studies (Metzler and Snell, 1952). Spectrophotometric analysis has played an exceptionally important role in determining the participating species in pyridoxal catalysis because of the high informational content of the pyridine derivative spectra. Very precise data on the pK's and individual ionic forms have been made possible by digital readout-computer analysis techniques developed by Metzler's group (Nagano and Metzler, 1967; Johnson and Metzler, 1970).

Infrared studies on pyridoxal phosphate (Anderson and Martell, 1964) have been valuable in confirming data produced by other methods, but the experimental difficulties involved in aqueous infrared studies have limited the amount of data available from this source.

X-ray crystallography has found some usefulness (Fujiwara and Tomita, 1969).

Recently, nuclear magnetic resonance has been employed in the analysis of Schiff's base formation in pyridoxal systems (Gansow and Holm, 1968; Abbott and Martell, 1969, 1970).

Model systems vs. enzymic systems

No matter how elegant model systems become, their true worth should be evaluated in how closely they approximate
biological conditions. Model systems are merely tools which simplify our analyses of enzymes.

At the present time there is little reason to doubt that the Braunstein-Snell hypothesis will be the basis for the understanding of the mechanism of vitamin $B_6$-dependent enzymes. But it is equally true that the systems and knowledge upon which that hypothesis was based differ somewhat from the enzymic systems. Thus, modifications, not really of the hypothesis, but rather the examples which are used to describe the hypothesis, have been necessary to reconcile the putative hypothesis with experimental fact.

That there are major differences between enzymic and non-enzymic catalysis is apparent from the comparison of rates or selectivity. Vernon (1964) has estimated transamination proceeds $10^9$ times as fast with glutamic-aspartic transaminase as with a model system. And when provided the correct substrate, the enzyme is immeasurably more accurate in producing the correct product. Discrepancies this large demand that the pathways of the two reactions be significantly different—different in the sense that the enzymic pathway is an enhanced version of the non-enzymic.

Even though the mechanism of reactions mediated by vitamin $B_6$ has been known for almost twenty years, not one enzymic mechanism has yet been elucidated. This is not too surprising, considering the complexity of enzymic systems, but it is discouraging from the point of view that so much excellent
research has been done with no sign of an imminent breakthrough.

What little is known about the active sites of vitamin B$_6$-dependent enzymes is discussed in some detail here because of the usefulness of the information in designing new analogs.

A disappointing finding has been that few pyridoxal phosphate-dependent enzymes require metals (Fasella, 1967). This means that much of the work on model systems must be treated with skepticism inasmuch as the profound effect of the metallic ions upon catalysis cannot be of the same nature as that of the amino acid side chains at the active site. A reversible change in the pH dependence of the electronic spectrum of some enzymes after prolonged dialysis suggests that perhaps an anion is present at the active site (Morino et al., 1966; Fasella, 1966).

The active site of vitamin B$_6$-dependent enzymes

Certainly the most definitive finding regarding the structure of the active site (Figure 6a) is the identification of a covalent bond between pyridoxal phosphate and the enzymes requiring it. Jenkins and Sizer (1957) were the first to suggest the existence of an imine form of pyridoxal phosphate (in aspartate aminotransferase). Their suggestion was based on the similarity of the electronic spectra of the enzyme and those of the model system imines (Metzler, 1957).
It had been shown, in the case of model systems, that pyridoxal (and hence its phosphate) has an absorption at about 25.6 kK. This is a significantly lower energy band than that produced by pyridine systems which do not possess an exocyclic double bond (greater than 30.3 kK), but not as low as the band observed in the enzymic system (23.3 kK) in slightly acidic conditions. On the other hand, Metzler (1957) showed that the pyridoxal-valine imine absorbs at 24.2 kK around neutrality, which is only 0.9 kK from the enzymic system. A further similarity between the systems is the absorptions in basic conditions (27.2 kK for the pyridoxal-valine imine and 27.5 kK for the aspartate aminotransferase).

The existence of the imine was unequivocally demonstrated (Fischer et al., 1958) in the case of muscle phosphorylase by reduction of the imine with sodium borohydride and isolation of the stabilized product. In this manner it was shown that pyridoxal phosphate is bound by its carbonyl group to the ε-amino group of a lysyl residue (Figure 6a). Subsequently this same linkage has been shown to exist in at least nine enzymes (Fasella, 1967), including aspartate aminotransferase (Hughes et al., 1962).

The presence of an aldimine rather than aldehyde at the active site alters the mechanistic picture to some extent. The aldimine and aldehyde functions are electronically and stereochemically similar, but the imine is quite a bit more
Figure 6a. A speculative diagram of the active site of aspartate aminotransferase (after Ivanov and Karpeisky, 1969)

Figure 6b. Dunathan's (1966) proposal of reaction specificity determined by conformation. The bar indicates the pyridine ring plane viewed from the 2-carbon of the R-amino acid to the imine nitrogen. E represents the enzyme with the carboxylate binding site attached. The wavy line locates the bond fission which leads to decarboxylation (35a), racemization or transamination (35b), or 2,3-elimination (35c)
susceptible to nucleophilic attack by an amine (transaldimina-
tion) as has been shown by kinetic studies on analogous systems
(Jencks and Cordes, 1963).

Here, then is one point where the increased efficiency of
the enzyme can be rationalized in a very satisfactory manner.
But transaldimination cannot be the entire explanation for the
difference in activity of the artificial and natural systems
because it is not the rate-determining step.

If the enzyme containing the borohydride-treated imine is
subjected to enzymic degradation, the peptide sequence adjacent
to the lysyl residue can be established (Table 1). This has
been done for rabbit muscle phosphorylase (Nolan et al., 1964),
both the mitochondrial and extramitochondrial aspartate
aminotransferases from pig heart (Morino and Watanabe, 1969),
Escherichia coli glutamic acid decarboxylase (Strausbauch and
Fischer, 1970), and Escherichia coli tryptophanase (Kagamiyama
et al., 1970). There appears to be little similarity among the
sequences. Whether this reflects a lack of participation of
adjacent groups or implies instead a multitude of mechanisms is
not known, but the former possibility seems more likely at the
present time. This implies, of course, that secondary and
tertiary structure are significant in the maintenance of the
active site.
Table 1. Primary sequence adjacent to the pyridoxal phosphate-lysyl residue in some enzymes

<table>
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<th>Enzyme Type and Source</th>
<th>Sequence Details</th>
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<tbody>
<tr>
<td><strong>Escherichia coli glutamic acid decarboxylase</strong> (Strausbauch and Fischer, 1970)</td>
<td>Ser-Ileu-Ser-Ala-Ser-Gly-His-(PLP)Lys-Phe</td>
</tr>
</tbody>
</table>
| **pig heart muscle aspartate aminotransferase** (Morino and Watanabe, 1969) | extramitochondrial Ser-(PLP)Lys-Asn-Phe  
mitochondrial Ala-(PLP)Lys-Asn-Met |
| **rabbit muscle phosphorylase b/a** (Nolan et al., 1964) | (PLP)Lys-Phe |
| **Escherichia coli tryptophanase** (Kagamiyama et al., 1970) | Ser-Ala-Lys-(PLP)Lys-Asp-Ala-Met-Val-Pro-Met |
The fact that only the reduced pyridoxal phosphate-lysine residue bond remains intact during hydrolysis does not prove that only one covalent bond exists between the coenzyme and apoenzyme. But the fact that the pyridoxamine phosphate form of the enzymes is significantly easier to resolve than aldimine form suggests that, at very least, the imine bond is the strongest interaction.

Electrostatic forces are thought to be responsible for the binding of the phosphate to the apoenzyme (Figure 6a). Coenzyme analogs which have a negatively-charged group in place of the phosphate often bind to the apoenzyme (Hullar, 1969; Furbish et al., 1969). Inorganic phosphate competes with pyridoxal phosphate for the binding site of pig heart apoaspartate aminotransferase (Banks et al., 1968) and rat kidney kyneurenine aminotransferase (Mason, 1957), but the phenomenon is not general.

The effect upon binding of modifications in the 2-position of pyridoxal phosphate has been studied (Mühlradt et al., 1967; Ivanov and Karpeisky, 1969). The compound in which the 2-methyl is replaced by hydrogen is a more effective coenzyme than the natural one in tryptophanase and aspartate aminotransferase. In general, as the 2-group increases in bulkiness (hydrogen, methyl, ethyl, n-butyl) the binding becomes weaker. It can therefore be surmised that, at least in these two enzymes, some portion of the apoenzyme is in proximity to the
2-position of the coenzyme (Figure 6a).

It is generally assumed that some type of basic functional group on the apoenzyme is juxtaposed with the pyridinium nitrogen so that a proton may be shifted from one to the other readily.

Ivanov and Karpeisky (1969) state that the phenolic group is bound electrostatically to a group on the protein. They point out that N-methylation, which places a permanent positive charge on the nitrogen, does not lower the pK of the phenol to the extent observed in the enzyme (8.0 vs. 6.2).

The existence and approximate location of two binding sites complementary to the two carboxylate groups of the substrate can be inferred from binding studies with dicarboxylic acids and aspartate aminotransferase (Jenkins and Sizer, 1957; Jenkins et al., 1959).

An imidazole group has been implicated in the enzymic catalysis because selective photolysis of a histidyl residue destroys activity (Peterson and Martinez-Carrion, 1970). Should the presence of a histidyl residue at the active site be definitely proven, it would be a very important discovery; imidazole has been shown to catalyze model system reactions (Bruice and Topping, 1963). In fact, it intervenes at the rate-determining aldimine-ketimine tautomerization step (Figure 5, 30, 33, 34) so it could be of central importance in the enzymic mechanism.
The structure of the active site is thought to include hydrogen bonding between a proton on the imine nitrogen and the phenolate group by analogy with model systems (Metzler, 1957; Heinert and Martell, 1963; Martell, 1963). In any event, only three other conformations of the N-substituted imine are possible due to the requirement of maximum \( \pi \)-orbital overlap between the imine and the pyridine ring. Steric hindrance probably prevents perfect planarity from being achieved in the anti-cisoid case (because of \( R \), phenolate interaction) and the anti-transoid case (because of \( R \)-interaction with 5-substituents on the pyridine ring, for example, the 5-methylenephosphate of pyridoxal phosphate). All possibilities are shown in Figure 7. Note that only the syn-cisoid conformation involves a stabilizing hydrogen bond.

The requirement of maximum overlap also demands that the bond being formed or broken during catalysis by vitamin \( B_6 \) be oriented along the \( \pi \)-electron system (Dunathan, 1966). This positioning would place the two uninvolved groups of the amino acid outside of the extended pyridine ring plane (Figure 6b). Dunathan (1966) points out that an appropriate out-of-plane positioning of the carboxylate binding group could thus determine which bond was to be broken. This would account for the observed enzymic specificities. Preliminary work tends to confirm this hypothesis (Dunathan et al., 1968).
Figure 7. Possible conformations of the N-substituted imine at the active site

Syn and anti refer to the position of the R group with respect to the C-H hydrogen of the aldimine. Cisoid and transoid refer to the position of the imine with respect to the phenolato group.
An even more elaborate theory, dealing with the dynamic stereochemistry of the active site of aspartate aminotransferase, has been put forth (Ivanov and Karpeisky, 1969). The central feature of this proposal is the tilting of the coenzyme about its 2,5-C axis so that various catalytic groups on the apo-enzyme are available at the appropriate steps of the catalytic sequence. Unfortunately, the experimental data which supports this hypothesis is not of a particularly reliable nature (circular dichroism studies) and furthermore there are alternate interpretations of even that meager evidence (Martinez-Carrion et al., 1970).

Although it has been shown that 3-hydroxy-4-pyridine-carboxaldehyde, and even 4-nitro-2-hydroxybenzaldehyde, possess the minimum structural requirements for transamination in model systems (Ikawa and Snell, 1954), they do not begin to duplicate the complexity of the active site.

In summary, many groups have been implicated, in a more or less decisive manner, as playing some role in catalysis (Figure 6a). Of these, the imine bond between pyridoxal phosphate and the ε-amino group of a lysyl residue is at present the most significant.

Organic synthesis of analogs

Organic synthesis of vitamin B₆ analogs has been prodigious, encouraged perhaps by the firm mechanistic theory upon which novel structures may be planned and by the central
and ubiquitous metabolic roles which B₆ occupies. B₆ analogs are currently being synthesized for pharmaceutical evaluation, enzyme studies, and model system use. No matter what the purpose, the central theme of the syntheses has been the retention of desirable and modification of the undesirable attributes of the molecule.

One of the tenets of a synthesis of B₆ analogs is that they start with pyridoxol. The reasons for this are straightforward: it is inexpensive and yet has most of the structural features of the coenzyme forms. In a very few cases (labeling, modification of unreactive groups) it has been desirable to begin with aliphatic precursors (Osbond, 1964; Mühlradt et al., 1967; Doktorova et al., 1969).

For the majority of syntheses using pyridoxol as the starting material the 5-position is modified. It was pointed out previously that the 1-, 3-, and 4-groups of pyridoxal are necessary for a "minimum structure" (Guirard and Snell, 1964) while the 2- and 6-positions are too unreactive for most synthetic purposes. On the other hand, the 5-hydroxymethyl group of pyridoxal is dysfunctional; it interferes with the catalytic ability of the 4-aldehyde position by formation of a hemiacetal (18b, Figure 3) (Guirard and Snell, 1964). Thus the 5-position is a logical and frequent choice for modification.
An obvious problem in using pyridoxol as a starting material is the almost identical reactivities of the 4- and 5-hydroxymethyl side chains. The ideal protective group would protect the 4-position and leave the 5-position free. The first synthetic route used for this purpose gave the 4-methoxy-methyl derivative of pyridoxol in 12% yield (Harris, 1940). The contemporary method of protection is the isopropylidene attachment on the 3- and 4-positions (Korytnyk and Wiedeman, 1962; Iwata, 1968). This group is stable in aqueous base but can be hydrolyzed completely in 1N hydrochloric acid at 100° for 15 min. It is noteworthy that the formation of the 3,4 derivative is critically dependent on the concentration of the hydrogen chloride gas in the acetone solvent. If the concentration is low, the 4,5-isopropylidene, a seven-membered cyclic ketal, is formed instead (Korytnyk, 1962).

After formation of the 3,4 isopropylidene derivative, the 5-position is usually activated by forming the chloro (Iwata, 1968) or carbonyl (Brooks, 1961, p 51; Korytnyk et al., 1964) group.

Reduction of 5'-chloro isopropylidene pyridoxol gives access to the 5-deoxy series of pyridoxol derivatives which have been used extensively in model system studies. 5-Deoxypyridoxal and 5-deoxypyridoxamine, as well as 5-deoxypyridoxol, are most reliably synthesized by this route.
The 5-side chain can be elongated by one carbon unit by reaction of the chloro derivative with an alkali metal cyanide in acetone-water (Tomita et al., 1966) or dimethyl sulfoxide (Korytnyk et al., 1967) of which the latter method is superior. The isopropylidene isopyridoxal can be condensed with nitromethane (Nef reaction) to yield the same carbon chain alteration (Korytnyk et al., 1967).

Two-carbon elongations are accomplished by Knoevenagel condensations (Korytnyk, 1965) or malonic ester syntheses (Tomita et al., 1966).

A synthetic difficulty which has not been solved as elegantly as selective protection occurs when it is desired to convert the deprotected pyridoxol derivative to pyridoxal. Most oxidizing agents over-oxidize to the pyridoxic acid analog because aldehydes are more easily oxidized than alcohols. Manganese dioxide is the only agent in common use which gives the aldehyde predominantly, although pyridoxic acid is formed to some extent even with this reagent (Brooks, 1961, p 68). Manganese dioxide is not an ideal reagent: it must be prepared periodically, it is unreliable in its action, it is difficult to manipulate. Furthermore, the lack of knowledge about its mode of oxidation makes planning or modifying an oxidation using it quite treacherous.

Manganese dioxide prepared by various methods can be either reasonably active or totally inactive toward a certain type of compound and have a totally different activity toward
another. Currently, the variation in activity among the types ("regular", "A", and "B") of manganese dioxide is ascribed to trace impurities which act as catalytic centers for the surface reactions (Evans, 1959). The fact that the reaction is heterogeneous and the impurities are immeasurably sparse has hampered mechanistic investigations (Evans, 1959).

Physical techniques of analysis other than uv spectroscopy have just recently come into use in vitamin B₆ chemistry but are now considered essential by the organic chemist. The pioneering work in nmr was done by Korytnyk; many of his publications contain extensive tables of the nmr spectra of derivatives (Korytnyk and Singh, 1963; Korytnyk et al., 1964; Korytnyk, 1965; Korytnyk et al., 1967) and deuterated derivatives (Korytnyk and Paul, 1965). Turchin et al. (1966) have also published an extensive nmr tabulation.

Mass spectrometry has great potential in the study of vitamin B₆ and its derivatives (DeJongh et al., 1966, 1968).

Chromatographic data being produced now will most certainly aid in the elucidation of vitamin B₆ metabolism. Investigations have been conducted utilizing thin layer chromatography (Dement'eva et al., 1968), thin layer chromatography and thin layer electrophoresis (Ahrens and Korytnyk, 1969), gas chromatography (Korytnyk et al., 1966; Prosser et al., 1967) and a combination of gas chromatography and mass
spectrometry (Richter et al., 1967). A tabulation of uv, ir, nmr, mp, tlc and other data has recently been compiled (Doktorova et al., 1969). Future syntheses in this area will involve the use of sophisticated protective groups, as presaged by the modification of the 4-position of pyridoxol (Korytnyk and Paul, 1970) and the synthesis of pyridoxdial (Sattsangi and Argoudelis, 1968). A simple method for protecting the aldehyde of pyridoxal would be a very desirable synthetic development. Such a method, reported recently in the literature (Prosser et al., 1967) has since been refuted (Paul and Korytnyk, 1969).
EXPERIMENTATION

Instrumental analysis

Routine nuclear magnetic resonance (nmr) spectra were obtained on either the Varian A-60 or the Hitachi-Perkin Elmer R-20B. High resolution spectra and decoupling experiments were done on the Varian HA-100. All samples were run at probe temperature (~ 40°). Chemical shifts are reported as parts per million downfield (δ) from tetramethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, Tiers' salt); the apparent coupling constant (J) is given in Hz. The notations: s, d, t, b, and vb mean singlet, doublet, triplet, broad, and very broad, respectively. In the one case in which the pD of the sample was adjusted by addition of concentrated sodium deuteride solution, the pD value was determined by adding 0.4 to the meter reading of a pH meter calibrated in the usual way (Lumry, Smith and Glantz, 1951). The sodium deuteroxide solution was prepared by cautious addition of metallic sodium to deuterium oxide. All mass spectra (ms) were obtained at 70eV using the solids inlet of the Perkin Elmer 270. The probe temperature was recorded during each run. The base peak is taken as the highest peak with m/e above 100 except in a few cases where other major peaks could be identified. The relative abundance (RA) of other species is tabulated as percentage of the base peak intensity. Reported peaks are at least twice the height of unreported peaks. The notations:
$M^+$ and $M^*$ indicate molecular ion and metastable ion, respectively. Replaceable hydrogen positions were labeled by repeatedly dissolving the compound in deuterium oxide and evaporating the solvent under reduced pressure.

Infrared (ir) spectra were obtained from potassium bromide pellets on the Perkin Elmer 21. Data are reported in kaysers ($1K = 1cm^{-1}$). The notations: s, m, w, b, vb, and d stand for strong, moderate, weak, broad, very broad, and doublet, respectively; these notations should be considered subjective.

Ultraviolet-visible (uv) spectra were measured on a Cary 15 or 1501, both equipped with Datex digital output. Spectra were obtained at room temperature, in 1 cm path length quartz cuvets, with the solvent as reference. Spectrophotometry was used in conjunction with a Corning 12 pH meter to establish the pH dependence of spectra. From these data the apparent pK's, and spectra of the pure ionic forms, were calculated by computer (Nagano and Metzler, 1967). Absorption maxima are reported in kilokaysers ($1kK = 10^4$/millimicrons or nanometers).

Elemental analyses (ea) were performed by Galbraith Laboratories Knoxville, Tennessee.

Melting points (mp) were determined on a hot stage microscope or a Thomas-Hoover oil bath, and are uncorrected.

Thin-layer chromatography (tlc) was done on 250 μ x 20 cm x 20 cm layers of silica gel H. Results are reported as the ratio of the distance of sample migration to solvent-front migration (Rf). Butanol - acetic acid - water (BAW) is the
top layer of a mixture 4 parts butanol to 1 part acetic acid to 5 parts water, by volume.

High-voltage electrophoresis (hve) was done on a Gilson Model D using Whatman No. 3 paper and pyridine--acetic acid--water buffer (1.0:3.4:409), pH 4.0 at 2 kV, for 1.0 hr. Migrations are reported as distances from the origin toward the cathode.

Compounds subjected to tlc or hve were detected in the following ways: A Transilluminator (a source of long-wave-length ultraviolet light) was used to detect fluorescence. The Gibbs test (Gibbs, 1927) (0.1 g of N,2,6-trichloro-p-benzoquinoneimine/100 ml benzene applied by spray, then sprayed with ammonium hydroxide vapor) detects the phenolic group para to an aromatic hydrogen by producing a blue spot. Isopropylidene derivatives do not react unless hydrolyzed (sprayed with hydrochloric acid vapor and placed in a 110° oven for ~ 0.25 hr) prior to application of Gibbs reagent. The ninhydrin test (0.4 g of 1,2,3-indantrione/500 ml of 95% ethanol applied by spray, then placed in a 110° oven for 0.25 hr) detects amines by producing an orange or pink spot. Compounds listed in Table 5 gave the expected results with ninhydrin and Gibbs reagents.

The ea, nmr, ms, ir, and uv data for each compound are tabulated immediately after its preparation. Additional properties (mp, pK, tlc, hve) for all compounds are assembled in Table 5, p 83.
Manganese dioxide "B"  The following procedure is a modification of that published by Mancera, Rosenkranz, and Sondheimer (1953). A 3-liter, 3-necked round bottom flask was equipped with a dropping funnel, thermometer, heating mantle, and mechanical stirrer. Manganous sulfate monohydrate (60 g) was added to ~ 1 liter of water in the flask and the solution was heated to 90°. Small portions of potassium permanganate were dissolved in boiling water, transferred to the dropping funnel, and rapidly added to the stirred manganese sulfate solution. A total of 60 g of potassium permanganate in ~ 1.5 liters of water was added.

One-half hour after the addition of the last of the permanganate, heating was discontinued and the mixture was allowed to cool to ~ 60°. The mixture was filtered through a large, medium porosity sintered glass funnel and the black precipitate was washed with ~ 0.5 liter of boiling water. As much water as possible was extracted by vacuum. The precipitate was removed from the funnel in as few pieces as possible, air dried overnight, and stored in a 110° oven. The cakes were pulverized just before use. When stored in this manner, the manganese dioxide retains its activity indefinitely.  

Note: manganese dioxide stains may be removed by a warm, saturated aqueous solution of sodium bisulfite.
Synthesis of analogs lacking the imine ring (Figure 8)

2,5-Dimethyl-3-hydroxy-4-pyridinecarboxaldehyde oxime (5-deoxypyridoxal oxime) (41)  2,5-Dimethyl-3-hydroxy-4-pyridinecarboxaldehyde (5-deoxypyridoxal, 40) (Mühlradt and Snell, 1967; Iwata, 1968) (0.76 g, 5 mmol) was dissolved in 10 ml of 95% ethanol. The solution was magnetically stirred while a solution of 0.45 g (5.5 mmol) of sodium acetate and 0.38 g (5.5 mmol) of hydroxylamine hydrochloride in 5 ml of water was added. A white precipitate formed almost immediately. When the precipitation was complete (~10 min) the mixture was filtered with suction and the precipitate was dried over calcium chloride, under vacuum. The yield was 0.74 g (89%).

The analytical sample was recrystallized from 95% ethanol.

\[ \text{ea} \ (C_{8}H_{10}O_{2}N): C, 58.05 (57.82) ; H, 6.16 (6.06) ; N, 16.85 \% (16.86\%) \]

\[ \text{nmr} \ \text{(saturated dimethyl sulfoxide-d}_{6}, \ TMS): \delta 2.33 (s, 6/2; 2- or 5-CH_{3}) , 2.37 (s, 6/2, 2- or 5-CH_{3}) , 7.83 (s, 1, 6-H) , 8.49 (s, 1, -CH=NNOH) , 12.08 ppm (vbs, 1, =NOH); \text{ (acidic deuterium oxide, DSS):} \delta 2.52 (s, 3, 2- or 5-CH_{3}) , 2.67 (s, 3, 2- or 5-CH_{3}) , 8.02 (s, 1, 6-H) , 8.66 ppm (s, 1, -CH=NNOH) \]

\[ \text{ms} \ (70eV, 25^\circ C): m/\text{e166 (RA100, M}^{+}, 149 (53, -OH), 122 (16), 121 (21, 149-CO), 120 (20), 119 (40\%) \]
Figure 8. Synthesis of compounds lacking an imine ring
ir (KBr): ν 2630 (v); 1616 (C=N); 1389 (s); 1325 (m); 1285 (m);
1247 (s); 1080 (m); 1045, 1025 (d); 942; 890 (b); 765, 760 (d);
~ 738, 729 K (bd)

uv (95% ethanol): ν_{max}^{30.7} (ε 4370), 38.1 K (11 800)

4-Aminomethyl-2,5-dimethyl-3-pyridinol dihydrochloride
(5-deoxypyridoxamine dihydrochloride) (42) Finely powdered
5-deoxypyridoxal oxime (41) (0.33 g, 2 mmol) was suspended in
10 ml of 1 N hydrochloric acid by vigorous magnetic stirring.
Zinc dust (0.98 g, 15 mmol) was added in one portion. The
mixture was stirred for 15 min or until the 5-deoxypyridoxal
oxime had completely dissolved. After the mixture had been
filtered through a medium-porosity sintered glass filter and
washed with 5 ml of water, the filtrate was applied to an ion-
exchange column to remove divalent zinc. The column of Dowex
AG 50 W-X8 (7.1 X 2.2 cm) had been previously equilibrated with
2.0 N hydrochloric acid. The zinc was removed by washing the
column with 2.0 N hydrochloric acid (~ 150 ml); the amine was
isolated by raising the eluent normality to 4.0 and pooling all
fractions having significant absorption at 34.0 K K. The
resulting solution was evaporated to dryness under reduced
pressure (50° bath). The solid was recrystallized from
methanol --ether to yield 0.31 g (69%) of the amine.

The column fractions containing divalent zinc were
identified by modification of a standard qualitative test (Yoe,
1938, p 176): 3 drops of sample, 20 drops of concentrated
ammonium hydroxide, and 3 drops of 0.1 M resorcinol in 95%
ethanol were mixed thoroughly and let stand several hours. Zinc produced a blue solution. A blank and a standard were run concurrently.

The amine (42) has previously been synthesized by other methods (Heyl et al., 1953; Kuroda, 1964).

\[
\text{ea} \quad (C_8H_{14}Cl_2N_2O) : C, 42.49(42.68); H, 6.33(6.27); N, 12.35\% (12.44) \\
\text{nmr (deuterium oxide, DSS)} : \delta 2.53(s,3,5-CH_3), 2.71(s,3,2-CH_3), 4.44(s,2,4-CH_2), 8.14 ppm(s,1,6-H) \\
\text{ms (70eV, 25°C)} : m/e 152(RA41, M^+), 135(53, -NH_3), 107(48, 135-CO), \nu 84.7(M^+, 135-107), 66(74, 107-CH_3CN), 38(39, H^3Cl), 36(100%, HCl) \\
\text{ir (KBr)} : \nu 2680(\nu b), 1534, 1486, 1364, 1107, 978, 914, 876, 751K \\
\text{uv (0.1 M phosphate, pH 6.96)} : \nu_{max} 31.0(\varepsilon 8120), 40.2kK(3780)
\]

**Attempted direct synthesis of the 5-membered imine ring analog** (Figure 9)

5-[(Aminomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride (isopyridoxamine dihydrochloride, 22) This procedure is similar to that used by Testa and Fava (1957).

Isopropylidene isopyridoxal oxime (45) (Brooks, Laakso and Metzler, 1966) (0.33 g, 1.8 mmol) was suspended in 4.1 ml of vigorously-stirred glacial acetic acid. Zinc dust (0.55 g, 8.4 mmol) was added in small portions so that the temperature
Figure 9. Attempted direct synthesis of the 5-membered ring imine
of the suspension did not rise above 50°. The suspension was stirred for an additional 0.33 hr after the last portion of zinc dust had been added. The filtrate resulting from suction filtration of the suspension was evaporated under reduced pressure (60° bath) to a yellowish oil. Hydrochloric acid (1 N, 5 ml) was added and the solution was placed on a steam bath for 0.5 hr. The hydrochloric acid solution was cooled and applied to a 8.0 X 2.3 cm Dowex AG 50W-X8 column equilibrated with 2.0 N hydrochloric acid. After the zinc had been washed through the column with 2.0 N hydrochloric acid (see p 44), the desired product was washed from the column with 4.0 N hydrochloric acid. Fractions having absorbance around 34.5 Kk were pooled, evaporated to dryness under reduced pressure (50° bath), and dried under vacuum, over magnesium sulfate. Dissolving the precipitate in warm methanol, treating with Norite, adding diethyl ether, and carefully cooling, yielded 0.29 g (81%) of slightly beige crystals.

\[
\text{ea } (C_{8}H_{14}Cl_{2}N_{2}O_{2}) : C, 39.98(39.85); H, 5.81(5.85); N, 11.67\%(11.62%) \\
\text{nmr (deuterium oxide, DDS)}: \delta 2.73(s, 3,2-CH_{3}), 4.48(s, 2,5-CH_{2}), 5.10(s, 2,4-CH_{2}), 8.36 \text{ ppm(s, 1,6-H)} \\
\text{ms (70eV, 110°C): } m/e 168(\text{RA9, } M^+ 151(100, -\text{NH}_3)) 150(47), 122(53), 94(53\%) \\
\text{ir (KBr): } \tilde{\nu} 2900(\text{s, vb}), 1543, 1486, 1277, 1230, 1055, 962, 88(s), 739 \text{ K(s)}
\]
uv (0.1 M phosphate, pH 7.00): $\nu_{\text{max}} = 30.7(\epsilon5770), 38.9 \text{ kK (4330)}$

6-Methyl-3H-pyrrolo[3,4-c]pyridin-7-ol (46b), attempted by oxidation

Manganese dioxide method 5-(Aminomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride (22) was oxidized in a manner suggested by Brooks (1961, p 36) for pyridoxol: 24 mg of the reactant was dissolved in 10 ml of 1:1 tetrahydrofuran -- water solution. Freshly powdered manganese dioxide (0.10 g) was added and the reaction suspension was vigorously stirred. The reaction was conducted for 1 hr at room temperature and 1 hr at reflux. Monitoring was done every 0.5 hr by observing the uv spectrum in 0.1 M acetate buffer, pH 5.0.

Similarly, the reaction was done in water and in 0.01 N hydrochloric acid.

Alcohol dehydrogenase method A solution of 1 ml of 250 $\mu$M 5-(aminomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride, 1 ml of 100 $\mu$M nicotinamide adenine dinucleotide, 200 $\mu$M triethanolamine buffer (pH 8.0), and 1 mg of either horse liver or yeast alcohol dehydrogenase was prepared and monitored at 27.8 kK. Appropriate blanks were used. No significant change was observed.
Attempted indirect synthesis of the 5-membered imine ring analog (Figure 10)

5-(N-Acetylaminomethyl)-2,2,8-trimethyl-4H-m-dioxino-[4,5-c]pyridine (47) 2,2,8-Trimethyl-4H-m-dioxino[4,5-c]-pyridine-5-carboxaldehyde oxime (isopropylidene isopyridoxal oxime, 45) (Brooks, Laakso and Metzler, 1966) (1.11 g, 5 mmol) was dissolved in a mixture of 20 ml of glacial acetic acid and 5 ml of acetic anhydride. Immediately after the oxime had completely dissolved, 3.27 g (50 mmol) of zinc dust was added to the vigorously-stirred solution. The suspension was allowed to cool to room temperature (~0.33 hr) before stirring was ceased. The suspension was then filtered through a medium porosity sintered glass filter into a stirred mixture of 200 ml of water, 600 ml of crushed ice, and 28.4 g of sodium hydroxide. The precipitate was washed with 5 ml of acetic acid, the filtrate and washing were combined, and the resulting solution was extracted 5 times with 50 ml portions of chloroform. The chloroform layer was dried (Drierite) and evaporated under reduced pressure (50° bath). The oily residue, which contained some solid, was stored overnight at 4°, during which time the desired product crystallized. Drying the crystals over Drierite, under vacuum, overnight gave 0.85 g (68%) of crude product contaminated with a small amount of isopropylidene isopyridoxamine. The crude residue was recrystallized from acetone--diethyl ether to give the analytical sample.
Figure 10. Attempted indirect synthesis of the 5-membered ring imine analog
ea (C₁₃H₁₈N₂O₃): C, 62.31 (62.38); H, 7.37 (7.25); N, 11.02% (11.19%)

nmr (v 0.1 N deuterium chloride, pD1.0, DSS): δ1.66 (s, 6, (CH₃)₂C=), 2.11 (s, 3, CH₃C=O), 2.57 (s, 3, 2-CH₃), 4.38 (s, 2, 5-CH₂), 5.10 (s, 2, 4-CH₂), 8.04 ppm (vbs, 1, 6-H);
(chloroform-d₃, TMS): δ1.54 (s, 6, (CH₃)₂C=), 1.99 (s, 3, CH₃C=O), 2.33 (bs, 3, 2-CH₃), 4.23 (d, 2, J=5.5 Hz, 5-CH₂), 4.80 (s, 2, 4-CH₂), 6.83 ppm (vbs, 1, NH)

ms (70eV, 25°C): m/e250 (RA13, M⁺), 192 (67,-acetone), 151 (53, 192-acetyl), 150 (100%, 192-ketene), ν 117.4 (M⁺, 192-150)

ir (KBr): 3205, 3030, 1661 (C=O), 1555, 1412, 1376, 1280, 1255, 1217, 1139, 1089, 1060, 946, 863, 791, 769, 687, 664

uv (95% ethanol): νmax 35.5kK (e5830)

5-(N-Acetylaminomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol hydrochloride (48) 5-(N-Acetylaminomethyl)-2,2,8-trimethyl-4H-2,3-dioxino[4,5-c]pyridine (47) (1.25 g, 5 mmol) was dissolved in 10 ml of 1.0 N hydrochloric acid and kept at 27.5°. To determine the progress of the hydrolysis, 10 microliter samples were periodically removed from the reaction solution, diluted to 50 ml with 0.1 M phosphate buffer (pH = 7.0), and examined at 25.7 kK. At 40 hr the hydrolysis of the isopropylidene moiety was 99% complete. Thin layer chromatography and ninhydrin analysis of the reaction solution revealed only a negligible amount of
ninhydrin-positive compound. The reaction solution was evaporated under reduced pressure (30° bath) to an oil which crystallized on standing. Recrystallization from anhydrous methanol and drying (Drierite, vacuum) gave 1.04 g (85%) of the desired material. Mathematical analysis of the experimental data gave a half-life, $t$, equal to $5.9 \pm 0.3$ hr and a pseudo first-order rate constant, $k_1$, of $0.12 \pm 0.01$ hr$^{-1}$.

\[
eq (C_{10}H_{15}CLN_2O_3): \text{C, 48.90 (48.69); H, 6.21 (6.13); N, 11.34% (11.36)}
\]

nmr (deuterium oxide, DDS): \(\delta 2.11 (s, 3, \text{CH}_3\text{C}=0), 2.68 (s, 2, 2-\text{CH}_3), 4.54 (s, 2, 5-\text{CH}_2), 5.03 (s, 2, 4-\text{CH}_2), 8.08 \text{ ppm (s, 1, 6-H)}\)

ms (70eV, 25°C): \(m/z 210 (\text{RA}43, M^+), 192 (80, -\text{water}), 151 (92, -\text{acetamide}), 150 (100, 192-\text{ketene}), 149 (99, 192-\text{acetyl}), 139 (50), 123 (64), 122 (87), 60 (47, \text{CH}_3\text{C}(=0)\text{NH}_2^+), 43 (88\%), \text{CH}_3\text{C}=0^+\)

ir (KBr): \(3185, 3049, 2703, 2584, 2008 (w), 1623 (s), 1285 (s), 1220 (s), 1099, 1031, 923 (w), 897 (m), 779, 671, 647 K\)

uv (0.1N hydrochloric acid): \(\nu_{\text{max}} = 34.3 kK (\epsilon 8810)\)

5-(N-Acetylaminomethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde hydrochloride (50), attempted

Manganese dioxide method

5-(N-Acetylaminomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol hydrochloride (48)

(0.062 g, 0.25 mmol) was dissolved in 5 ml of pyridine and 0.25 g of freshly powdered manganese dioxide was added in one portion to the vigorously stirred solution. The mixture was
refluxed for 1.0 hr. At 0.25 hr intervals, a small amount of the reaction suspension was filtered through Whatman No. 1 fluted filter paper to give a clear filtrate, 10 microliters of which was diluted to 5.0 ml with 0.1 M phosphate buffer, pH 7.0, and examined by uv spectrophotometry. As the reaction progressed, a peak at 27.4 kK became prominent, but decreased somewhat after 0.75 hr of reaction. Preparative tlc (BAW) was utilized to separate minor products from the desired compound which had an Rf of 0.35 and blue fluorescence and gave a positive Gibbs test. Elution with water of selected bands on the tlc plate revealed that only the major band at Rf 0.35 possessed an absorption maximum at 27.4 kK under conditions used for monitoring the reaction. If the buffer solution was maintained at pH 7.0 on addition of hydroxylamine hydrochloride, the isolated compound showed a shift of +0.9 kK in its uv spectrum; no change was observed on addition of phenylhydrazine under the same conditions. Hve under the usual conditions caused the compound to migrate 3.5 cm.

Using similar procedures, oxidations were conducted at room temperature and at reflux in the following solvents: dioxane; acetic acid; water; 0.1 N hydrochloric acid; 1 M phosphate buffer, pH 2.0; and 1 M formate buffer, pH 4.0.

Two procedures found useful for the oxidation of pyridoxol to pyridoxal were also tried. One involves neutral water--tetrahydrofuran and reflux (Brooks, 1961, p 36), the other, dilute sulfuric acid and room temperature (Ahrens and Korytnyk,
Chromium trioxide method  In a similar manner, 0.025 g of chromium trioxide was used instead of manganese dioxide. The reactions were carried out in either glacial acetic acid or anhydrous pyridine at 25° and 70°.

Generally, the oxidations were monitored for 20 hr or until appreciable degradation was observed.

Alcohol dehydrogenase method  Under conditions identical to those used for the attempted enzymic oxidation of 22 (see p 52), the N-acetyl derivative was tested.

Of the above attempted reactions, only oxidation by manganese dioxide in refluxing acetic acid or pyridine yielded significant amounts of material absorbing at a frequency lower than that of the reactant.

6-Methyl-3H-pyrrolo[3,4-c]pyridin-7-ol dihydrochloride (46b), attempted by deprotection  To 0.5 ml of filtrate obtained from the reaction of compound 48 with manganese dioxide in pyridine at reflux (0.5 hr) was added 4.5 ml 1 N HCl. This solution was kept at 75° for 8 hr while being monitored by uv in a manner similar to that employed in the oxidation. The absorption maximum shifted to 28.7 kK and diminished in intensity to about 60% that of the filtrate. The product exhibited no yellow color under visible or ultraviolet light, nor displayed any uv shift in the presence of hydroxylamine.
Synthesis of precursors of the 6-membered imine ring analog (Figure 11)

5-(2'-Aminoethyl)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride (52) The compound was prepared from pyridoxol hydrochloride (10) by the reaction sequence shown in Figure 11. Compounds 36, 37, 51, and 52 were synthesized by the methods of Iwata (1968), Tomita et al. (1966), Korytnyk et al. (1967), and Tomita et al. (1966), respectively.  

\[ \text{ea (} C_{11}H_{16}Cl_2N_2O_2\text{): } C, 40.96, 41.05(42.37); H, 6.47, 6.46(6.33); Cl, 27.02, 26.94(27.80); N, 10.64, 10.70(10.98%) \]

\[ \text{ms (70eV, 50°C): } m/z182(37,M^+), 164(12, - water), 163(10,153(-CHO)), 135(41,153-water), \omega128.6(182-153), 124(31), \omega119.4(M^*,153-135), 107(21), 106(178)\omega100.5(M^*,153-124), \omega85.2(M^*,135-107) \]

Synthesis of the 6-membered imine ring analog and its derivatives (Figure 12)

7,8-Dihydro-3-methyl-2,6-naphthyridin-4-ol dihydrochloride monohydrate (53) 5-(2'-Aminoethyl)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride (52) (0.51 g, 2 mmol) was dissolved in 50 ml of water and vigorously stirred by a magnetic stirrer. Freshly pulverized manganese dioxide (1.0 g) was added all at once. The progress of the reaction was monitored by withdrawing 0.1 ml of the reaction suspension and diluting this aliquot to 50 ml with 0.1 M acetate buffer, pH 5.0. The sample was then filtered through Whatman no. 1 filter paper.
Figure 11. Synthesis of the precursors of the 6-membered imine ring analog
Figure 12. Synthesis of the 6-membered imine ring analog and its derivatives
after which absorbance at 23.3 kK was measured. When the
23.3 kK peak reached its maximum (≈ 0.25 hr), the reaction
was complete. Subsequent operations were performed in the dark.
The reaction suspension was filtered through Whatman no. 1
filter paper, and the residue of manganese dioxide was washed
with two 25 ml portions of water. The clear, yellow-orange
solution was then evaporated under reduced pressure in a 50°
water bath to a volume of about 3 ml. The solution was applied
to an 8.9 X 2.75 cm column of Dowex AG 50W X 8 and eluted at a
rate of 2 ml/min with 2 N hydrochloric acid to remove the
divalent manganese and starting material. The fluorescent
yellow band was collected and was evaporated to dryness under
reduced pressure (50° water bath). The residue was dried over
magnesium sulfate, under vacuum, with protection from light to
yield 0.43 g (85%) of yellow-white solid. Note: detectable
decomposition occurs on exposure of this compound to tempera-
tures above 50° or to room lighting for more than 1 hr.

ea (C₈H₁₄Cl₂N₂O₂): C,42.80,42.51(42.70); H,5.27,5.62
(5.58); Cl,27.53(28.01); N,11.04(11.07); O,12.60%
(12.64%)
nmr (deuterium oxide, pD3.7, DSS): δ2.57(s,3,3-CH₃),
ν3.17(t,2,J=7.8 Hz,8-CH₂),ν4.03(t,2,J=7.8 Hz,7-CH₂),
7.37(bs,1,1-H),9.07 ppm(bs,1,5-H)
The results of high resolution and spin-decoupling
experiments are given in Figure 13.
Figure 13. Nmr spectrum of cyclic imine 53 in D$_2$O, pD 3.7
DETAILS OF 100 MHz SPECTRUM

IRRADIATION AT 83.37 ppm

IRRADIATION AT 89.07 ppm

60 MHz SPECTRUM
See Table 2.

**ir** (KBr): 3300; 2550 (b); 1669 (C=O); 1543; 1319, 1297 (d); 1245; 927; 717K

**uv** (0.1 M formate, pH 3.70): $\lambda_{\text{max}} = 33.8(2090)$, 41.3 kK (11 700)

Computer analysis of the dependence of the molar extinction coefficients on the wavenumber and pH (Nagano and Metzler, 1967; Johnson and Metzler, 1970) is shown in Figures 14 and 15, respectively.

### Table 2. Mass spectra of 7,8-dihydro-3-methyl-2,6-naphthyridin-4-ol dihydrochloride monohydrate (53) and its 4-$d_1$ and 7,7-$d_2$ derivatives at 70 eV, 30°C

<table>
<thead>
<tr>
<th>m/e</th>
<th>Unlabeled RA(%)</th>
<th>4-$d_1$ RA(%)</th>
<th>7,7-$d_2$ RA(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>164</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>163</td>
<td>100</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>162</td>
<td>100</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>161</td>
<td>19 (-H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td></td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>134</td>
<td>7 (-CO)</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>14 (-CHO)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>107</td>
<td>8 (134-vinyl)</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>106</td>
<td>8 (133-vinyl)</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Above a probe temperature of 50°C, a major peak is observed at m/e 150 (-H$_2$).
Figure 14. Molar extinction coefficients of four ionic forms of $\underline{53}$ plotted against wave number.

$H_3P$ represents the most highly protonated form and $P$ the completely deprotonated form.
Figure 15. Absorbance of 53 at 23.3 (O), 27.8 (A), and 34.5 kK ([]) plotted against pH.

The calculated curves (———) are based on the spectra in Figure 14 and pH values of 1.35, 5.99, and 8.86.
Equilibrium constants for the reactions of 53 with hydroxylamine and with sodium bisulfite at pH 5.0 (0.05 M acetate buffer) were evaluated spectrophotometrically by observing the intensity of the 23.3 kK peak in ~ 10^{-4} M solutions of 53. The carbonyl reagent concentration was varied from 10^{-1} to 10^{-5} M. The equilibrium constant for the reaction of 2,5-dimethyl-3-hydroxy-4-pyridinecarboxaldehyde (5-deoxy-pyridoxal, 40) was determined in a similar manner for comparison with the imine 53.

The imine (10^{-4} M) showed no tendency to participate in a transamination reaction with L-alanine (10^{-2} M) when incubated for up to 40 hr within a pH range of 1-9, or for up to 20 hr under the same conditions except for the presence of KAlSO_{4} (10^{-3} M) as a catalyst (Metzler and Snell, 1952). Absence of transamination was inferred from the lack of change in the electronic spectrum. Optical rotatory dispersion studies of similar solutions showed no racemization of the amino acid.

5-(2'-Aminoethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde oxime hydrochloride (54) Hydroxylamine hydrochloride (0.04 g, 0.6 mmol) was added to a stirred solution of 53 (0.12 g, 0.5 mmol) in 2 ml of water. The solution was passed through a Millipore filter, the pH was adjusted to 5.0 with concentrated sodium hydroxide, and the preparation was stored overnight at 4°. The beige crystals were isolated by filtration and dried under vacuum, over magnesium sulfate; yield, 0.073 g (67%).
Addition of another 0.04 g of hydroxylamine hydrochloride to the supernatant resulted in only 0.003 g of additional material.

\[\text{ea } \left(\text{C}_9\text{H}_{14}\text{ClN}_3\text{O}_2\right) : \text{C}, 46.78(46.66); \text{H}, 6.14(6.09); \text{Cl}, 15.15(15.30); \text{N}, 18.02\% (18.14\%)
\]

\[\text{nmr (dimethyl sulfoxide-d}_6\text{TMS)}: \delta 2.52(t, 2, J = 2Hz, \text{-CH}_2 - \text{N}^+\text{D}_3), \nu 2.98(t, 2, J = 2Hz, 5-\text{CH}_2), 7.90(s, 1, 6-\text{H}), 8.78 \text{ ppm(s, 1, CH = NOH)}\]

\[\text{ms (70 eV, 70°C): } m/e 195(6, \text{M}^+), 166(31, \text{-CHO}), 162(100, \text{-hydroxylamine}), 161(45), 149(87, \text{-OH}), 133(62\%)
\]

\[\text{ir (KBr): } 3420, 2970(b), 1618(\text{C=N}), 1460, 1395, 1263, 1144, 1042, 945, 876, 732 \text{ K(b)}\]

\[\text{uv (saturated hydroxylamine hydrochloride, pH 1.38): } \nu_{\text{max}} 30.6(e7200), 36.1 \text{ kK(9160)}\]

3-Methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4-ol dihydrochloride (56) Compound 53 (0.12 g, 0.5 mmol) was dissolved in 2 ml of 1.0 M formate buffer, previously adjusted to pH 3.7. Sodium borohydride (0.06 g, 1.6 mmol) was cautiously added to the vigorously stirred solution in ~ 0.01 g portions. The original yellow color of the solution eventually disappeared. The reaction solution was then applied directly to a 26 x 1.2 cm column of Dowex AG 50W-X8. Approximately 500 ml of 2 N hydrochloric acid was passed through the column at a rate of 0.3 ml/min. The eluent was then changed to 4 N hydrochloric acid, and 5 ml fractions were collected for the next 200 ml.
The larger of the peaks absorbing at 35.7 kK was isolated and evaporated to dryness under reduced pressure, at which time crystals formed. The sample (0.092 g, 82%) was kept overnight, under vacuum, over magnesium sulfate.

The analytical sample was recrystallized from methanol--ether.

\[
\text{ea (C}_9\text{H}_14\text{Cl}_2\text{N}_2\text{O): C, 45.33(45.59); H, 5.90(5.95); Cl, 29.77(29.90); N, 11.69\% (11.81\%).}
\]

\[
\text{nmr (deuterium oxide, DSS): } \delta 2.70 (s, 3, S-CH}_3\text{), } \delta 3.28 (t, 2, J=5.3 \text{ Hz, 8-CH}_2\text{), } \delta 3.65 (t, 2, J=5.3 \text{ Hz, 7-CH}_2\text{), 4.60 (s, 2, 5-CH}_2\text{), 8.22 ppm (s, 1, 1-H).}
\]

\[
\text{ms See Table 3}
\]

\[
\text{ir (KBr): 3410, 2670 (b), 1541, 1198, 983, 308, 902 K}
\]

\[
\text{uv (0.1 N sodium hydroxide, pH 12.46): } \nu_{\text{max}} 33.5 (e 7560), 40.7 \text{ kK (6940). The spectra of the various ionic forms are given in Figure 16.}
\]

5-(2'-Aminoethyl)-4-aminomethyl-2-methyl-3-pyridinol trihydrochloride (55), attempted Sodium borohydride (0.05 g, 1.3 (mmol) was dissolved in 2 ml of water to which 1 drop of \nu 1 N sodium hydroxide had been added. Finely powdered 54 (0.11 g, 0.5 mmol) was added to the vigorously stirred borohydride solution, and the reaction solution was stirred for 1 hr. Concentrated hydrochloric acid was added dropwise until the bubbling ceased. The solution was then applied to a Dowex column, and a procedure similar to that used for the isolation
Table 3. Mass spectra of 3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4-ol dihydrochloride (56) and its 4,6-d_2 derivative at 70 eV

<table>
<thead>
<tr>
<th>m/e</th>
<th>Unlabeled, 50°C</th>
<th>4,6-d_2, 80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>166</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>164</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>163</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>100 (-CHO)</td>
<td>36</td>
</tr>
<tr>
<td>108</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>107</td>
<td>42 (-ethylene)</td>
<td>24</td>
</tr>
<tr>
<td>106</td>
<td>23 (107-H)</td>
<td></td>
</tr>
<tr>
<td>~105.1</td>
<td>M*(135-107)</td>
<td></td>
</tr>
<tr>
<td>~85.6</td>
<td>M*(107-106)</td>
<td></td>
</tr>
</tbody>
</table>

of 56 was followed. Thin layer chromatography, mass spectrometry, and mixture melting point determination indicated that the product was 56 rather than the anticipated 55.

4-Hydroxy-3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-5-one hydrochloride (57) The procedure for the preparation of 56 was followed except that the very small peak (the peak area was about 1/100 that of 57 at 35.7 kK) which eluted immediately before 56 was isolated. The yield of 57 was
Figure 16. Molar extinction coefficients of four ionic forms of cyclic amine 56

$H_2P$ represents the most highly protonated form and $P$ the completely unprotonated amine.
estimated by spectrophotometry to be 1.5 mg. The solution was evaporated to dryness under reduced pressure and dried under vacuum, over CaCl₂. The material was sufficient for measurement of the mass spectrum and electronic spectra at several values of pH.

Attempts to increase the yield of 57 by a longer oxidation with MnO₂ led only to a complex mixture of products containing a small amount of 57.

From electronic spectra at 15 values of pH, the spectra of the three ionic forms were evaluated: H₂P, v_max 31.2; HP, 31.4; P, 28.5 kK.

Table 4. Mass spectra of 4-hydroxy-3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-5-one hydrochloride (57) and its 4,6-d₂ derivative at 70 eV

<table>
<thead>
<tr>
<th>m/e</th>
<th>Unlabeled, 35°C RA(%)</th>
<th>4,6-d₂, 80°C RA(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>179</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>178</td>
<td>28 (M⁺)</td>
<td>15</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>149</td>
<td>63 (-CHO)</td>
<td>32</td>
</tr>
<tr>
<td>148</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>122</td>
<td>73 (149-vinyl)</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>96</td>
<td>18</td>
</tr>
<tr>
<td>104</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
6-(2'-Hydroxy-2'-propyl)-3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4,5-diol dihydrochloride, "acetone adduct" (58)

7,8-Dihydro-3-methyl-2,6-naphthyridin-4-ol dihydrochloride monohydrate (53) (0.25 g, 1 mmol) was dissolved in 10 ml of anhydrous methanol. Anhydrous acetone (10 ml) was added and the solution was refluxed for 1 hr. During the reaction the solution was protected from moisture and light. The solution was evaporated to dryness under reduced pressure (50° bath) and dissolved in a minimum amount of warm methanol. Addition of anhydrous diethyl ether precipitated a white solid which was isolated by suction filtration and recrystallized from methanol—ether to give 0.17 g of crystals.

ea (C₁₂H₂₀Cl₂N₂O₃): C, 46.52, 46.09 (46.31); H, 6.18, 6.39 (6.48); Cl, 23.03 (22.78); N, 8.96, 8.80 (9.00); O, 15.23% (15.42%)

nmr (deuterium oxide, DSS): 51.97 (bs, 3), 2.65 (s, 3, 3-CH₃)
3.37 (t, 2, J=5.8 Hz, 7-CH₂), 3.75 (t, 2, J=5.8 Hz, 8-CH₂)
5.02 (vbs, ~1,5-H), 8.20 ppm (s, 1, 1-H)

ms (70 eV, 50°C): m/e 220 (RA15), 163 (95), 162 (100, 220-acetone), 133 (32%, 162-CHO)

ir (KBr): 3401, 3145, 2899, 2600 (b), 1543, 1393, 1234, 1098, 1054, 856, 805 K

uv (0.1 N sodium hydroxide, pH 12.51): vₘₐₓ 33.1, 40.5 kK
5-(2'-Aminoethyl-2',2'-d$_2$)-3-hydroxy-2-methyl-4-pyridinemethanol dihydrochloride (see 52) A procedure previously used for the preparation of the non-deuterated compound (Tomita, Brooks, and Metzler, 1966) was modified to give a higher yield. The nitrile was dried over phosphorus pentoxide; ether was refluxed over lithium aluminum hydride for several days and distilled immediately before use. The reaction was conducted under a dry nitrogen atmosphere. Ether (55 ml) was placed in a 3-necked reaction flask equipped with a powerful stirrer and cooled in an ice bath. Lithium aluminum deuteride (purchased from Alfa Inorganics, Beverly, Mass.) (1.0 g) was added to the cold ether. The nitrile, dissolved in 55 ml of ether, was added to the hydride slurry through a constant-pressure dropping funnel at a rate of ~2 ml/min. The reaction mixture was kept at 0° for 0.5 hr after the last portion of nitrile had been added, slowly brought to reflux (1 hr), and refluxed for 2 hr. After the slurry had again been cooled in an ice bath, 5 ml of water and then 5 ml of 20% sodium hydroxide were cautiously added. The mixture was filtered to remove the inorganic salts, dried over sodium sulfate, refiltered, and evaporated to an oil under reduced pressure (50° bath). To the oil was added 20 ml of 1 N hydrochloric acid. The resulting solution was placed on a steam bath for 0.25 hr, evaporated to a yellow oil under reduced pressure, and dried over Drierite and sodium hydroxide,
under vacuum. Yield: 1.03 g (81%) of yellowish solid. Repeated recrystallizations from methanol—ether gave the 0.23 g of white crystals used in further experiments.

The compound was identical to authentic, non-deuterated compound (Korytnyk et al., 1967) with respect to mp, uv (0.1 N sodium hydroxide), and Rf(BAW) and gave a single orange spot with ninhydrin and a single blue spot with Gibbs reagent.

7,8-Dihydro-3-methyl-2,6-naphthyridin-4-ol-7,7-d$_2$ dihydrochloride monohydrate (see 53) The deuterated compound was synthesized from its correspondingly-deuterated precursor by the method used for the non-labeled analog. The mass spectrum and nmr spectrum differed in the expected ways from the authentic non-labeled compound; the uv spectrum was identical to that of the non-deuterated imine.
Table 5. Additional properties of the synthesized compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>mp(°C)</th>
<th>pK</th>
<th>tlc($R_F$), solvent</th>
<th>hve (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>196-6.5</td>
<td>0.17</td>
<td>BAW</td>
<td>20.1</td>
</tr>
<tr>
<td>41</td>
<td>238-9.5</td>
<td>0.44</td>
<td>BAW</td>
<td>16.2</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>0.15</td>
<td>BAW</td>
<td>20.3</td>
</tr>
<tr>
<td>47</td>
<td>118.5-20</td>
<td>0.36</td>
<td>BAW</td>
<td>14.8</td>
</tr>
<tr>
<td>48</td>
<td>177-9</td>
<td>4.84,8.74</td>
<td>BAW</td>
<td>14.5</td>
</tr>
<tr>
<td>52</td>
<td>204-5.5</td>
<td>3.80,~7.75,~10.03</td>
<td>AA</td>
<td>23.1</td>
</tr>
<tr>
<td>53</td>
<td>~200</td>
<td>1.35,5.99,8.86</td>
<td>AA</td>
<td>18.1</td>
</tr>
<tr>
<td>54</td>
<td>227-9</td>
<td>unstable in solution</td>
<td>AA</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>239-41</td>
<td>3.74, 8.48, 10.0</td>
<td>AA</td>
<td>20.3</td>
</tr>
<tr>
<td>57</td>
<td></td>
<td>4.53,10.1</td>
<td>AA</td>
<td>14.1</td>
</tr>
<tr>
<td>58</td>
<td>178-9.5</td>
<td>0.15</td>
<td>BAW</td>
<td>19.3</td>
</tr>
</tbody>
</table>

mp = melting point  
tlc = thin-layer chromatography  
hve = high voltage electrophoresis, migration toward cathode  
BAW = butanol - acetic acid - water (4:1:5)  
AA = 30% acetic acid  
For detailed experimental procedures, see the introductory remarks in Experimentation p 40.
Oxidation methods

It was apparent from the very beginning of this research effort that one of the major difficulties in the synthesis of vitamin B₆ analogs was the oxidation of alcohols to aldehydes. And the aldehyde analogs were clearly required for any model system. It therefore seemed appropriate to develop a dependable method of oxidation at the outset. Manganese dioxide oxidations were initially eliminated from consideration because of the variability of results, the difficulty in monitoring, and the lack of theoretical understanding on which to base modifications.

The first oxidizing agent to be tried was cerium (IV) which had been shown to give very high yields of benzyl aldehydes from alcohols under acidic aqueous conditions (Trahanovsky and Young, 1965). Use of this reagent was discontinued after results from Trahanovsky's laboratory indicated that the phenolic group interfered with the oxidation (W. S. Trahanovsky, Iowa State University, Ames, Iowa, personal communication, 1967).

Another promising oxidizing agent was diethylazo dicarboxylate (Yoneda et al., 1967). Although benzyl alcohol was oxidized to benzoaldehyde in only moderate yield with this reagent, no over-oxidation was observed. This was a valuable feature, because overoxidation is thought to be the principle
cause of low yields in the case of pyridoxal synthesis (Brooks, 1961, p 68). Considerable difficulty in preparing and characterizing the reagent was encountered. Trial oxidations using the small amount available failed to give the desired pyridoxal.

Electrooxidation of pyridoxol was attempted after extensive polarographic analysis indicated the feasibility of such a method. A small amount of product was obtained but the majority of the reactant was transformed into an intractable green tar.

Several oxidation methods of a more conventional nature (e.g., Oppenauer oxidation) were cursorily examined without any encouraging results.

At this point it was apparent that a prohibitive amount of research effort would have to be expended to discover a replacement for manganese dioxide, so research on oxidation was abandoned in the hopes that synthetic problems of less difficulty would yield more tangible results.

5-Deoxy analogs

To this end work was begun on seeking a convenient synthesis of 5-deoxypyridoxamine (42, Figure 8), a compound useful in model system studies. Hydrogenolysis of pyridoxal oxime (17, Figure 3) (Naito and Ueno, 1959 appeared to be the simplest (most direct) route to the desired product. Many attempts to reproduce the results obtained by Naito and Ueno, using a glass hydrogenation apparatus, a specially designed high capacity hydrogenator, or a modified (thermostated)
Parr apparatus, were all unsuccessful. Other workers have experienced the same difficulty, and one of them has suggested that a minor difference in the palladium-charcoal catalyst may be responsible (C. Iwata, Iowa State University, Ames, Iowa, personal communication, 1967).

The pathway which finally yielded results (Figure 8) was the reduction 5-deoxypyridoxal oxime (41) (Testa and Fava, 1957). Although this synthesis requires seven steps (from pyridoxol), the yields are high for each step if the last step is modified as indicated in the previous section. In this modification the lower yields and inconvenience associated with hydrogen sulfide precipitation of the Zn(II) are avoided by the use of an acidic Dowex 50W-X8 column.

The nmr of 5-deoxypyridoxal oxime (41) was obtained in two solvents. In dimethyl sulfoxide-d<sub>6</sub> it was run as a saturated solution; in that solvent the proton on either the oxygen of the oxime or the phenol appears far downfield as a very broad singlet. Inasmuch as Korytnyk and Paul (1965) did not observe the phenolic proton while examining pyridoxal derivatives in dimethyl sulfoxide-d<sub>6</sub>, the observed peak at δ 12.08 ppm can be assigned to the proton on the oxime. There is no experimental data for 5-deoxy compounds which would distinguish between the two methyl groups 0.04 ppm apart. However, in acidic deuterium oxide, the downfield methyl singlet is near the customary position of the 2-methyl group.
The ms exhibits the loss of OH radical typical of aromatic oximes (Budzikiewicz et al., 1967, p 374).

The infrared spectrum shows an absorption at 1616 K which is probably due to imine stretching mode.

The most outstanding feature of the instrumental analysis of 5-deoxypyridoxamine dihydrochloride (42, Figure 8) is its readily-interpretable mass spectrum. Every major peak can be rationalized. The two large peaks at low mass number (m/e 36 and 38) are attributable to hydrogen chloride caused by the breakup of the hydrochloride salts. The m-17 peak represents an unusual loss of ammonia which is made favorable in this case by an ortho effect decomposition (McLafferty, 1967, p 133). The remaining two fragmentations are expected on the basis of work by DeJongh et al. (1966).

Intermediates of the attempted syntheses of the 5-membered imine ring analog

Isopyridoxamine dihydrochloride (22, Figure 9) was prepared by the method perfected for 5-deoxypyridoxamine. The infrared spectrum was identical to that of authentic sample.

In the nmr spectrum, the assignment of the downfield methylene resonance to the neutral hydroxymethyl group rather than the protonated aminomethyl group conforms to the data on pyridoxamine (Korytnyk and Singh, 1963). The pyridoxamine data are difficult to rationalize however. Not only does the positively-charged group shift to a more shielded position
compared to pyridoxol, but the "unchanged" group shifts to a slightly less shielded position.

An unusually small molecular ion (9%) is observed in the ms. Also, the rare loss of ammonia is the dominant fragmentation. Why the loss of ammonia, which must rearrange via a 7-membered ring transition state should be a better loss than formation of a 6-membered ring and loss of water, is very puzzling, especially in view of the Lewis base character of the two heteroatoms.

The isopyridoxamine was prepared so that it could be oxidized to the 5-membered ring imine (46, Figure 9). The oxidation was first attempted under conditions identical to those which had been successfully used for the 6-membered ring imine. The only effect observed under these conditions was the disappearance of the ultraviolet spectrum. The same result was observed under several other conditions of oxidation. The compound was either adsorbed on the manganese dioxide or was degradatively oxidized. There is a sole instance of a primary benzylamine being oxidized by manganese dioxide but the conditions were quite different (Highet and Wildman, 1955).

Attempts to oxidize the compounds with the relatively non-specific alcohol dehydrogenases from horse and yeast also failed.

Actually, there is some doubt that the cyclic compound could exist at all. No compounds having the 3H-pyrrolo
[3,4-\text{c}]\text{pyridine ring system with an endocyclic double bond have been reported. Furthermore, compounds in which benzene replaces pyridine, the isoindoles, are in rapid tautomeric equilibrium with the isoindolenines. This type of compound is very unstable} (\text{Veber and Lwowski, 1963; Fletcher, 1966}). \text{These are not sufficient reasons to abandon the synthetic efforts, however; the open chain form (46a) of the compound would be an interesting compound with which to compare the cyclic imines.}

\text{Methods of protecting the amine group from oxidation were investigated. W. Korytnyk (Roswell Park Memorial Institute, Buffalo, New York, personal communication, 1969) suggested that the benzoyl be tried. Although the dibenzoylation of isopropylidene isopyridoxamine is thought to have proceeded rapidly (exothermic reaction), difficulty in obtaining pure starting material made the reaction products difficult to identify. Therefore, methods of reducing the corresponding oxime to isopropylidene isopyridoxamine which would give higher yields than the published method (32\%) were sought (Brooks et al., 1966). Sodium borohydride and hydrogen over palladium-charcoal were ineffective reagents, perhaps because acidic solutions had to be avoided due to lability of the isopropylidene group. Finally, zinc in acetic acid was tried with some success, but apparently the acidic conditions and the exothermic reaction produced enough biproducts to still make purification difficult. The use of anhydrous acetic acid}
While the benzoylation experiments were in progress, acetylation (Figure 10) was also being tried. The results were very encouraging, so the benzoylation experiments were discontinued. Actually, the acetylation reaction was a serendipitous discovery. One attempted preparation of isopropylidene isopyridoxamine had resulted in a good yield of a crystalline product which gave a negative ninhydrin test. The unexpected product, identified as the monoacetyl derivative, 47, (Figure 10) had been produced from the reaction of acetic anhydride with the expected product. Apparently, appreciable quantities of acetic anhydride had been produced in an attempt to make anhydrous acetic acid by distillation over phosphorus pentoxide.

Isolation of the acid-labile N-acetyl isopropylidene isopyridoxamine (47) from a relatively large quantity of acetic acid at first proved troublesome. Evaporation under reduced pressure was time-consuming, produced some hydrolysis of the isopropylidene group, and left a mixture of solvent and product which was difficult to purify further. Neutralization at 0°C and extraction of the product with chloroform was found convenient and efficient.

The nmr of the compound at pD 1.0, a solvent in which the compound slowly hydrolyzes, is routine. In deuterated chloroform, however, the compound presents a spectrum which is
difficult to interpret. The aromatic methyl and amino protons can be assigned readily by comparison with tribenzoylpyridoxamine (Paul and Konytnyk, 1969). The doublet centered at \( \delta 4.23 \) ppm must be the methylene group adjacent to the planar amide, so the process of elimination assigns the 4-methylene group to the downfield, 2-proton resonance. The puzzling aspects of this spectrum are the broadening of the usually sharp 2-methyl peak and the absence of the aromatic proton. These phenomena suggest a strong perturbation, probably at the pyridine nitrogen. Perhaps hydrogen bonding of the amide proton to the pyridine nitrogen is responsible for this unique effect.

The mass spectrum shows the expected loss of acetone (DeJongh et al., 1966). Likewise, the loss of acetyl (Budzikiewicz et al., 1967, p 342) and ketene (Budzikiewicz et al., 1967, p 350) have precedents. The loss of ketene is additionally supported by an appropriate metastable ion.

Selective hydrolysis of the isopropylidene group from N-acetyl isopropylidene isopyridoxamine proceeded unexpectedly smoothly to give 48 (Figure 10). TLC indicated that only a small amount (less than 5%) of the N-acetyl had been hydrolyzed. Recrystallization eliminated the impurity. The pseudo first-order rate constant and half-life were accurately determined.

The mass spectrum of compound 48 is a catalog of possible fragmentations: water is lost by ortho effect (McLafferty, 1967, p 133), the amide decomposes by McLafferty-type
rearrangement (McLafferty, 1967, p 123). Both ketene and acetyl can be lost from M – water. Ions resulting from the retention of the charge on the smaller fragment of the double hydrogen rearrangement, and acetyl loss, are observable in the low-mass region.

The N-acetyl function could be readily removed by heating a 1 N hydrochloric acid solution of the compound for 0.5 hr on the steam bath.

It would have been desirable to obtain the free base of N-acetyl isopyridoxamine so that manganese dioxide oxidation in chloroform could be tried. In order to know the neutralization point, the pK's of the compound were obtained by spectrophotometry and computation (Nagano and Metzler, 1967; Johnson and Metzler, 1970). The neutralization point was 6.79. Neutralization of a concentrated solution produced a precipitate which was not soluble in organic solvents such as acetone, chloroform or hexane. Neither was the neutralized compound extractable with chloroform. Chloride analysis (Willard et al., 1965, p 576) indicated that the compound still retained 26 to 29% of its original chloride content.

Attempts to oxidize the N-acetyl isopyridoxamine hydrochloride (48, Figure 10) proved futile. Most reaction conditions produced degradation of the starting material. Utilization of manganese dioxide in acetic acid, or better, pyridine, at reflux did produce a compound with a lower energy
absorption in good yield. Isolation of the compound from preparative tlc, and subsequent analysis, showed that it was unreactive with phenylhydrazine and reacted only with high concentrations of hydroxylamine (These data were obtained by spectrophotometry at pH 7.0.). The product had an unusually slow migration toward the negative electrode on hve and gave an intense blue fluorescence and a positive Gibbs test. From these data the unknown product was tentatively identified as 5-(N-acetylaninomethyl)-3-hydroxymethyl-2-methyl-4-pyridinecarboxylic acid (49, Figure 10).

In the hope that the compound was the aldehyde (Figure 10), an aqueous acidic solution of the compound was hydrolyzed by heating on a steam bath for 0.5 hr—a procedure previously shown to remove the acetyl group—but neutralization did not produce a solution with the required electronic spectrum.

Oxidation by alcohol dehydrogenases was unproductive.

The 6-membered imine ring analog and related compounds

The synthesis of the immediate precursor of the 6-membered ring imine was accomplished by the pathway shown in Figure 11. Slight modifications (Iwata, 1968) of the standard procedure (Korytnyk and Wiedeman, 1962) for the preparation of isopropylidene pyridoxol (36, Figure 11) make the preparation much more convenient and reliable.
The following step, the chlorination of the alcohol, generally proceeds in almost quantitative yield (Tomita et al., 1966).

Korytnyk's "Method B" for the preparation of the nitrile derivative (51, Figure 11) (Korytnyk et al., 1967) was far superior to any other method, probably because the reactants are reasonably soluble and stable in dimethyl sulfoxide. Oddly enough, the best crystals of the nitrile are formed by cooling a saturated solution quickly. Recrystallization from petroleum ether removes a small amount of ether-insoluble material but it is generally not necessary to have the sample in such pure form. It is curious that Korytnyk reduced the corresponding nitrovinyl compound, rather than the nitrile (Korytnyk et al., 1967), in order to obtain 5-(2'-aminoethyl)-3-hydroxy-2-methyl-4-pyridinemethanol, (52, Figure 11) even though the nitrile 51 was readily available to him whereas the nitrovinyl compound was not. The overall yields of the amine are similar for the two routes. An interesting feature of the nitrile reduction (Tomita et al., 1966) is the "pea soup green" color of the reaction solution; the color is a valuable indicator of whether the lithium aluminum hydride is sufficiently active.

The isopropylidene group was hydrolyzed, without isolation of the intermediate, to give the rather impure amine dihydrochloride (52, Figure 11). Recrystallization from methanol--ether (Korytnyk et al., 1967) is the preferred
method of purification. Authentic sample, kindly furnished by Dr. Korytnyk, was identical to the compound synthesized in this laboratory by the criteria of uv, mixture mp, and tlc (BAW). The surprisingly rapid formation of the 6-membered ring imine (53, Figure 12) under such mild conditions is the consequence of the ring stability, discussed later. Manganese (II) was shown by analysis (Pierce et al., 1958, p 407) of the eluent to precede the imine from the column. This testing procedure, which had an estimated sensitivity of $10^{-3}$ moles of manganese (II) per mole of imine, indicated that there was complete separation of the organic and inorganic products.

Compound 53 has not been recrystallized despite repeated efforts. It exists as an amorphous solid.

To obtain an interpretable nmr spectrum (Figure 13) it was necessary to adjust the pH to 3.7. At that pH only one ionic form is present in appreciable concentration; the peaks, particularly those at $\delta \sim 3.17$ and $\sim 4.03$ ppm, became much more distinct.

Three of the absorptions are readily interpreted. The sharp peak at $\delta 2.57$ ppm falls at approximately the same place as the methyl group of most other vitamin $B_6$ derivatives. Similar correlations identify the $\delta 7.37$ ppm peak with the one-aromatic proton. The extremely low-field, very broad absorption represents the aldimine proton. Double irradiation at 100 MHz made possible the assignment of the two apparent triplets.
Irradiation of the aldimine proton sharpened the triplet at \( \delta \) 4.03 ppm but had no effect upon the \( \delta \) 3.17 ppm peak. The \( \delta \) 4.03 ppm peak must represent the 7-methylene protons. Irradiation of the aromatic proton had negligible effect on the triplet at \( \delta \) 3.17 ppm, even though the aromatic proton would be expected to be broadened somewhat by allylic coupling to the 8-methylene protons. On the other hand, the coupling between the aldimine proton and the 7-methylene protons is quite apparent when the triplet is collapsed to a broad singlet by decoupling the other triplet at \( \delta \) 3.17 ppm. An nmr spectrum (\( pD \) 3.7) of 7,7-\( d_2 \) derivative of 53 confirmed the assignments by possessing no resonance at \( \delta \) 4.03 ppm.

In addition to the broadening effects on the two triplets by the downfield singlets, there is an additional amount of broadening caused either by ring puckering of the partially-saturated ring or deuteron exchange, or both. Inasmuch as the two methylene peaks form an \( A_2B_2 \) system, the chemical shifts are approximate.

In the mass spectrum (Table 2), the molecular ion is the base peak. Loss of hydrogen radical is most favored. Deuterium labeling in the 4-phenolic position and in the 7-methylene position shows that carbon monoxide or CHO radical are also lost from the molecular ion. Subsequent loss of 27 from these two products probably is caused by the splitting off of the vinyl radical. Scrambling of the deuterium is observed in
both the $4-d_1$ and $7,7-d_2$ derivatives. At temperatures above 50°C pyrolysis products become noticeable. The most salient change is the increase in the intensity of the m/e 160 peak (m - H$_2$) which represents the formation of the completely aromatic, 3,4- substituted 2,6-naphthyridine.

The infrared spectrum shows no carbonyl absorption band. Presumably, the moderate, sharp absorption a 1669 K is the imine stretching mode.

Detailed computer analysis of the pH dependence of the electronic spectrum (Nagano and Metzler, 1967; Siano and Metzler, 1969; Johnson and Metzler, 1970) of 53 revealed four distinct ionic forms of the compound. These forms differ from one another in their degree of protonation; they can be conveniently designated on this basis. Thus $H_3P$ represents the most protonated ionic form and $P$ the least protonated. Figure 17 shows the structures which are probably the most significant of the many possible subforms of each ionic form.

Reference to Figure 14 reveals the salient subform of the most highly protonated form, $H_3P$, to be the more aromatic subform $H_3P(1)$, rather than the hydrated imines absorbing at higher energy.

On the basis of work by Heinert and Martell (1963), the intense yellow-orange color of weakly acidic solutions of 53 can be assigned to the keto-enamine $H_2P(1)$. The unusually large magnitude of the absorption at 23.3 kK relative to characteristic absorptions of the enol-imines $H_2P(2)$ and
Figure 17. Some ionic forms of 53
H₂P(3) indicates the great stability of this form.

That more than one ionic form is responsible for the peak at 33.7 kK is apparent from resolution of the H₂P spectrum into lognormal distribution curves (Siano and Metzler, 1969). It can be seen that the half-width (5.70 kK) and skewness (1.094) of the 33.7 kK-band deviate significantly from the values expected for a band representing a single ionic species, e.g., the 23.3 kK-band (half width = 3.55 kK, skewness = 1.377). The deviation may be due to the presence of the lactam 57, a known impurity in 53.

Figure 14 shows that two subforms of approximately equal concentration contribute to the low-energy portion of the HP spectrum. These have been assigned structures HP(1) and HP(2) (Figure 17). A lognormal resolution of the two bands, plus the usual simplifying assumptions about the extinction coefficients, permit the calculation of the tautomeric ratio (R): HP(2)/HP(1) = 0.90. This value of R was used to evaluate the intrinsic pK's for the equilibria between H₂P(1), HP(1), HP(2), and P. A ring-opened ionic form, H₄P has been postulated to explain a shift in the spectrum in very acidic solutions.

The inactivity of 53 in the model non-enzymic transamination is probably a consequence of the rather high stability of the cycloaldimine ring. This hypothesis is supported by the observed difference in equilibrium constants for the reaction with hydroxylamine of 53 and a 5-deoxypyridoxal. The constants
at pH 5 are $1.2 \times 10^3$ and $38 \times 10^3$ l. mole$^{-1}$, respectively.
If all other differences between the two compounds are ignored, the standard free energy change for ring formation can be calculated from the ratio of these equilibrium constants as $\Delta G^o = -2.0 \pm 0.2$ kcal/mole.

It is not surprising that 53 does not bind specifically to the apoenzyme forms of muscle phosphorylase b (rabbit), aspartate aminotransferase (soluble, α-subform, pig heart), or glutamic decarboxylase (E. coli). The lack of binding may be, in part, a consequence of the stability of the cycloaldimine ring. However, even if 53 did react with the ε-amino group of the lysine at the binding site for pyridoxal phosphate, the ammonium group of 53, which would be released by this transaldimination reaction, would doubtless be repelled by any positively charged group which might be present at the phosphate binding site, thus inhibiting binding.

The electronic spectrum of 53 is strikingly similar to that of the pyridoxal phosphate dependent enzyme, aspartate aminotransferase (pig heart, soluble, α-subform). The absorption maximum of the enzyme is at 23.3 kK at low pH and shifts to 27.5 kK upon dissociation of a single proton with a pK of 6.3. From Figure 17 we see that form $H_2P(1)$ also absorbs at 23.3 kK and shifts to 27.0 kK with dissociation of a single proton of pK 6.3. This suggests that structures having the transoid conformation (Figure 7) must be considered for
vitamin B₆ containing enzymes. However, the identity of the pK's is probably fortuitous, because it is hard to believe that the presence of the phosphate group in the coenzyme will not have a substantial effect on the pK. It is also noteworthy that structure HP(1) (Scheme III) absorbs at 23.3 kK with a peak shift to 27.4 kK, even closer to that of the enzyme at high pH and a pK of 8.6. Thus, rather similar spectra are obtained independently of the state of protonation of the pyridine ring nitrogen. Care must be exercised in assuming that a particular state of protonation of the ring of the coenzyme occurs in the enzymes.

The oxime 54 (Figure 12) is readily prepared. Its nmr clearly shows the aldoxime proton in dimethyl sulfoxide-d₆, but apparently the N-OH proton is too broadened for observation in the poor signal-to-noise ratio of this spectrum.

The mass spectrum of the oxime is interesting because it is actually a superimposition of two spectra—the oxime itself and the cyclic imine 53. The oxime has been observed to revert to the cyclic imine in high vacuum and the resulting hydroxy-lamine can be readily detected in the mass spectrum. The initial step of oxime fragmentation is apparently the usual loss of CHO. The next loss, that of 27, would require more data for identification.

Attempts to synthesize 5(2'-aminoethyl)-4-aminomethyl-2-methyl-3-pyridinol trihydrochloride (55, Figure 12) from oxime 54 resulted in formation of 56 instead. This is due to
the instability of the oxime. It readily loses hydroxylamine in aqueous solution or under high vacuum to give 53. If a reducing agent is present, 53 is then reduced in the expected manner (Figure 12).

Compound 53 is reduced by either sodium borohydride or hydrogen on 10% palladium - charcoal to yield amine 56 (Figure 12).

The mass spectrum (Table 3) shows a loss of CHO to give the base peak at m/e 135. A metastable ion, and deuterium labeling in the 6-position confirm the loss of ethylene (some scrambling) as the next step. Another M indicates a loss of one proton from the m/e 107 fragment. Figure 16 displays the computer-calculated electronic spectra (Nagano and Metzler, 1967; Johnson and Metzler, 1970) of the four ionic forms of the amine.

If 53 (Figure 12) existed in part as the ring-opened free aldehyde, some 52 (Figure 11) should be produced under reducing conditions. A careful search for 52 following reduction of 53 revealed only one component other than the expected 56. This minor component was found to be the lactam 57 (Figure 12). Lactam 57 cannot be separated from 53 chromatographically, but it is resistant to reduction and can be isolated from 56, the reduction product of 53.

Because only a small quantity of lactam 57 was available structural determination was based on ms, uv, pK, and Gibbs test.
Fragmentation of the lactam (Table 4) follows the loss of CHO radical, then loss of 27 pattern characteristic of this ring system. The base peak is produced by a loss of the N-H moiety plus an unidentifiable portion of the molecule.

Support for the lactam structure is also provided by the number (2) and values (4.53, 10.1) of the pK's as well as the uv spectrum itself.

A positive Gibbs test indicates the compound is not radically different from the other derivatives.

A plausible structure for the acetone adduct is shown (58, Figure 12). The elemental analysis is consistent with this product and the mass spectrum can be rationalized in the following way:

The expected molecular ion could not be observed, even at 10 eV. The loss of water to give an ion at m/e 220 is certainly plausible. The base peak is produced by loss of acetone from the dehydrated ion. After loss of acetone to give the cyclic imine, the fragmentation follows the expected course. Both uv and nmr confirm that the adduct reverts, in acidic, aqueous solutions, to the imine. The difficulty in assignment of a structure to the adduct arises from the anomalous nmr. It unequivocally yields a 3:3 ratio between the aromatic methyl protons and the apparent acetone methyl group. This ratio should be 3:6, of course. The synthesis of crystalline hydrobromide salt of the adduct would permit X-ray
analysis.

The 7-membered imine ring analog

Work on the 7-membered imine ring (Figure 18), using a known route (Korytnyk, 1965) for the initial phase, proceeded smoothly until the acid 60 was reached. The acid possessed the correct mp, uv, ir, and Gibbs reactions, and had a pK in the acid region as shown by uv. However, it failed to react with diazomethane to form the methyl ester (Korytnyk et al., 1967). The acid could be reduced to the corresponding alcohol 61 by lithium aluminum hydride (Korytnyk, 1965) as shown by mp and ir determinations. It can reasonably be expected that replacement of the alcohol by an amine (Gabriel synthesis), hydrolysis of the isopropylidene group, and manganese dioxide oxidation will yield the 7-membered imine ring 64 (Figure 18). Unlike the 5-membered cyclic imine, the 7-membered ring can be expected to be reasonably stable, for the benzene analogs are known (Goldman et al., 1969).
Figure 18. Proposed synthesis of the 7-membered imine ring analog
1) HCl / acetone 0°C
2) neut.

1) SOBr
2) potassium phthalimide

1 N HCl / 100°C

MnO₂ / water

malonic acid / pyridine 100°C
In conclusion, it can be said that a reasonable start has been made on the synthesis of an important series of homologs. Synthesis of additional members of the series must be left to others. The choice of the 6-membered imine ring as a thesis project appears to have been exceedingly fortuitous.

It is hoped that the results of this five year research project will in some small way aid those who will eventually elucidate the mechanism of a vitamin $B_6$-dependent enzyme.
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