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Vitamin B6 derivatives and related compounds

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VITAMIN B_6 DERIVATIVES AND RELATED COMPOUNDS

by

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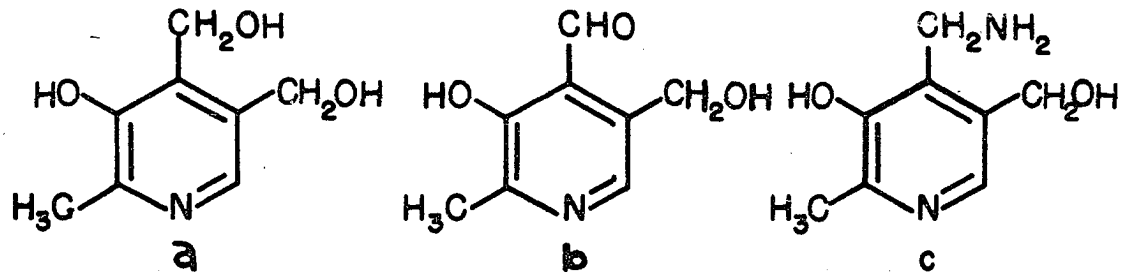
INTRODUCTION

Several years before the actual isolation of the pure crystalline vitamin, Gyorgy (1) in 1934, defined vitamin B_6 as "that part of the vitamin B complex responsible for the cure of a specific dermatitis developed by rats on a vitamin-free diet supplemented with vitamin B_1 and lactoflavin". Investigations dealing with vitamin B_6 activity and its purification by several groups at that time were impeded by the lack of an assay which would differentiate between riboflavin (vitamin B_2) and vitamin B_6, a factor which is extremely important since all plants and animals appear to contain these two materials. In 1936 Birch and Gyorgy (2) reported some very significant purification studies on vitamin B_6 and described the behavior of this material toward various chemical reagents. Subsequent to this disclosure, progress on the purification and isolation of vitamin B_6 was rapid, and within the first five months of 1938, five research teams reported the isolation of the crystalline substance. Keresztesy and Stevens (3) and Lepkovsky (4) published the isolation of crystalline vitamin B_6 in February; Kuhn and Wendt (5) and Gyorgy (6) made similar announcements in April, and Ichiba and Michi (7) described the hydrochloride of vitamin B_6 in June.

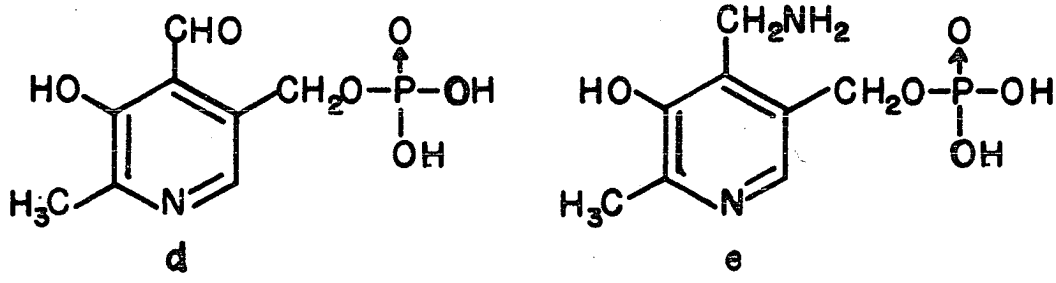
In each instance the compound isolated by these investigators was shown to be 2-methyl-3-hydroxy-4,5-di-(hydroxy-

methyl)-pyridine (Figure 1,a) by degradation (8, 9, 10, 11) and synthesis (12, 13), and was designated pyridoxine. This compound, having been the first isolated, was considered by many to be the only active form of vitamin B_6. Subsequent investigations by Snell and his associates (14) revealed the additional active companion compounds, 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine (Figure 1,b) and 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine (Figure 1,c), named respectively pyridoxal and pyridoxamine. Therefore, it seems apparent that this study under the leadership of Snell was partly responsible for the findings that pyridoxal-5-phosphate (Figure 1,d) (codecarboxylase) and pyridoxamine-5-phosphate (Figure 1,e) are important coenzyme forms. The activity of the other forms is probably explained by the existence of certain enzymatic systems which convert them to 2-methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid or pyridoxal-5-phosphate.

In addition to its role in the enzymic decarboxylation of α-amino acids, vitamin B_6 (pyridoxal phosphate) catalyzes many other important biochemical amino acid transformations. Schlenk and Snell (15) afforded the first clue to the concept that phosphorylated pyridoxal was also a coenzyme for the enzymic transamination of L-α-amino acids. In a rather interesting manner they observed that the tissue of rats raised on a vitamin B_6-deficient diet had lower transaminase activity



Pyridoxine Pyridoxal Pyridoxamine



Pyridoxal-5-Phosphate Pyridoxamine-5-Phosphate

Figure 1. The forms of vitamin B_6

than that of rats supplied with sufficient amounts of vitamin B_6. Furthermore, the transaminase activity of tissue from vitamin B_6-deficient rats was stimulated by the conjoint addition of pyridoxal and adenosine triphosphate. Snell (16) also reported the nonenzymic interconversion of pyridoxal and pyridoxamine by heating glutamic acid with pyridoxal or by heating α-ketoglutaric acid with pyridoxamine, and cited these reactions as evidence for the role of a pyridoxal derivative in enzymic transamination.

A phosphorylated pyridoxal derivative was prepared in a rather ambiguous synthetic fashion (81, 96) as early as 1945, and was shown by several investigators to act as the coenzyme of a number of aminopherases isolated from animal tissues and bacterial cells (17, 18, 97, 98). During the same year this vitamin B_6 derivative was identified with codecarboxylase (99, 100), that is to say, the coenzyme of L-tyrosine decarboxylase from S. faecalis (81), and other bacterial α-amino acid decarboxylases (101).

Phosphorylated pyridoxal has been shown to activate the apoenzyme of the transaminase isolated from the microorganism S. faecalis. This transaminase catalyzes the glutamic acid-aspartic acid transamination system (17). Pure glutamic acid-aspartic acid transaminase and pure glutamic acid-alanine transaminase have been isolated and shown to be different enzymes (18). In both systems, however, pyridoxal phosphate

was found to be the responsible coenzyme (17, 18, 19, 20). Pyridoxal phosphate was also found (21) to be the coenzyme for enzymic transamination between α-ketoglutaric acid and the L-α-amino acids, aspartic acid, alanine, valine, leucine, norleucine, tryptophan, tyrosine, phenylalanine, and methionine, as well as transamination systems with α-keto acids such as pyruvic acid, α-ketobutyric acid, and α-ketoisocaproic (22). It is also the coenzyme responsible for the enzymic racemization of D- or L-alanine (23) and D- or L-glutamic acid (24).

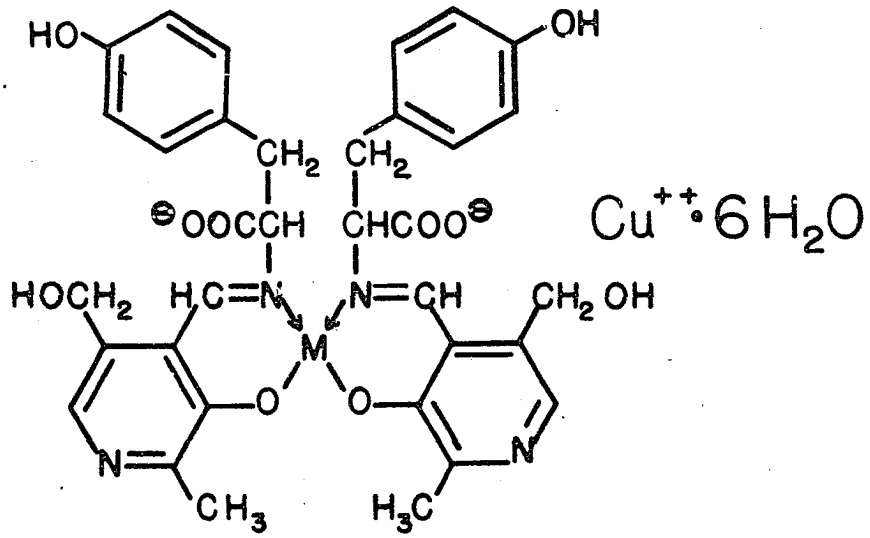
Enzymes containing pyridoxal phosphate catalyze such α,β-elimination reactions as the dehydration and subsequent deamination (25) of serine and threonine, the desulfhydration and subsequent deamination (26) of cysteine, and the cleavage of tryptophan to indole, pyruvic acid and ammonia.

In such cases involving the α, γ-elimination reactions as the enzymic dehydration and subsequent deamination (27) of homoserine, and the enzymic desulfhydration and subsequent deamination (26) of homocysteine, vitamin B_6 is also necessary. Another group of reactions in this category is the enzymic cleavage of cystathionine to cysteine and α-ketobutyric acid or to homocysteine and pyruvic acid (28, 29). The literature contains reports of several other investigations dealing with similar vitamin B_6-dependent enzymes (30, 31, 32, 33, 34, 35, 36, 37), however, it appears that this

coenzyme is also involved in fatty acid metabolism (38) and has been isolated from muscle phosphorylase (39), although its exact functions in the two latter instances are unknown.

The modern concept and interpretation of the mechanism of vitamin B_6 catalysis in α-amino acid biochemistry began with the observation by Metzler and Snell (35) on trace metal ion (Cu^{++}, Fe^{+++}, Al^{+++}) catalysis of the nonenzymic pyridoxal-catalyzed transamination of α-amino acids.

Baddiley (40) described the preparation of water-soluble, green copper chelates of pyridoxal with the α-amino acids glycine, alanine, serine, lysine, isoleucine, valine, threonine, and methionine. This investigator was able to isolate and characterize on the basis of analysis the copper chelate compound (I) of tyrosine and pyridoxal (Figure 2). In view of these facts, Baddiley (Figure 2) assumed the non-enzymic transamination of α-amino acid by pyridoxal as proceeding through the formation of a chelate complex (II) containing one molecule each of pyridoxal, pyridoxamine, the α-amino acid, the α-keto acid and one metal ion. Applying this mechanism, the transamination would be accomplished by an electron-pair displacement in the complex (II) to give (III), which upon hydrolysis would afford the deaminated amino acid and aminated keto acid. During this period the transaminases had not been isolated in the required states of purity as to permit an accurate determination of their metal ion content and need;



(I)

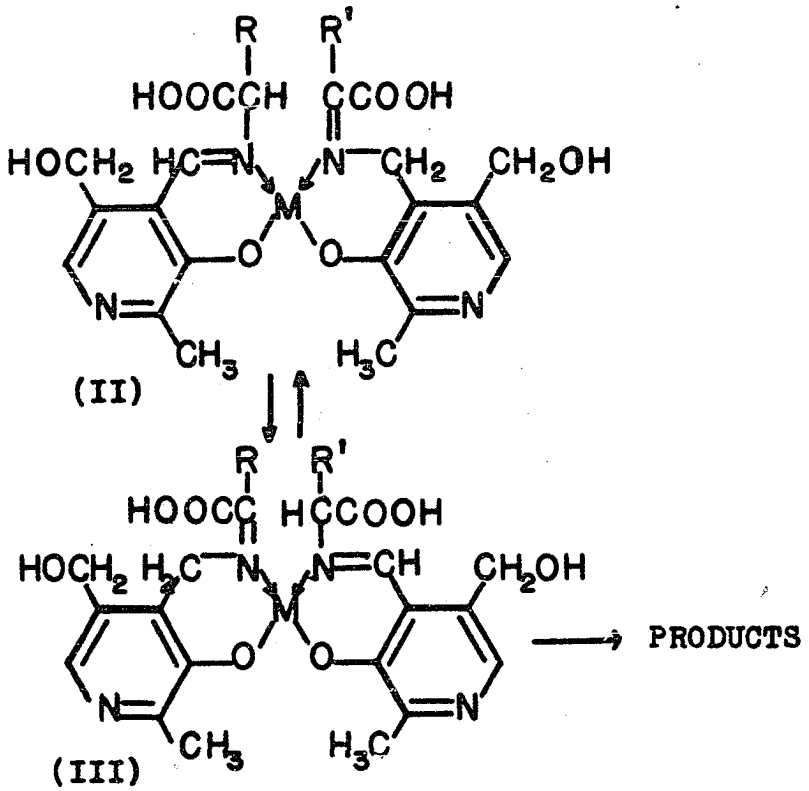


Figure 2. Baddiley mechanism of nonenzymic amino acid transformation (40)

therefore, this particular mechanism could not be extended to biological systems without certain reservations.

Several enzymic amino acid conversions have also been reproduced in nonenzymic systems with pyridoxal and a metal ion as catalysts. A few rather significant conversions of this type are transamination (35, 36), serine deamination (25), cysteine desulfhydration (25), racemization (37), and decarboxylation (41). It has been assumed that both types of reactions follow similar mechanisms, since the nonenzymic reactions parallel very closely the enzymic reactions (33, 42).

It is at this point interesting to note, that on the basis of the information presented thus far, the only structural features of pyridoxal appearing to be absolutely essential for nonenzymic catalysis are the 4-formyl group, 3-phenolic group, and the basic-ring nitrogen atom. The 5-hydroxymethyl group although apparently unnecessary in nonenzymic catalysis, is quite important physiologically as the point of attachment of phosphate (33). The 2-methyl group is likewise considered important only from a physiological standpoint.

A general mechanism for pyridoxal catalysis of α-amino acid transformations has been proposed by the two independent groups of Braunstein and Shemyakin (94, 95) and of Metzler *et al.* (33). The first step of which is the formation of the

Schiff's base (Figure 3, IV) from the amino acid and pyridoxal. This conjugated intermediate is stabilized by the metal ion which induces planarity in the conjugated system through chelate-ring formation to give the complex (Figure 3, V). A concerted electrophilic action by the basic-ring nitrogen atom of the pyridoxal moiety and the chelated metal ion is transmitted through the conjugated system of the chelate (V). This enhances the displacement of an electron pair from the α-carbon atom of the amino acid (VI). One of the three electron pairs can be displaced. However, displacement of the electron pair between the α-carbon atom and the hydrogen atom can lead to racemization, transamination, β-elimination, or γ-elimination reactions. Decarboxylation is promoted by release of the electron pair between the α-carbon atom and the carboxyl group. Release of the electron pair between the α-carbon atom and the alkyl group causes degradation to a lower amino acid homolog. The nature of the amino acid and also the pH, solvent, or catalyst combination determine the nature of the electron pair involved and ultimately which of the three α-carbon bonds is cleaved. A recent review article on the general subject of vitamin B_6 and its enzymes has been written by Braunstein (43).

With the nonenzymic vitamin B_6 catalyzed transformation fairly well investigated and established, Fischer and his associates (44, 45) have directed their attention to the prob-

lem of studying the active sites in several enzymes, with emphasis on the mode of attachment of the pyridoxal phosphate. These workers have revealed that aside from the attachment through the phosphate group, pyridoxal is also bound to the enzyme as the imine of the ε-amino group of lysine. This was accomplished by reducing phosphorylase b with sodium borohydride at pH 4.5, hydrolysis of the reduced enzyme with chymotrypsin, separation of the peptide containing the vitamin B_6 derivative, followed by complete acid hydrolysis of the peptide and isolation and identification of the coenzyme fragment as ε-N-pyridoxyllysine. By similar treatment of cystathionase, and glutamic-aspartic transaminase the identical pyridoxylamine derivative was isolated and characterized (45).

Having presented in a rather concise fashion the work which has been done in the area of the role of vitamin B_6 in amino acid metabolism, it seems only reasonable and proper to mention at this point an investigation which suggests that the present mechanistic approach to the action of pyridoxal is invalid. Gonnard and co-workers (46, 47, 48) have studied the effect of phosphopyridoxal isonicotylhydrazone on 3-(3,4-dihydroxyphenyl) alanine decarboxylase and glutamic-aspartic transaminase and have found this substance behaves as an activator. They find its activity to be greater than that of the natural cofactor, pyridoxal phosphate, and have indicated that this hydrazone does not intervene by splitting off pyridoxal

phosphate. Furthermore, it is decalred that the complete molecule takes part in the reaction, and it can be considered a real coenzyme. It is the opinion of the author that these results although slightly ambiguous do not require a new or different mechanistic theory, but merely fortify that which is presently acknowledged when one considers the recent work of Fischer (44, 45).

In view of these recent observations presented in the preceding discussion this investigation may be considered as having had two objectives. However, its main or primary purpose was the synthesis of a series of model compounds whose structures would incorporate into the individual molecules some of those features presented in the mechanistic models (Figure 3), together with those indicated by the work of Fischer (44, 45), on the mode of binding of vitamin B_6 to the enzyme. These compounds are in other words merely vitamin B_6 derivatives which may possess greater activity in nonenzymic catalysis of amino acid transformations than does pyridoxal and are to be studied later in this manner. A secondary goal of this work was one of possibly obtaining a series of rather potent enzyme inhibitors through a replacement of the hydroxyl group of the 5-hydroxymethyl unit in pyridoxine and pyridoxal, with certain other organic substituents. A study of the inhibitory effect is also to be undertaken in a later investigation.

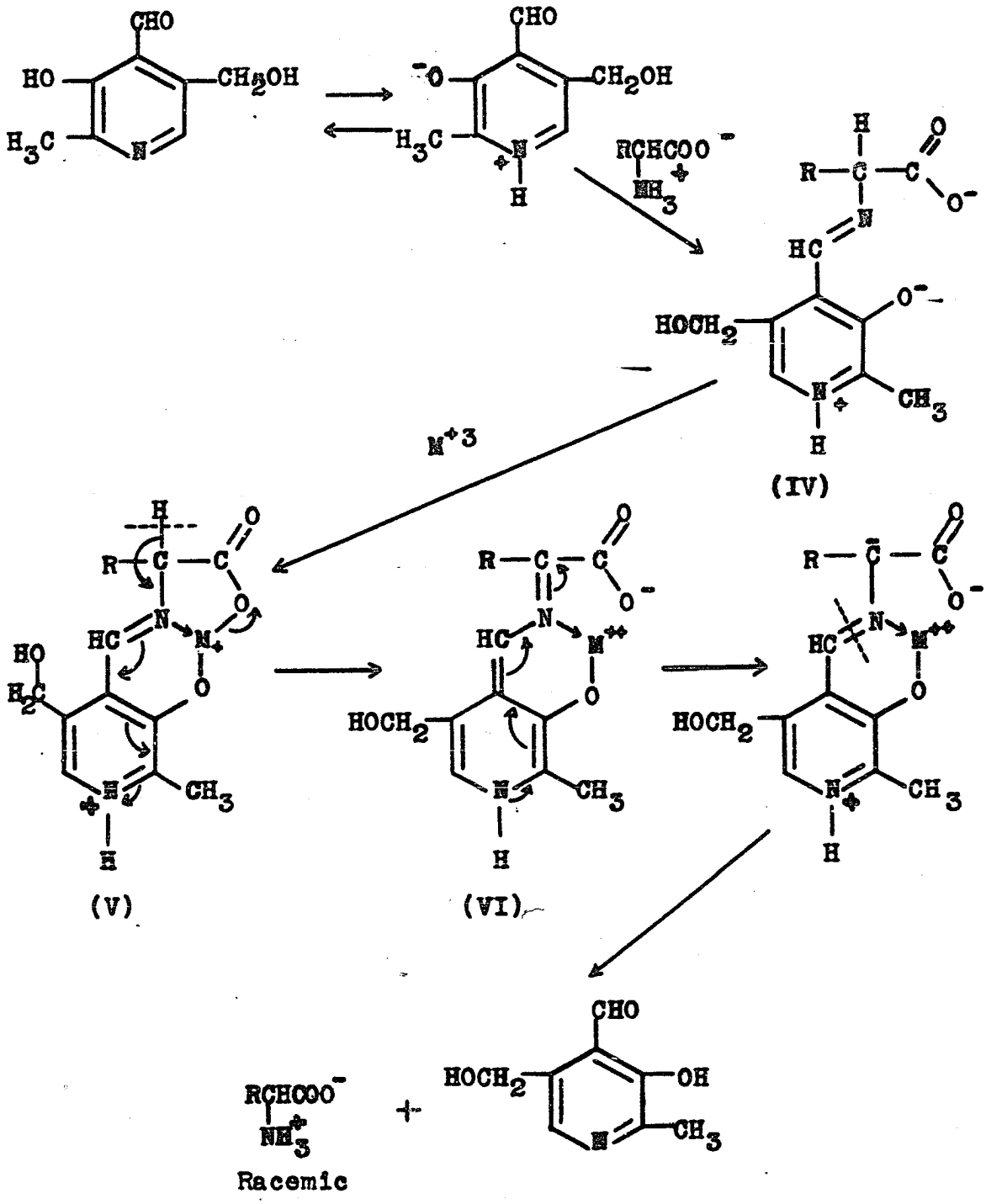


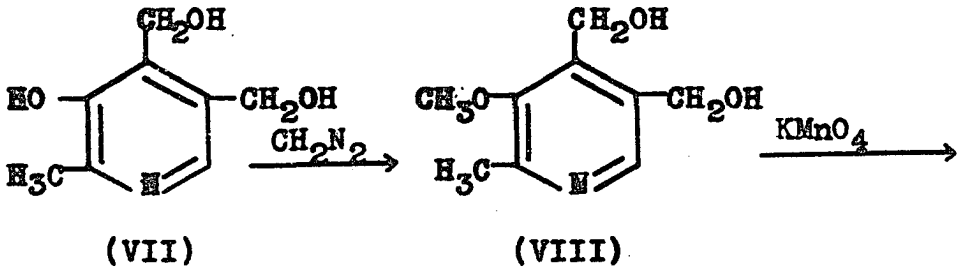
Figure 3. Mechanism of nonenzymic racemization of amino acids

HISTORICAL

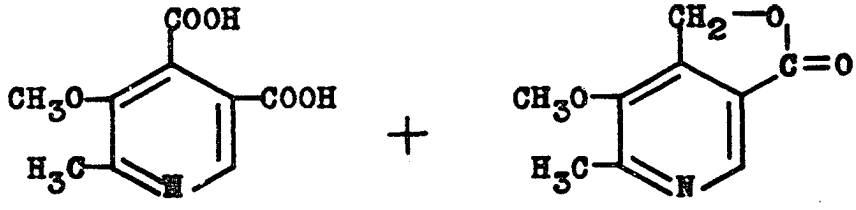
Structure, Synthesis and Derivatives of Pyridoxine

Pyridoxine was initially isolated from yeast (5, 6) and rice bran (3, 4, 7, 49). The vitamin was isolated as the hydrochloride $C_8H_{12}ClNO$ (m.p. 204-206° C., decomp.) of the base $C_8H_{11}NO_3$ (m.p. 160° C.) as was determined by elemental analysis. The empirical formula also represents the molecular formula, a fact based on the observation that the titration curve of the hydrochloride contained only one break. The base was optically inactive and stable to both acid and alkaline hydrolysis, nitrous acid and Fehling's solution. It contained three active hydrogen atoms, a C-methyl group and a phenolic group, however, the evidence indicated the absence of any alkoxy or alkylamino groups. These data suggested that the free-base had the character of a cyclic tertiary amine. Treatment of pyridoxine with ferric chloride gave a red color, quite reminiscent of the color resulting from the action of ferric chloride on 3-hydroxypyridine. The ionization constant of the vitamin and the variation of the ultraviolet absorption spectrum with pH was analogous to that of several 3-hydroxypyridine derivatives. It was at this point that pyridoxine was considered a substituted 3-hydroxypyridine.

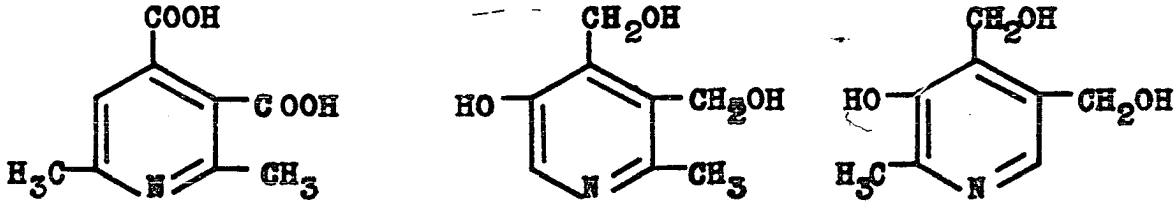
The character and position of the other functional groups were corroborated by oxidation (Figure 4). The methyl ether



(VII) (VIII)



(IX) (X)



(XI) (XII) (VII)

Figure 4. Structure of pyridoxine proposed by Stiller (11)

of pyridoxine (VIII) was obtained by reaction of the vitamin with diazomethane. Upon oxidation, this compound was observed to consume approximately four equivalents of potassium permanganate, yielding a lactone (X) and dibasic acid (IX). The similarity between the dibasic acid and 2,6-dimethylcinchomeronic acid (XI) was evident by a comparison of their pK_a values and ultraviolet absorption spectra. Evidence for the vicinal relationship of the carboxyl groups of the dibasic acid (IX) was afforded by the production of a yellow fluorescent phthalein when the acid was fused with resorcinol. The fact that neither of the carboxyl groups (IX) were in the alpha-position, was indicated by the failure of the dibasic acid to give a color on treatment with ferric chloride. On the basis of these data it was reasonably assumed that the vicinal carboxyl groups were in the 4- and 5-positions of the 3-hydroxypyridine nucleus of the dicarboxylic acid.

The dibasic acid having been derived from pyridoxine by the loss of four hydrogen atoms and the addition of two oxygen atoms, it was properly concluded that hydroxymethyl groups occupied the 4- and 5-positions of pyridoxine. At this stage, the structure of the vitamin was restricted to only two possibilities (VII) and (XII). Evidence for the attachment of the methyl group in the 2-position (VII) was obtained by the formation of a blue color upon treatment of pyridoxine with 2,6-dichloroquinone-chloroimide (Gibb's reagent) in alkaline

solution. Since a blue color is not normally produced by para-substituted phenols, it was inferred that the 6-position of pyridoxine was unsubstituted and structure (VII) was assigned the vitamin by Stiller et al. (11).

Harris and co-workers (12) confirmed the structure of pyridoxine by the synthesis of the dicarboxylic acid (IX) and the lactone (X) from cyanoacetamide (XIV) and α-acetyl-α'-ethoxyacetone (XIII, Figure 5).

Proof of the structure of pyridoxine was also independently afforded by Kuhn and his group (9, 10, 50, 51) (Figure 6,a) obtained through mild permanganate oxidation of the methyl ether (VIII) in neutral solution to the lactone (X). This reaction was considered sufficient proof for a vicinal relationship of the original hydroxymethyl groups in pyridoxine. By alkaline potassium permanganate oxidation of the methyl ether (VIII) to the tricarboxylic acid (XV), which gave a red color on treatment with ferrous sulfate, the C-methyl group of pyridoxine was located in the 2-position of the pyridine nucleus. It was apparent that at least one carboxyl group of the tricarboxylic acid was alpha to the pyridine nitrogen atom. When compound (XV) was monodecarboxylated and converted to the anhydride (XVI), the product failed to give a red color with ferrous sulfate. It was, therefore, concluded that the tricarboxylic acid had only one carboxyl group alpha to the pyridine nitrogen atom. The tricarboxylic acid having been

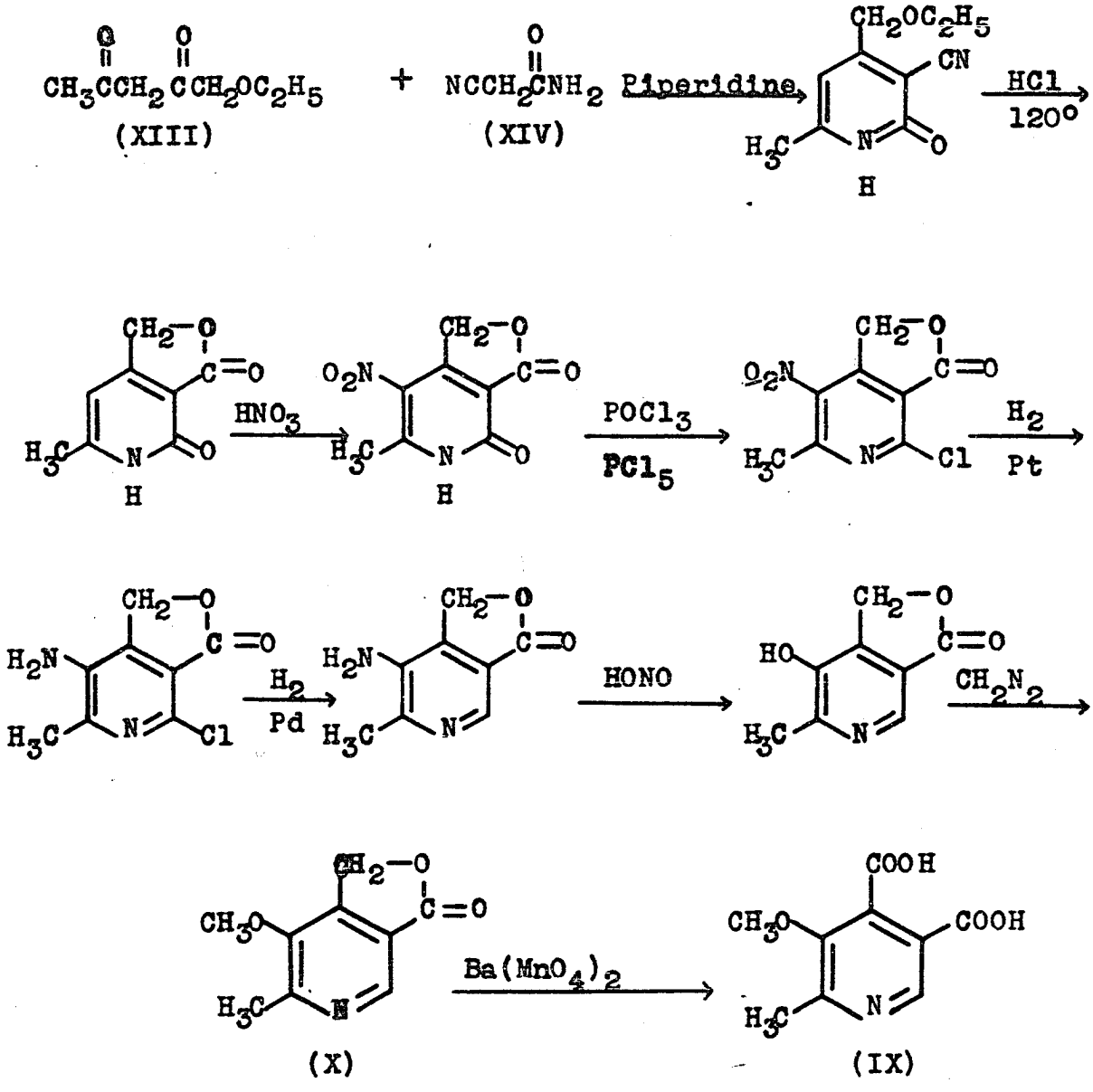


Figure 5. Proof of the structure of pyridoxine (12)

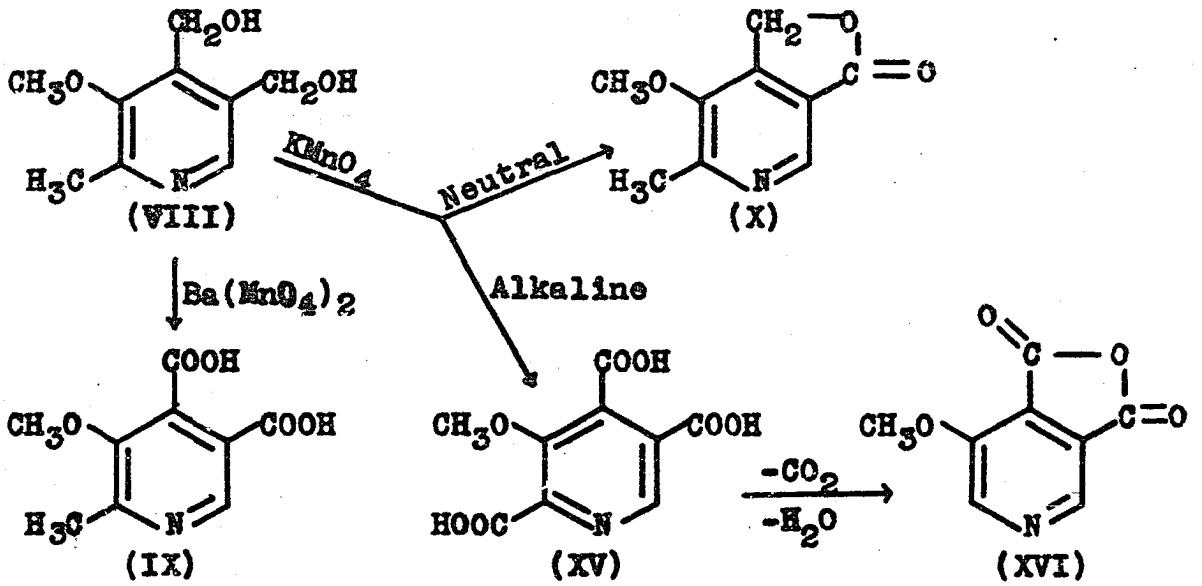


Figure 6a. Proof of the structure of pyridoxine (9, 10, 50, 51)

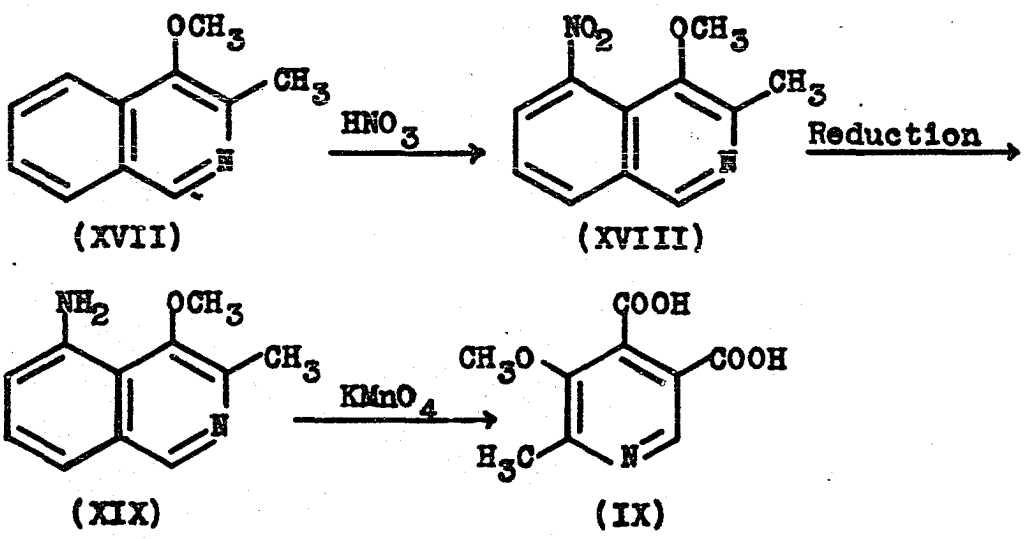


Figure 6b. Proof of the structure of pyridoxine (13)

obtained only by vigorous oxidation, led to the conclusion that its α-carboxyl group had been derived from a 2-methyl group on the pyridine ring.

Utilization of the Folin-Dennis reagent fixed the phenolic group of pyridoxine in the 3-position of the pyridine ring, by the formation of a deep blue color. It had been previously established that 3-hydroxypyridine derivatives with this reagent, give a deep blue color, which is not given by 2- and 4-hydroxypyridine compounds.

In a rather interesting manner Kuhn (13) was also able to confirm the structure of pyridoxine by the synthesis of the dibasic acid (IX, Figure 6,b). By the reaction of 3-methyl-4-methoxyisoquinoline (XVII) with nitric acid, a mononitro compound was obtained, probably the 5-nitro derivative (XVIII), which was then reduced to the corresponding amine (XIX). Oxidative degradation of this amine afforded the dibasic acid (IX).

Ichiba and Michi (52, 53, 54) accomplished the synthesis of the key compound, 3-methoxy-2-methylpyridine-4,5-dicarboxylic acid (IX) in a slightly different manner (Figure 7). After obtaining 1-chloro-3-methoxy-2-methylisoquinoline (XX) it was reduced to 3-methoxy-2-methylisoquinoline (XXI), which yielded on oxidation the desired dicarboxylic acid (IX).

Harris and Folkers (55, 56, 57, 58) in a series of papers described a synthesis of pyridoxine (Figure 8), which began

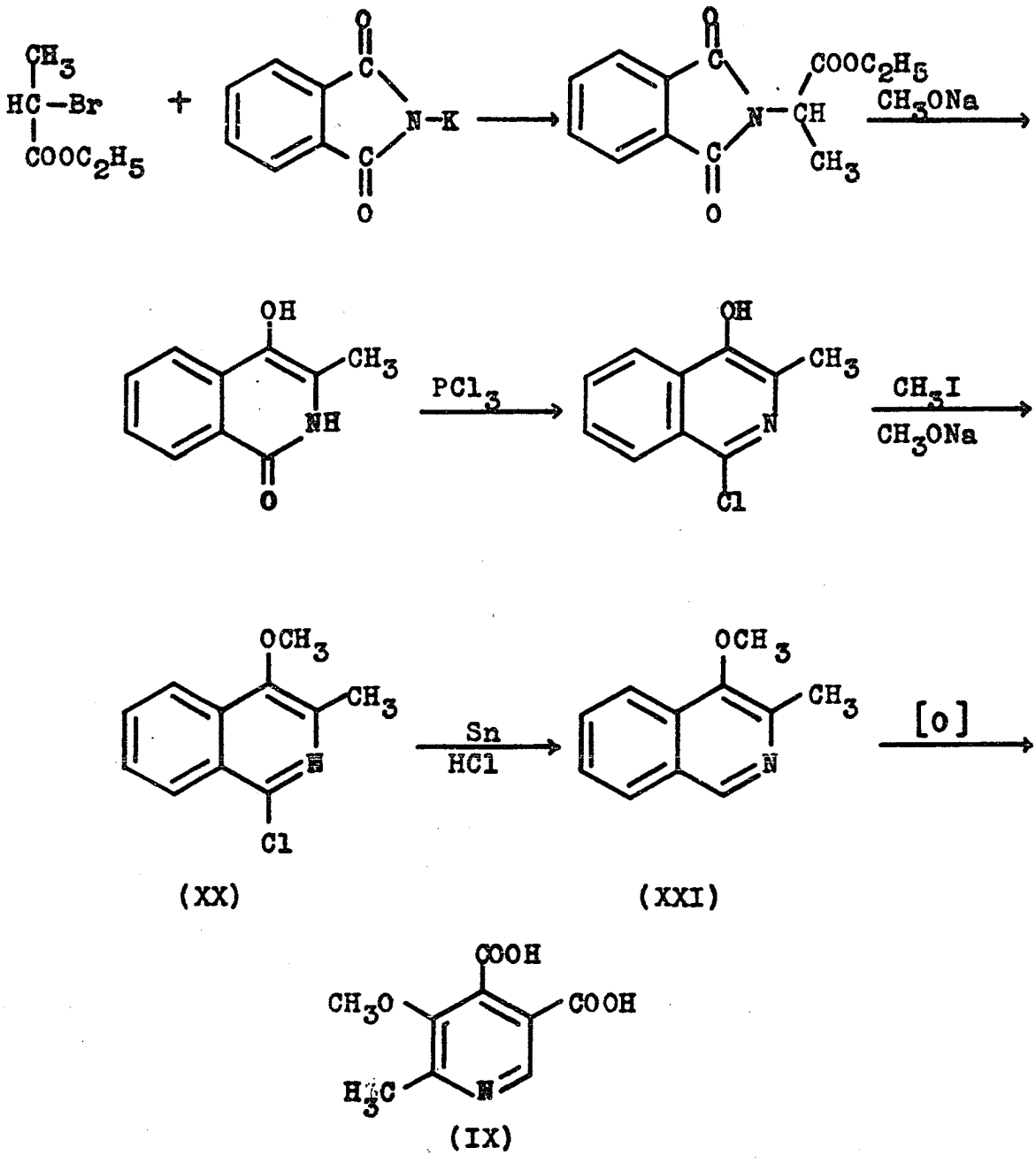


Figure 7. The synthesis of 3-methoxy-2-methylpyridine-4,5-dicarboxylic acid (52, 53, 54)

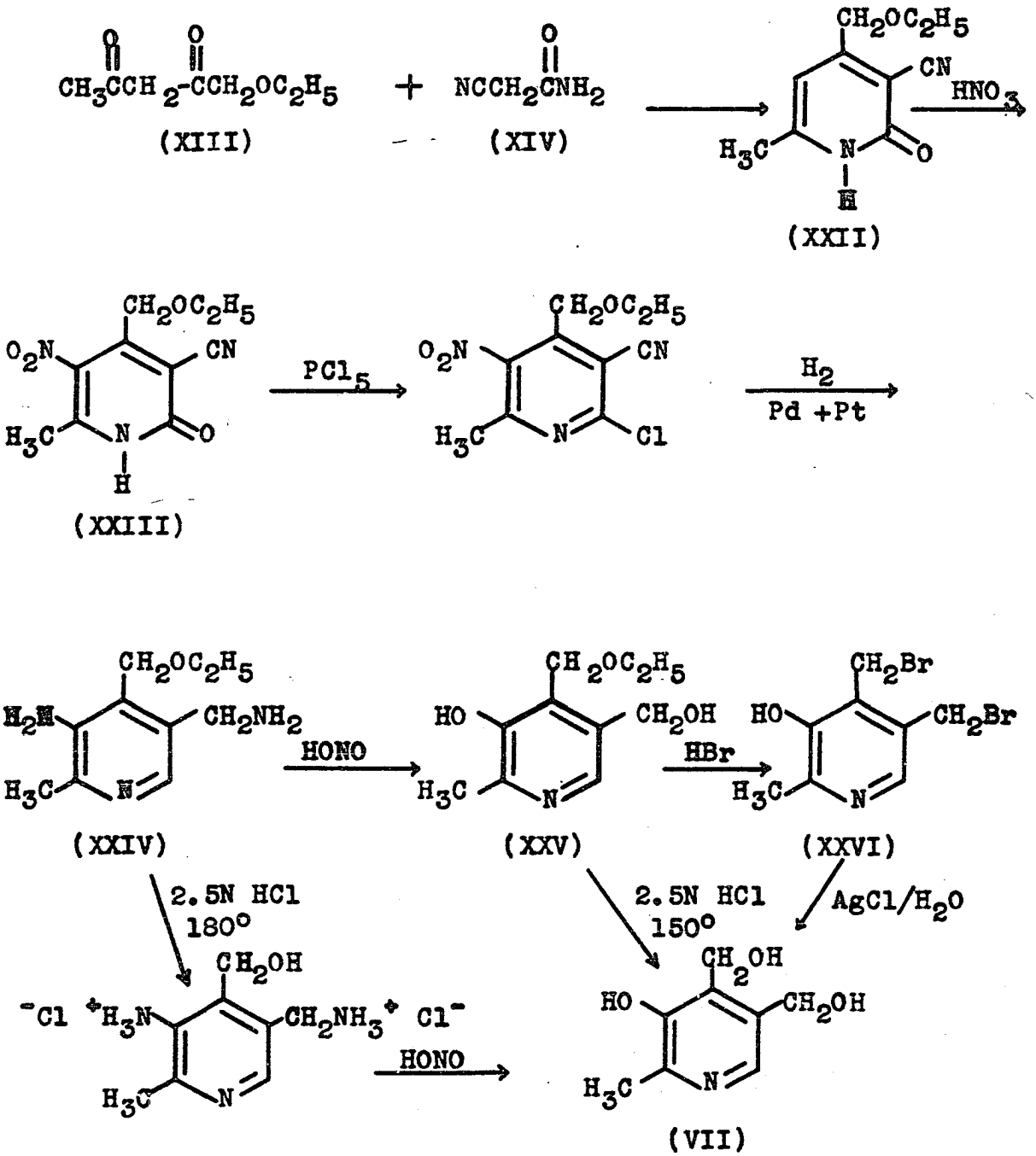


Figure 8. The synthesis of pyridoxine (55, 56, 57, 58)

with the condensation of α-acetyl- α'-ethoxyacetone (XIII) and cyanoacetamide (XIV). The condensation product, 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone (XXII), with nitric acid gave the nitropyridone (XXIII), which upon chlorination and reduction was converted to 3-amino-5-aminomethyl-4-ethoxymethyl-2-methylpyridine (XXIV). Conversion of this diamino compound with nitrous acid to 4-ethoxymethyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine (XXV), followed by hydrolysis, led directly to pyridoxine (VII). Certain variations and improvements (58) utilizing the key starting material, 3-amino-5-aminomethyl-4-ethoxymethyl-2-methylpyridine (XXIV), eliminated the use of constant-boiling hydrobromic acid for ether cleavage, wherewith, the necessity for subsequent hydrolysis of the dibromide (XXVI) was also eliminated. Another consequence of the modified process was a more facile isolation and purification of the intermediate products.

During the same period Kuhn et al. (13) described a synthesis of pyridoxine (VII) using as the starting material the dibasic acid (IX, Figure 9). The sequence of reactions involved conversion of the carboxyl groups to nitrile groups, followed by reduction to aminomethyl groups, and then conversion of the aminomethyl groups to hydroxymethyl groups through diazotization. The final steps of the synthesis involved cleavage of the O-methyl group with hydrobromic acid with subsequent hydrolysis of the dibromide in the presence of

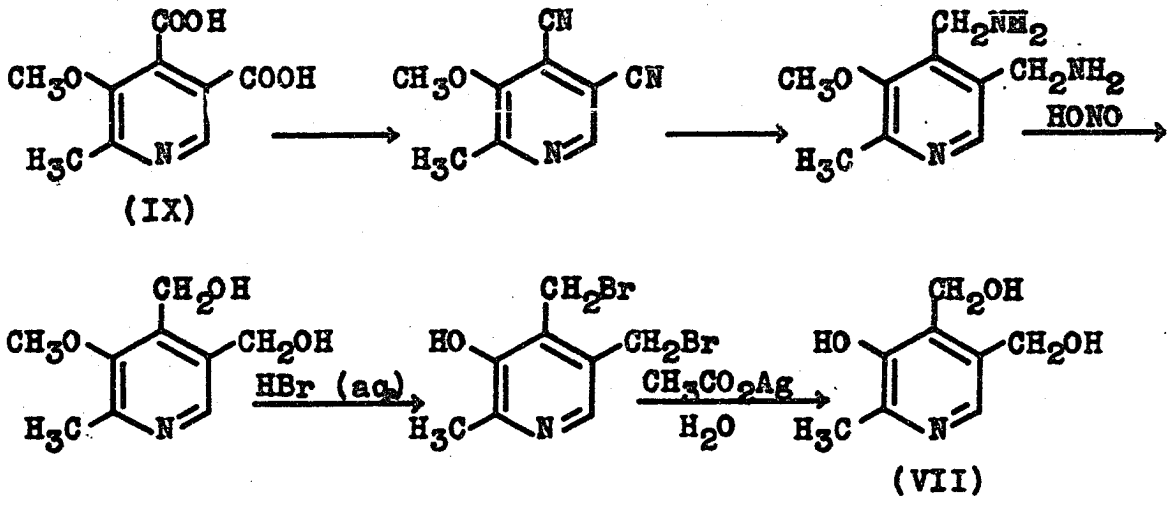


Figure 9. The synthesis of pyridoxine (13)

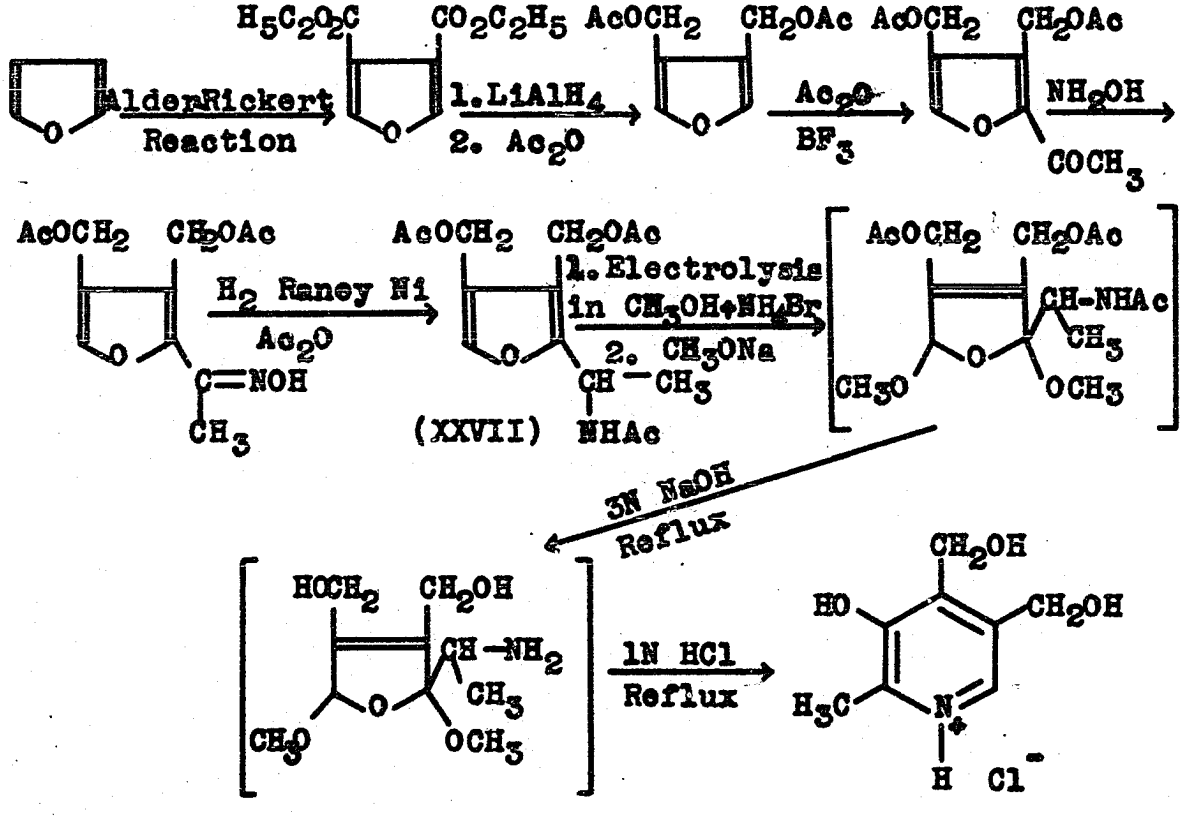


Figure 10. The Elming and Clauson-Kaas synthesis of pyridoxine (61)

silver acetate.

Of interest is the fact that, until 1951, at least one of the two hydroxymethyl groups of pyridoxine was derived from a nitrile group by reduction and subsequent diazotization. The nitrile groups were either introduced in the initial condensation or obtained by conversion of carboxyl groups. Several studies (59, 60, 61, 62) of the lithium aluminum hydride reduction of vicinal diesters of heterocyclic compounds have revealed the rather elegant route of rendering the hydroxymethyl groups accessible in one step from carboxylic acids or esters, thus simplifying considerably the synthesis of pyridoxine. Other workers (63, 64) have employed with amazing success a mixture of sodium borohydride and aluminum chloride in ethylene glycol dimethyl ether.

A more recent communication by Elming and Clauson-Kaas (61) which describes the synthesis of pyridoxine is shown in Figure 10. Probably the most unique feature of this method is the extremely efficient electrolytic 2,5-dimethoxylation of a substituted furan (XXVII) and subsequent opening of the furan ring with simultaneous formation of the pyridine nucleus.

Investigations involving pyridoxine derivatives appear to be almost nonexistent, except for its esters, their preparation (5, 53, 65, 66, 67, 68, 69, 70), and reactions such as hydrogenolysis (71, 72) and oxidation (73). Aside from

these ester derivatives the only others seem to be a group of vitamin B_6 analogs of thiamine (74), wherein the pyrimidine nucleus of thiamine has been replaced with the pyridine nucleus of pyridoxine.

Pyridoxamine and Pyridoxal

During the course of an investigation of the growth-promoting activity of vitamin B_6 (pyridoxine) with lactic acid bacteria, Snell and associates (14) suggested the existence of one or more pyridoxine-like compounds in natural materials which possessed even greater growth-promoting properties than pyridoxine. As a result of this proposal, it was subsequently shown (75, 76, 77) that rather mild amination or oxidation reactions greatly improved pyridoxine as a growth promoter for lactic acid bacteria. In complete accord with these observations 4-aminomethyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine (pyridoxamine, XXVIII), and 4-formyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine (pyridoxal, XXIX) were shown by synthesis (66, 78) to be the biologically active amine and aldehyde (Figures 11 and 13).

The synthesis of pyridoxamine (XXVIII) was first described by Harris et al. (66, 78) (Figure 11). The method involved the amination of either pyridoxine diacetate (XXX) or 3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine (XXXI) with ammonia in methanol at 140° C. Further confirma-

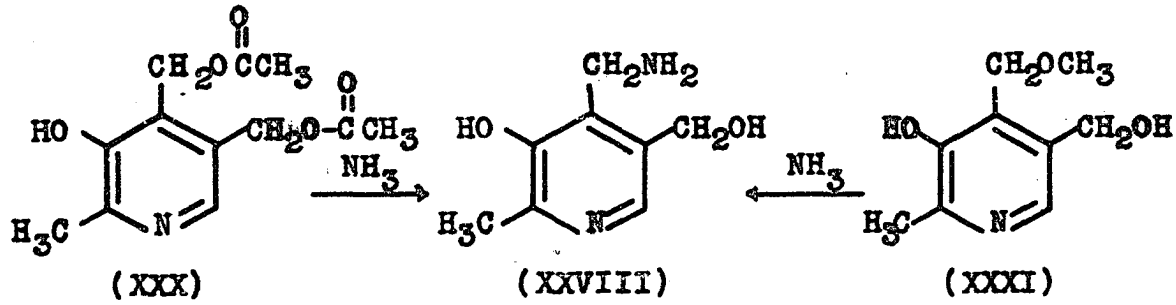


Figure 11. The synthesis of pyridoxamine (66, 78)

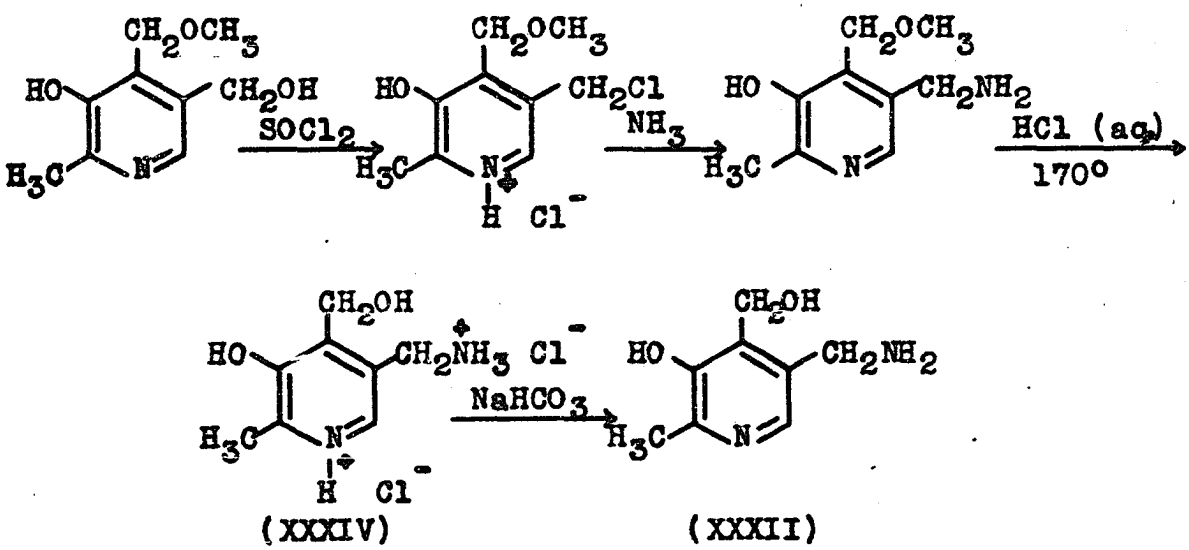


Figure 12. The synthesis of isopyridoxamine (66, 78)

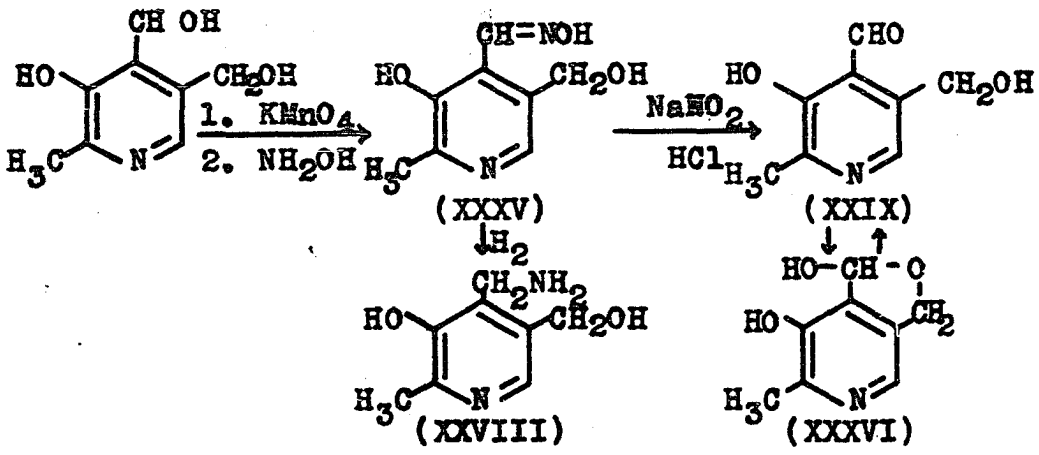


Figure 13. The synthesis of pyridoxal (66, 78)

tion of the structure of pyridoxamine was afforded by the synthesis (Figure 12) of the biologically inactive isomer, 5-aminomethyl-3-hydroxy-4-hydroxymethyl-2-methylpyridine (isopyridoxamine) (XXXII). This compound was prepared by the conversion of 3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine (XXXI) to the 5-chloromethyl derivative (XXXIII), amination with subsequent cleavage of the methoxyl group of the 4-substituent gave the dihydrochloride (XXXIV). Neutralization with sodium bicarbonate yielded (XXXII). As a growth promoter for lactic acid bacteria this isomer had no significant activity and also differed in chemical properties from pyridoxamine.

These investigators (66, 78) (Figure 13) also accomplished the synthesis of pyridoxal (XXIX) by careful potassium permanganate oxidation of pyridoxine, and isolated it as the oxime (XXXV). Treatment of the oxime with sodium nitrite and hydrochloric acid gave pyridoxal, which may exist either as the aldehyde (XXIX) or as the cyclic acetal (XXXVI), but mainly as the latter. Fixation of the formyl group of pyridoxal in the 4-position was established by the catalytic hydrogenation of pyridoxal oxime (XXXV) to pyridoxamine (XXVIII), a conversion which has been recently performed (79) with zinc and acetic acid. The oxidation of pyridoxine to pyridoxal has been greatly improved by the utilization of manganese dioxide and aqueous sulfuric acid (88, 90). Further proof of the

structure of pyridoxal was made by the synthesis of isopyridoxal (XXXVII, Figure 14) (66).

A most interesting and also unique conversion of pyridoxamine to pyridoxine has been reported by Sakuragi and Kummerow (73). These workers prepared several triacyl pyridoxamine derivatives and revealed that treatment of these substances with a refluxing mixture of isoamyl nitrite and acetic acid, converted them to the corresponding triacyl pyridoxine derivatives which upon saponification with alcoholic base yielded pyridoxine. The reaction was also performed on several mono-N-acyl pyridoxamine derivatives with the same results. The synthesis of a few other triacyl, mono-N-acyl, and N-alkyl pyridoxamines was undertaken (80).

Pyridoxal Phosphate and Pyridoxamine Phosphate

Pyridoxal phosphate is now recognized as the coenzyme, codecarboxylase, responsible for all nonoxidative enzymic amino acid transformations. Codecarboxylase-containing enzymes catalyze such reactions as decarboxylation, transamination, racemization, β-elimination, and γ-elimination in amino acid biochemistry.

Pyridoxal phosphate was initially synthesized in low yield through the reaction of phosphorous oxychloride on pyridoxal in the presence of water (81). The isolation of pyridoxal phosphate from such a reaction mixture as the cal-

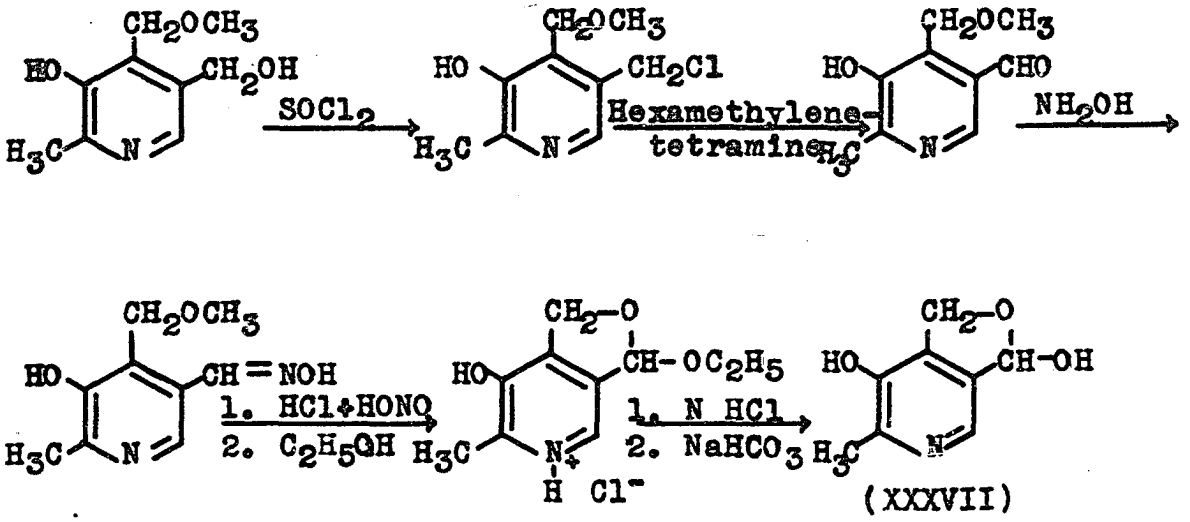
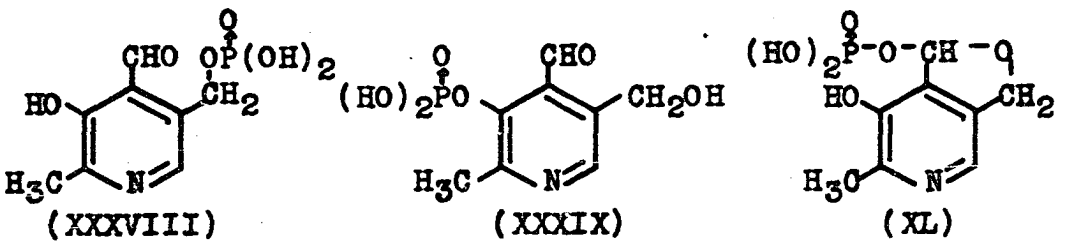


Figure 14. The synthesis of isopyridoxal (66)



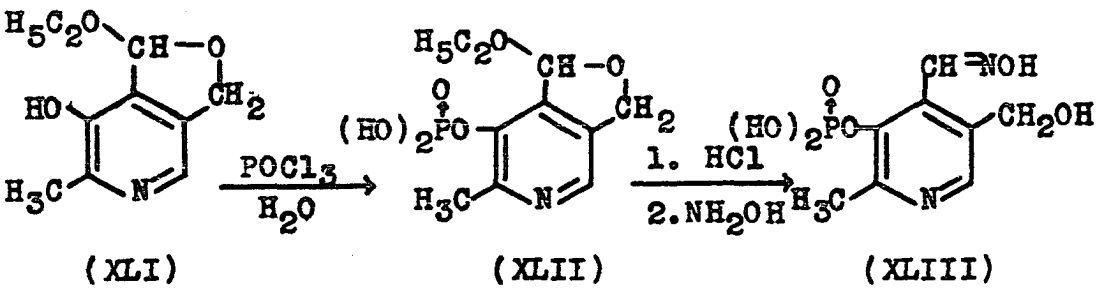


Figure 15. Proof of the structure of pyridoxal phosphate (83, 84, 85)

cium salt and confirmation of the structure of the coenzyme as 4-formyl-3-hydroxy-2-methyl-5-pyridylmethylphosphoric acid (XXXVIII) was reported by Heyl et al. (82). These workers also prepared the methiodides and methochlorides of these vitamin B_6 derivatives.

Pyridoxal phosphate could have had either of three structures, ascertainable from the analytical data (Figure 15) (XXXVIII), (XXXIX), and (XL). The structure with the phosphorylated phenolic group (XXXIX) was eliminated as a result of a negative ferric chloride color test for a free phenolic group, although positive with both the calcium salt and the oxime of pyridoxal phosphate, and the synthesis (83, 84, 85) of 3-pyridoxal phosphoric acid oxime (XLIII), which was not identical with codecarboxylase oxime. By the conversion of pyridoxal monoethyl acetal (XLI) to the 3-phosphorylated pyridoxal monoethyl acetal (XLII), the synthesis of the 3-phosphorylated material was accomplished. The acetal group was removed with hydrochloric acid in the presence of hydroxylamine.

The structure (XL) with a phosphorylated C-4 hemiacetal carbon atom, was excluded from further consideration because the same oxime of pyridoxal phosphate (XLIV) was obtained either by phosphorylation of pyridoxal oxime or by the oxidation of pyridoxime phosphate (XLV) and subsequent conversion to the oxime (Figure 16).

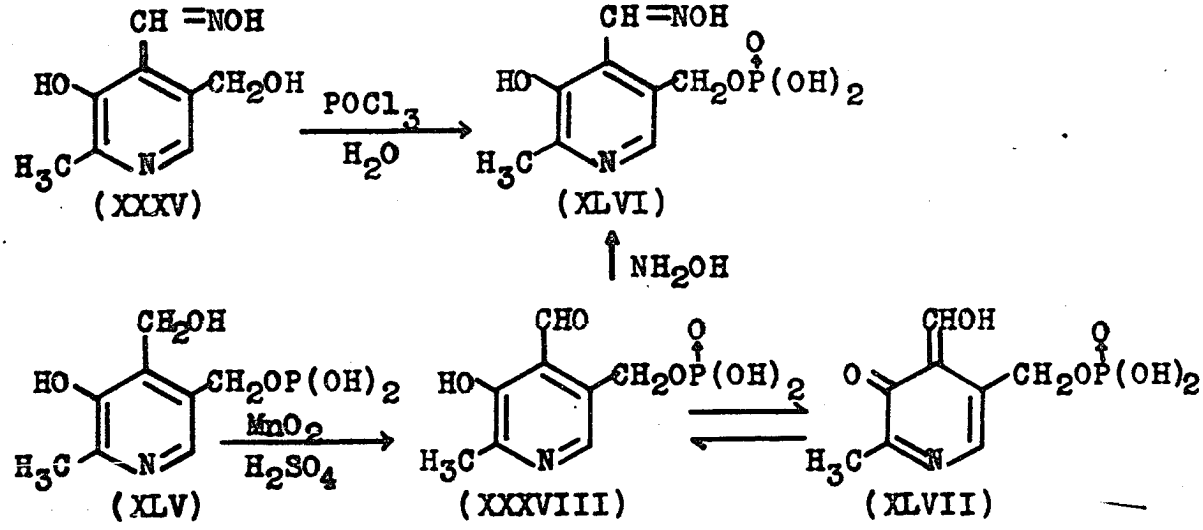


Figure 16. Proof of the structure of pyridoxal phosphate (83, 84, 85)

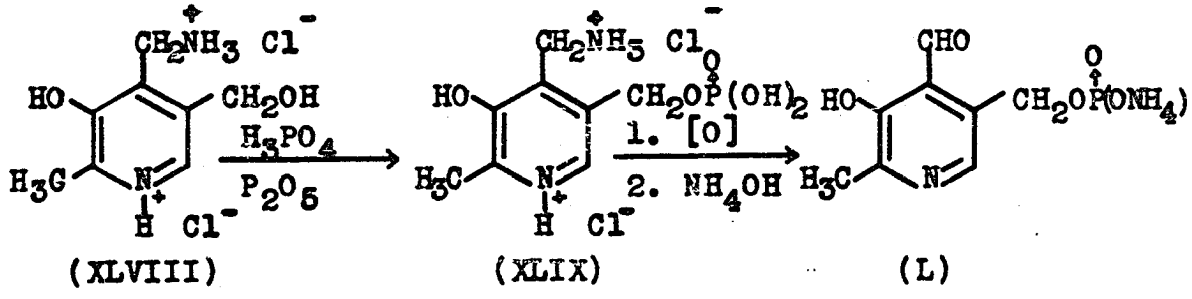


Figure 17. The synthesis of the ammonium salt of pyridoxal phosphate (86)

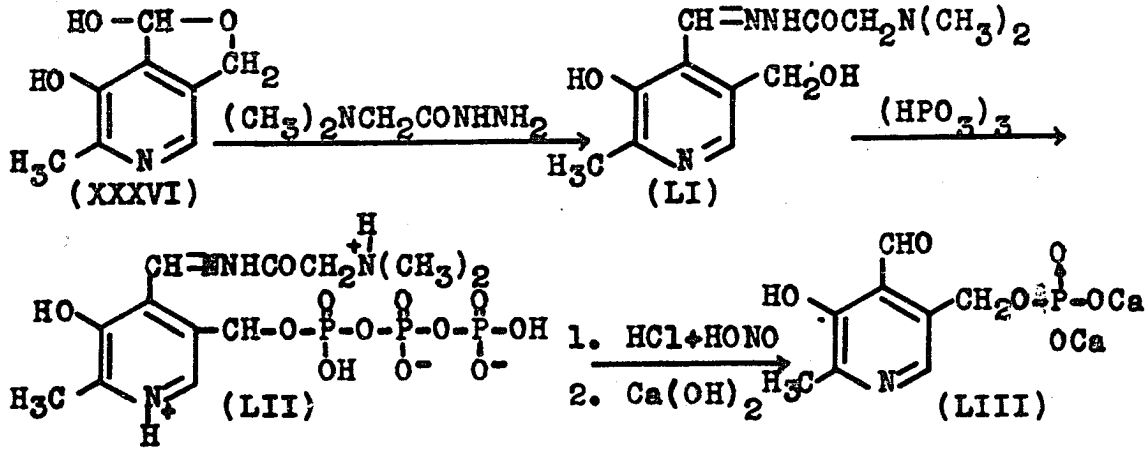


Figure 18. The synthesis of the calcium salt of pyridoxal phosphate (88)

The remaining structure (XXXVIII) must then account for codecarboxylase, however, two unusual properties of this material required further study. Pyridoxal phosphate also gave a negative Gibbs phenol test, which was an indication that the phenolic group is pyridoxal phosphate and is not "free" in the usual sense, but is obviously influenced by the 4-formyl group. A shift of the maxima of the ultraviolet absorption spectrum of pyridoxal phosphate in alkaline solution to longer wavelengths than those of pyridoxal was the character of the second anomaly. The properties were explained on the basis of the existence of a keto form (XLVII) at certain pH's.

The synthesis of pyridoxal phosphate by Wilson and Harris (86) (Figure 17) was achieved by phosphorylation of pyridoxamine dihydrochloride (XLVIII) with anhydrous phosphoric acid, and the product (XLIX) was adsorbed on a column of activated charcoal. Oxygen, adsorbed on the charcoal, converted the amino group of the phosphorylated product to a formyl group so that, on elution with ammonium hydroxide the ammonium salt (L) of pyridoxal phosphate was obtained.

Peterson and Sober (87) in a similar fashion oxidized the aminomethyl group to a formyl group with manganese dioxide. The product (pyridoxal phosphate) was purified by elution from a column of Amberlite XE-64 resin.

The synthesis of pyridoxal phosphate by Viscontini et al. (88) involved first the conversion of pyridoxal (XXXVI) to the

N,N-dimethylglycylhydrazone (LI) (Figure 18), which then was phosphorylated with metaphosphoric acid. The 5-triphosphoric acid ester (LII) produced was hydrolyzed with hydrochloric acid in the presence of nitrous acid, giving pyridoxal phosphate, which was isolated as the calcium salt (LIII).

An unambiguous synthesis of codecarboxylase was described by Baddiley and Mathias (89) (Figure 19). These workers condensed pyridoxine (VII) with acetone in the presence of zinc chloride, and obtained the resulting OO-isopropylidene pyridoxine (LIV). This compound was phosphorylated with phosphoric anhydride in phosphoric acid (LV), and the isopropylidene residue was removed by careful acid hydrolysis giving pyridoxine-5-phosphate (XLV), which was oxidized by the method of Heyl et al. (90) to pyridoxal phosphate (LXVI). Similarly Tanaka (91) has recently reported the synthesis of pyridoxal phosphate and pyridoxamine phosphate using diphenylchlorophosphite as the phosphorylating agent.

Heyl et al. (90) may also be credited with the synthesis of pyridoxamine phosphate by the direct phosphorylation of pyridoxamine in aqueous solution with phosphorous oxychloride. The product was isolated and characterized as the crystalline di-p-toluenesulfonyl derivative.

In addition to those books and papers previously mentioned the literature contains other excellent reviews of the chemistry of vitamin B_6 (92, 93).

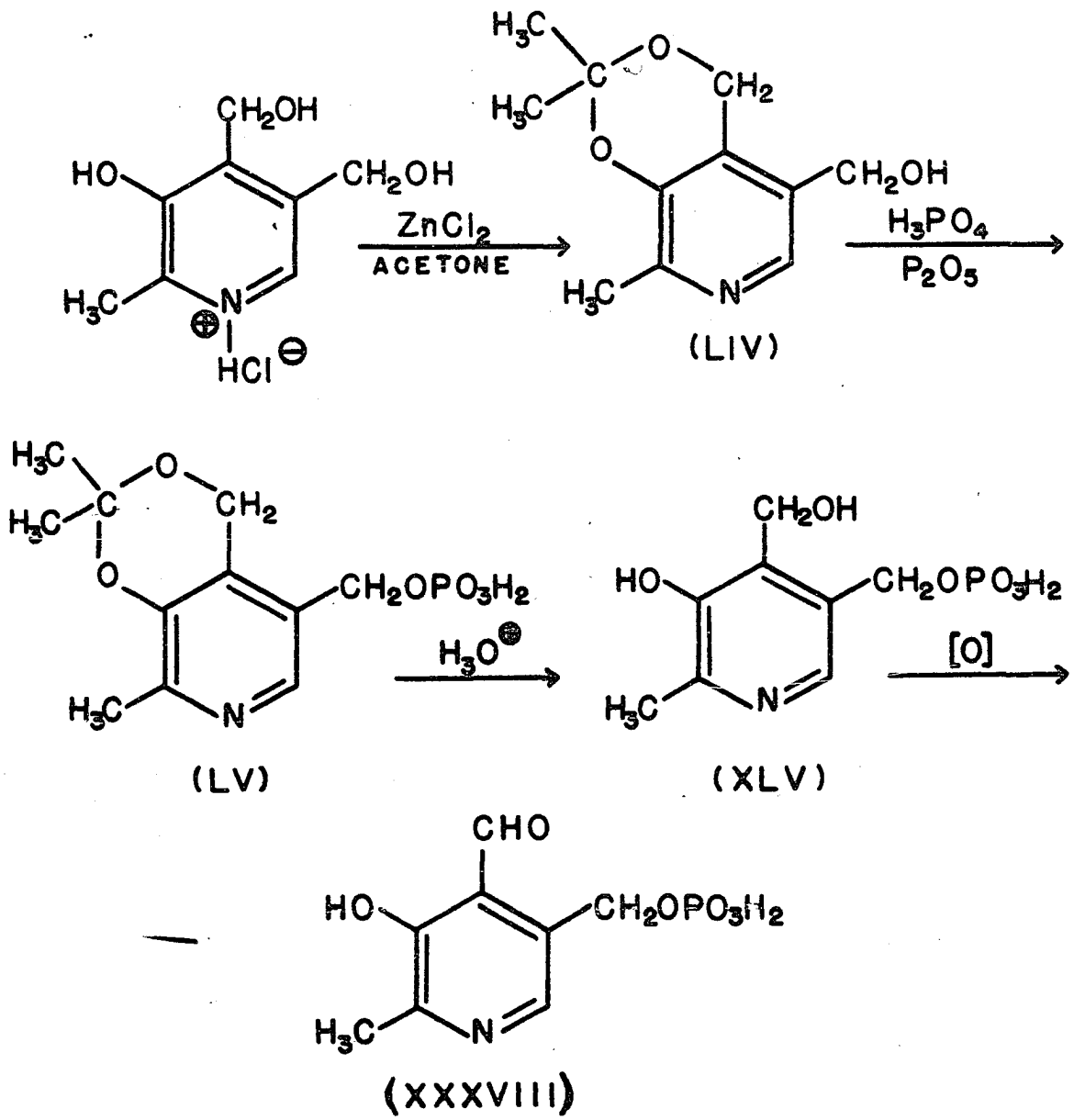


Figure 19. An unambiguous synthesis of pyridoxal phosphate (89)

EXPERIMENTAL

The pyridoxine hydrochloride used in this investigation was kindly furnished through the generosity of the American Cyanamid Company, Hoffmann-LaRoche Incorporated, and Merck and Company, Incorporated. Unless otherwise specified, all compounds used as starting materials were of the highest purity commercially obtainable. All melting points are uncorrected. Reactions involving the use of lithium aluminum hydride were carried out under an atmosphere of dry nitrogen. The elemental analyses of the compounds described herein were performed by Dr. Ing. A. Schoeller, Mikroanalytisches Laboratorium, Kronach, West-Germany. Infrared spectra have been prepared of each of the pure compounds described as well as of many impure fractions. The original copies of these spectra are filed with Dr. David E. Metzler, Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa.

Synthesis of Pyridoxal and N-Substituted Pyridoxal Derivatives

Manganese dioxide "B" This form of manganese dioxide was prepared according to the procedure of Harnfeist et al. (102), and was termed manganese dioxide "B" by these workers. One pound of manganous carbonate was placed (spread to a thickness of approximately one inch) in a porcelain dish and

heated at 280-300° in the presence of air for 20 hours and cooled to room temperature. The product was a very finely powdered manganese dioxide, which was called manganese dioxide "A" by Harnfeist et al. This material was then mixed with 1 l. of a solution of 150 ml. of nitric acid (sp. gr. 1.42; 70-71%) and 850 ml. of water, stirred for 5 minutes and filtered with suction. The acid treated oxide was washed with warm (50°) water until the washings attained a pH of about 6. The wet cake was pressed on the suction filter to remove excess water and was then dried at 230-250°. The friable black cake was crushed to a fine powder and stored in a tightly sealed bottle.

Manganese dioxide (activated) A more active form of manganese dioxide prepared according to Attenburrow et al. (103) was obtained in the following manner. To a hot (90°) solution of 96 g. (0.61 mole) of potassium permanganate in 600 ml. of water there was simultaneously added, with gentle stirring a solution of 84.15 g. (0.5 mole) of manganous sulfate monohydrate in 150 ml. of water and 117 ml. of a 40% sodium hydroxide solution, during a period of 1 hour. The dark brown precipitate was collected on a suction filter and washed with warm (50°) water to remove the last trace of permanganate. The damp cake was pressed on the filter and then dried at 100°. The dried material was pulverized and stored in a tightly sealed bottle.

3-Hydroxy-5-(hydroxymethyl)-2-methylpyridine-5-carboxaldoxime (Pyridoxal oxime) (XXXV) To a stirred suspension of 50 g. of manganese dioxide "B" (102) in 100 ml. of tetrahydrofuran there was added a solution 23.65 g. (0.115 mole) of pyridoxine hydrochloride (VII) and 4.59 g. (0.115 mole) of sodium hydroxide in 100 ml. of warm (70°) water. The mixture was stirred at reflux for 45 minutes, and filtered with suction. The filtrate was concentrated to 100 ml. by distillation under reduced pressure, during which a small amount of the pyridoxal precipitated from solution. The manganese dioxide was washed thoroughly with three 100 ml. portions of hot water and the concentrated filtrate and washing were combined and treated with 9 g. (0.13 mole) of hydroxylamine hydrochloride and 21.3 g. (0.26 mole) of sodium acetate. The oxime which precipitated almost immediately was collected on a filter and dried in a vacuum desiccator over anhydrous calcium chloride. The yield of the pure product was 15 g. (71.5%); m.p. 224-226° with decomposition.

Anal. Calcd. for $C_8H_9N_2O_3$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.88; H, 5.55; N, 14.78.

Following essentially the same procedure described above several other solvents were used with manganese dioxide "B" (102) and an activated manganese dioxide (103). The reaction has been carried out under acidic, basic and neutral conditions and in all cases unless specified otherwise sufficient water

was added in order to dissolve the pyridoxine. The results are shown in Table 1.

Pyridoxal hydrochloride (XXIV) To a solution of 27.07 g. (0.15 mole) of pyridoxal oxime (XXXV) in 430 ml. of 1N HCl there was added dropwise with stirring a solution of 11.6 g. (0.16 mole) of sodium nitrite in 50 ml. of water. The reaction mixture then was heated for 1.5 hours with occasional stirring on a steam-bath in order to remove the last traces of nitrous acid as indicated by testing with starch-potassium iodide paper. This solution was decolorized with Norit-A and evaporated to dryness under reduced pressure on a water-bath. The residue was extracted with three 100 ml. portions of boiling absolute ethanol and these extracts were filtered. The filtrate was evaporated under reduced pressure to a very viscous mass, which was dissolved in 200 ml. of 1N HCl and decolorized with Norit-A. The decolorized solution was concentrated under reduced pressure to a very thin sirup (approximately 70 ml.) and treated with 120-150 ml. of cold acetone. Upon cooling in an ice-bath and triturating, the crystalline product was caused to precipitate from solution. The yield of the desired substance was 26 g. (86%); m.p. 173-4° with decomposition.

Anal. Calcd. for $C_8H_{10}ClNO_3$: C, 47.19; H, 4.95; N, 6.88. Found: C, 47.23; H, 5.02; N, 6.80.

Table 1. Effect of reaction media and conditions on the oxidation of pyridoxine to pyridoxal by manganese dioxide[a]

Moles of pyridoxine	Ratio of MnO_2 "B" to pyridoxine (by wt.)	Medium	°C	Time, hrs.	Yield,[b] %
0.05 Hydrochloride	1:1	150 ml. H_2O + 0.05 mole H_2SO_4	62	3	61
0.24 Hydrochloride	2:1	250 ml. H_2O	25-30	1	40
0.06 Free base	4:1	13 ml. H_2O + 5 ml. t-BuOH[c]	25-30	27	28
0.03 Free base	4:1	100 ml. pyridine + 5 ml. H_2O	25-30	22	29
0.03 Free base	4:1	100 ml. THF[d] + 10 ml. H_2O	25-30	18	65
0.03 Free base	4:1	200 ml. THF + 10 ml. H_2O	62-64 25-30	7 13	30
0.06 Free base	3:1	200 ml. THF + 15 ml. H_2O	62-64	5	37
0.05 Hydrochloride	1:1	200 ml. THF + 0.05 mole H_2SO_4 in 10 ml. H_2O	62-64	4	55

[a]In several cases when a more active MnO_2 (103) was used under the conditions described the yield was always 10-15% less.

[b]The yield represents pyridoxal oxime isolated.

[c]t-BuOH represents tert.-butanol.

[d]THF represents tetrahydrofuran.

Table 1. (Continued)

Moles of pyridoxine	Ratio of MnO_2 "B" to pyridoxine (by wt.)	Medium	°C	Time, hrs.	Yield, %
0.05 Hydrochloride	3:1	75 ml. THF + 25 ml. H_2O + 13 ml. 10% NaOH	25-30	1	64
0.25 Hydrochloride	1:1	400 ml. THF + 0.24 mole NaOH in 200 ml. H_2O	62-64	1	69[a]
0.12 Hydrochloride	2:1	100 ml. THF + 0.12 mole NaOH in 100 ml. H_2O	62-64	0.75	72[a]

1,3-Dihydro-7-hydroxy-1-methoxy-5,6-dimethylfuro[3,4-c]-pyridinium iodide (Monomethyl acetal of pyridoxal methiodide) (LVIII) This compound was prepared according to the method of Heyl et al. (90). A solution of 5 g. (0.024 mole) of pyridoxal hydrochloride in 100 ml. of absolute methanol was refluxed for 15 minutes. After cooling, 3.36 g. (0.04 mole) of solid sodium bicarbonate was added and the mixture was refluxed for an additional 1.5 hours. The mixture of undissolved salts was removed by filtration and washed well with 20 ml. of cold methanol. The combined filtrate and washings were added to 200 ml. of benzene and again filtered to remove a slight turbidity. This benzene-methanol solution was treated 30 ml. of methyl iodide and refluxed for 16 hours. This solution then was evaporated to dryness under reduced pressure on a water-bath. The yellow residue which was obtained, was dissolved in 15 ml. of warm (60°) dimethylformamide, to which boiling ethyl acetate was added until a very slight turbidity was produced. By slow cooling, the pure product was caused to crystallize from this solution. After drying in a vacuum desiccator the yield was 7.52 g. (95%); m.p. 179-180° with decomposition.

1,3-Dihydro-1,7-dihydroxy-5,6-dimethylfuro[3,4-c]-pyridinium chloride (Pyridoxal methochloride) (LIX) A solution of 7.52 g. (0.023 mole) of compound (LVIII) (monomethyl acetal of pyridoxal methiodide) dissolved in 50 ml. of

water was mixed with 6.6 g. (0.046 mole) of freshly prepared silver chloride and stirred mechanically for 3.5 hours at room temperature. The mixture was filtered and the silver halides were washed with three 25 ml. portions of hot (80°) 1N HCl. The combined filtrate and acidic washings were decolorized with Norit-A and evaporated to dryness on a water-bath (60°) under reduced pressure. The solid residue was dissolved in 5 ml. of water and recrystallized by the slow addition of acetone while cooling in an ice-bath. After drying in vacuum over anhydrous calcium chloride, 4.8 g. (91%) of the pure product was obtained which decomposes slowly above 160° without melting.

Anal. Calcd. for $C_9H_{12}ClNO_3$: C, 49.66; H, 5.56; N, 6.44. Found: C, 50.53, 50.41; H, 5.71, 5.80; N, 6.25, 6.25.

1,3-Dihydro-7-hydroxy-1-methoxy-5,6-dimethylfuro[3,4-c]-pyridinium chloride (Monomethyl acetal of pyridoxal N-p-nitrobenzyl chloride (LX) A solution of 3 g. (0.015 mole) of pyridoxal hydrochloride dissolved in 35 ml. of absolute methanol and 15 ml. of absolute methanol saturated with anhydrous hydrogen chloride was refluxed for 15 minutes, and evaporated to dryness under reduced pressure on a water-bath. The residue was dissolved in 50 ml. of anhydrous methanol and treated with 5 g. (0.059 mole) of sodium bicarbonate, then refluxed for 1 hour. The cooled (10°) solution was filtered and the salts were washed with 15 ml. of cold (0°) anhydrous

methanol. To the combined filtrate and washings there then was added 10 ml. absolute methanol and 2.53 g. (0.015 mole) of p-nitrobenzyl chloride and the mixture was heated at reflux for 16 hours. Subsequent to filtration the solution was concentrated by evaporation under reduced pressure, to half its original volume, and upon cooling in an ice-bath the crystalline product separated. After two recrystallizations from absolute methanol 2.4 g. (46%) of the pure product was obtained; m.p. 158-159°.

Anal. Calcd. for $C_{16}H_{17}ClN_2O_5$; C, 54.55; H, 4.87; N, 7.95. Found: C, 54.81; H, 4.82; N, 8.28.

1,3-Dihydro-1,7-dihydroxy-6-methyl-5-p-nitrobenzylfuro-/3,4-c/pyridinium chloride (Pyridoxal N-p-nitrobenzyl chloride) (LXI) Exactly 2 g. (0.006 mole) of the monomethyl acetal of pyridoxal N-p-nitrobenzyl chloride (LX) was dissolved in 15 ml. of warm (65°) 1N HCl. Upon cooling slightly the crystalline product separated from solution and was removed by filtration and dried in vacuo over anhydrous calcium chloride. The yield of pure product was 1.92 g. (99%); m.p. 192-193° with decomposition.

Anal. Calcd. for $C_{15}H_{15}ClN_2O_5$: C, 53.19; H, 4.46; N, 8.27. Found: C, 53.27, 53.31; H, 5.08, 4.94; N, 7.88, 8.00.

5-Benzyl-1,3-dihydro-1,7-dihydroxy-6-methylfuro/3,4-c/-pyridinium chloride (Pyridoxal N-benzyl chloride) (LXII) This compound was prepared from 3 g. (0.015 mole of pyridoxal

hydrochloride and 1.87 g. (0.015 mole) of benzyl chloride in exactly the same manner as that described for the preceding compound, pyridoxal N-p-nitrobenzyl chloride (LXI). In this case, however, the monomethyl acetal derivative was not isolated and characterized. Upon recrystallization form dilute HCl (1N), 2.5ᵹ g. (59%) of the product was obtained; m.p. 194-195° with decomposition.

Anal. Calcd. for $C_{15}H_{16}ClNO_3$: C, 61.33; H, 5.49; N, 4.77. Found: C, 61.1ᵹ, 61,37; H, 5.53, 5.65; N, 4.94, 4.93.

Pyridoxine and Pyridoxamine Derivatives

5-(Hydroxymethyl)-ᵹ,ᵹ,8-trimethyl-4H-m-dioxino[4,5-c]-pyridine (Isopropylidene pyridoxine) (LIV) This substance was prepared according to the method of Baddiley and Mathias (89) with, however, several important modifications. To a filtered solution of 95.41 g. of anhydrous zinc chloride dissolved in 650 ml. of anhydrous acetone there was added 41 g. (0.ᵹ mole) of pyridoxine hydrochloride (VII). This solution then was maintained at reflux with the exclusion of moisture for 1ᵹ hours and then allowed to stand at room temperature for an additional 1ᵹ hours. Approximately 400 ml. of the solvent was removed by distillation under reduced pressure on a water-bath (60°) and the viscous residue was poured slowly with stirring into a solution of 65 g. (1.63 mole) of sodium hydroxide in 400 ml. of a mixture of ice and water and

placed in an ice-bath. During the neutralization of the acidic reaction mixture the temperature was not allowed to rise above 10°. By the careful addition of 6N H_2SO_4 the pH was adjusted to 8.5 and carbon dioxide was bubbled into the mixture until a pH of 7.5 was attained. The precipitate was removed by suction-filtration and washed with 400 ml. of boiling acetone and 200 ml. of boiling chloroform. The aqueous filtrate was discarded and the combined acetone and chloroform washings were evaporated to a gummy mass which partially solidified upon cooling. Subsequent to extraction of this semi-solid residue with boiling acetone, and filtration, the filtrate was treated with 20 ml. of water and evaporated to dryness under reduced pressure. Recrystallization of the solid residue from cyclohexane afforded 38.4 g. (92%) of the desired product; m.p. 109-110°.

Recrystallization of this material from water gave a hydrate with a melting point of 93.94°, which when dried *in vacuo* (0.3 mm.) at 80° over anhydrous magnesium perchlorate or again recrystallized from cyclohexane melts at 109-110°. No attempt was made to determine the amount of water of hydration.

Anal. Calcd. for $C_{11}H_{15}NO_3$: C, 63.14; H, 7.23; N, 6.70. Found: C, 63.20; H, 7.24; N, 6.73.

Isopropylidene pyridoxine hydrochloride was prepared by bubbling anhydrous hydrogen chloride through a solution of

1 g. (0.004 mole) in 50 ml. of dry benzene until precipitation of the salt had ceased. The salt was removed by filtration, and recrystallized by dissolving it in 10 ml. of boiling absolute 2-propanol to which 3 ml. of boiling ethyl acetate then was added. By allowing the solution to cool slowly to room temperature a quantitative yield of the hydrochloride was obtained which melts with decomposition at 197-198°.

Anal. Calcd. for $C_{11}H_{16}ClNO_3$: C, 53.77; H, 6.56; N, 5.70. Found: 53.84; H, 6.24; N, 5.70.

5-(Chloromethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]-pyridine hydrochloride (5-Chloromethylisopropylidene pyridoxine hydrochloride) (LXIV) To a solution of 10.5 g. (0.05 mole) of isopropylidene pyridoxine in 100 ml. of warm (65°) anhydrous benzene there was added with stirring and all at once a solution of 6.54 g. (0.055 mole) of thionyl chloride in 20 ml. of dry benzene. The product precipitated almost immediately and the mixture was heated just to the boiling-point of the solvent, cooled and filtered with suction. The yield of the vacuum dried crude product was 13 g. (98%); m.p. 188-190°. Recrystallization from anhydrous acetone afforded 12.57 g. (95%); m.p. 191-192°.

Anal. Calcd. for $C_{11}H_{15}Cl_2NO_2$: C, 50.01; H, 5.72; N, 5.30. Found: C, 49.99; H, 5.61; N, 5.31.

5-(Cyanomethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]-pyridine (5-Cyanomethylisopropylidene pyridoxine) (LXV) To a mechanically stirred suspension of 2.64 g. (0.01 mole) of 5-chloromethylisopropylidene pyridoxine hydrochloride (LXIV) in 50 ml. of acetone there was added a solution of 4.55 g. (0.07 mole) of potassium cyanide in 17 ml. of water and the stirred mixture was refluxed for 16 hours. The solvent then was removed by distillation under reduced pressure on a water-bath and the oily residue partially solidified on standing at room temperature for 2 hours. Recrystallization of this semi-solid first from water and then from petroleum ether (Skelly B, b.p. 60-71.2°) afforded 1.88 g. (86%) of the pure nitrile; m.p. 90-91°.

Anal. Calcd. for $C_{12}H_{14}N_2O_2$: C, 66.04; H, 6.46; N, 12.85. Found: C, 66.25; H, 6.47; N, 12.69.

5-Hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridineacetic acid (LXVI) A solution of 2.18 g. (0.01 mole) of 5-cyanomethylisopropylidene pyridoxine (LXV) in 5 ml. of concentrated hydrochloric acid was heated at 40° on a water-bath for 45 minutes. A small amount of a crystalline substance which separated was redissolved upon the addition of 5 ml. of cold water. This acidic solution was carefully neutralized to pH 6.0 with 10% aqueous sodium bicarbonate and evaporated to dryness under reduced pressure on a water-bath (60°). The residue was extracted with hot ethanol and the extracts were

evaporated to dryness. The resultant solid was recrystallized twice from a mixture of n-propanol-ethyl acetate. The yield of the acid was 1 g. (50%); m.p. 214-215°.

Anal. Calcd. for $C_9H_{12}N_2O_3$: C, 54.54; H, 6.10; N, 7.07. Found: C, 54.55; H, 6.01; N, 7.18.

Several attempts were made to obtain the amide (LXVI) from the nitrile (LXV) by use of alkaline hydrogen peroxide, however, the material produced by these reactions could not be purified to a constant melting point.

5-(2-Aminoethyl)-3-hydroxy-4-(hydroxymethyl)-2-methylpyridine dihydrochloride (LXVII) To a stirred solution of 0.57 g. (0.015 mole) of lithium aluminum hydride in 50 ml. of anhydrous ether (0°) there was added dropwise, during a period of 30 minutes a solution of 3.27 g. (0.015 mole) of 5-cyanomethylisopropylidene pyridoxine (LXV) in 70 ml. of anhydrous ether and the mixture was stirred for an additional hour at 0°. With continued cooling and stirring 2 ml. of ice water was added cautiously and after 10 minutes 2 ml. of 20% aqueous sodium hydroxide was added, followed by 5 ml. of water. The ether layer was decanted and the moist granular inorganic hydroxides were washed with two 50 ml. portions of ether. The washings were combined with the decanted ether layer and dried over anhydrous sodium sulfate. Subsequent to the removal of the drying agent, and evaporation of the ether under reduced pressure an oily residue was obtained which partially

crystallized on standing. The semi-solid material was dissolved in 40 ml. of 1N HCl, heated on a steam-bath for 15 minutes and evaporated to dryness under reduced pressure. This residue then was dissolved in 150 ml. of hot (75°) absolute ethanol and concentrated by distillation under reduced pressure to 50 ml., which upon standing in a refrigerator overnight (14 hours) afforded a portion of the crystalline product. The concentration process was repeated until 1.5 g. (45%) of the product was obtained; m.p. 190-191°.

Anal. Calcd. for $C_9H_{16}Cl_2N_2O_2$: C, 42.37; H, 6.32; N, 10.98. Found: C, 42.52; H, 6.43; N, 11.34.

5-(2,2-Dicarboxyethyl)-2,2,8-trimethyl-4H-m-dioxino-/4,5-c/pyridine diethyl ester hydrochloride (Diethyl malonate derivative of 5-chloromethylisopropylidene pyridoxine hydrochloride) (LXVIII) To a stirred solution of 0.92 g. (0.04 g. at.) (0.04 mole of sodium ethoxide) of sodium in 50 ml. of absolute ethanol there was added 6.4 g. (0.04 mole) of diethyl malonate and after a period of 15 minutes there was added 5 g. (0.019 mole) of solid 5-chloromethylisopropylidene pyridoxine hydrochloride (LXIV) and 1 g. of potassium iodide. The mixture was stirred at room temperature (25-30°) for 48 hours. The solvent was nearly all removed by distillation under reduced pressure and the oily residue was treated with a solution of 2 g. of sodium bicarbonate in 50 ml. of water, then extracted with three 75 ml. portions of ether. The com-

bined ether extracts were dried over anhydrous sodium sulfate. The drying agent was removed by filtration and anhydrous hydrogen chloride was bubbled through the ethereal solution during a period of 30 minutes. An oil which initially separated, slowly crystallized on standing. The solid was removed by filtration and after three recrystallizations from ethyl acetate the yield of the pure product was 2.5 g. (34%); m.p. 148-149°.

Anal. Calcd. for $C_{18}H_{26}ClNO_6$: C, 55.81; H, 6.76; N, 3.61. Found: C, 56.01; H, 6.76; N, 3.58.

5-(2-Acetamido-2,2-dicarboxyethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]pyridine diethyl ester (Diethyl acetamidomalonate derivative of 5-chloromethylisopropylidene pyridoxine) (LXIX) To a stirred solution of 0.92 g. (0.04 g. at.) (0.04 mole of sodium ethoxide) of sodium dissolved in 50 ml. of absolute ethanol there was added 8.7 g. (0.04 mole) of diethyl acetamidomalonate, and after a period of 15 minutes there was added 5 g. (0.019 mole) of 5-chloromethylisopropylidene pyridoxine hydrochloride (LXIV) and 1 g. of potassium iodide. The mixture was stirred at room temperature (25-30°) for 48 hours. The solvent was removed by distillation under reduced pressure and the oily residue was treated with 50 ml. of 2% aqueous sodium hydroxide, then extracted with four 50 ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The solid residue was recrystallized from petroleum

ether (Skelly B, b.p. 60-71.2°), yielding 5 g. (65%) of pure air-dried product; m.p. 122-123°.

Anal. Calcd. for $C_{20}H_{28}N_2O_7$: C, 58.81; H, 6.91; N, 6.86. Found: C, 58.83; H, 6.75; N, 6.86.

Diethyl[5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridylmethyl]malonate (LXX) A solution of 1.9 g. (0.005 mole) of 5-(2,2-dicarboxyethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]pyridine diethyl ester hydrochloride (LXVIII) in 10 ml. of 1N HCl was heated on a steam-bath for 15 minutes, then cooled and neutralized to pH 8 with 20% aqueous sodium hydroxide. The precipitated crystalline product was air-dried and recrystallized from 80% ethanol. The yield of the desired compound was 1.2 g. (80%); m.p. 134-135°.

Anal. Calcd. for $C_{15}H_{21}NO_6$: C, 57.87; H, 6.80; N, 4.50. Found: C, 57.76; H, 6.67; N, 4.43.

Diethyl acetamido[5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridylmethyl]malonate (LXXI) A solution of 3 g. (0.007 mole) of 5-(2-acetamido-2,2-dicarboxyethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]pyridine diethyl ester (LXIX) in 15 ml. of hot (70°) 1 NHCl was heated on a steam-bath for 15 minutes, cooled and neutralized to pH 8 with 20% aqueous sodium hydroxide. The precipitated crystalline air-dried product was recrystallized from n-propanol, yielding 2.5 g. (93%); m.p. 187-188°.

Anal. Calcd. for $C_{17}H_{24}N_2O_7$: C, 55.79; H, 6.51; N,

7.54. Found: C, 55.13; H, 6.20; N, 7.47.

5-Hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridinepropionic acid hydrochloride (LXXII) A solution of 1 g. (0.003 mole) of diethyl[5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridyl-methyl]malonate in 10 ml. of concentrated hydrochloric acid was heated under reflux for 4.5 hours, then evaporated to dryness under reduced pressure on a water-bath. The residue was recrystallized from a mixture of ethylene glycol monoethyl ether and ethyl acetate, yielding 0.5 g. (68%) of the product; m.p. 188-190°.

This substance (1.18 g., a 25% yield) was obtained also by the acid hydrolysis of the crude compound (LXVIII) without any of the intermediates being isolated. A mixed melting point of the material obtained from each procedure showed no depression (mixed m.p. 188-190°).

Anal. Calcd. for $C_{10}H_{14}ClNO_4$: C, 48.49; H, 5.70; N, 5.66. Found: C, 48.76; H, 5.69; N, 5.86.

2,2,8-Trimethyl-4H-m-dioxino[4,5-c]pyridine-5-carboxaldehyde (Isopropylidene-5-pyridoxal) (LXXIII) A mixture of 20 g. of manganese dioxide "B" (10%), 5 g. (0.024 mole) of isopropylidene pyridoxine (LIV) in 50 ml. of chloroform was stirred at room temperature (25-30°) for a period of 22 hours. The reaction mixture then was diluted with 100 ml. of chloroform and filtered with suction through a one-half inch layer of "Celite". The manganese dioxide was washed with five 50

ml. portions of boiling chloroform and finally with 50 ml. of boiling methanol. The combined filtrate and washings were evaporated under reduced pressure on a water-bath to an oily residue which was caused to crystallize by triturating and cooling in an ice-bath. The solid was decolorized by placing it on a column of alumina (4 inches long and 3/4 inch in diam.) and eluting either with petroleum ether (Skelly B, b.p. 60-71.2°) or a 1:1 mixture of chloroform and petroleum ether (Skelly A, b.p. 26.6-31.1°). The eluent was then evaporated to an oil which solidified on standing. The crude solid was twice recrystallized from petroleum ether (Skelly A, b.p. 26.6-31.1°), wherewith, 4.3 g. (87%) of the pure vacuum-dried product was obtained; m.p. 62-63°.

Anal. Calcd. for $C_{11}H_{13}NO_3$: C, 63.79; H, 6.32; N, 6.76. Found: C, 63.50; H, 6.21; N, 6.72.

2,2,8-Trimethyl-4H-m-dioxino/4,5-c/pyridine-5-carboxaldoxime (Isopropylidene-5-pyridoxal oxime) (LXXIV) To a solution of 2.07 g. (0.01 mole) of isopropylidene-5-pyridoxal (LXXIII) in 15 ml. of 95% ethanol there was added a solution of 0.76 g. (0.011 mole) of hydroxylamine hydrochloride and 0.44 g. (0.011 mole) of sodium hydroxide in 5 ml. of water. The crystalline product separated from solution almost immediately. There was obtained 2.13 g. (96%) of the pure product; m.p. 205-206° with decomposition.

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N,

12.61. Found: C, 59.75; H, 6.08; N, 12.49.

5-(Aminomethyl)-2,2,8-trimethyl-4H-m-dioxino[4,5-c]-pyridine (Isopropylidene pyridoxamine) (LXIII) To a stirred solution of 0.54 g. (0.012 mole) of lithium aluminum hydride in 25 ml. of anhydrous ether maintained at -40° in a "Dry Ice"-acetone bath there was slowly added a suspension of 2 g. (0.009 mole) of isopropylidene-5-pyridoxal oxime (LXXIV) in 100 ml. of anhydrous ether. The cooling bath was removed and mixture was allowed to warm up to room temperature, then refluxed for 10 hours. The mixture was again cooled in a "Dry-Ice"-acetone bath and 10 ml. of water was slowly added followed by 4 ml. of 20% aqueous sodium hydroxide. Subsequent to separating and drying the ether layer over anhydrous sodium sulfate, then was removed by evaporation under reduced pressure to a semi-solid residue. Recrystallization of this residue from cyclohexane afforded 0.6 g. (32%) of the product; m.p. 89-90°.

Anal. Calcd. for $C_{11}H_{16}N_2O_2$: C, 63.44; H, 7.74; N, 13.45. Found: C, 63.51; H, 7.48; N, 13.26.

1,3-Dihydro-3,7-dihydroxy-6-methylfuro[3,4-c]pyridine (Isopyridoxal) (XXXVII) A solution of 1.04 g. (0.005 mole) of isopropylidene-5-pyridoxal (LXXIII) in 10 ml. of 1N HCl was heated on a steam-bath for 15 minutes. The solution was cooled and neutralized carefully to pH 6.5 with 10% aqueous NaOH. Upon standing the crystalline product separated. There

was obtained 0.81 g. (97%) of the vacuum-dried substance, which decomposes slowly above 150° without melting.

Anal. Calcd. for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.35; H, 5.36; N, 8.07.

3-Hydroxy-4-(hydroxymethyl)-2-methylpyridine-5-carboxaldoxime (Isopyridoxal oxime) (LXXVI) A solution of 2.2 g. (0.01 mole) of isopropylidene-5-pyridoxal oxime (LXXIV) in 15 ml. of 1N HCl was heated on a steam-bath for 15 minutes, cooled and neutralized to pH 6.5 with 10% aqueous NaOH. Upon standing the oxime separated. The yield of the pure product was 1.8 g. (99%); m.p. 191-192° with decomposition.

This substance was also prepared in a slightly different manner. A solution of 1.67 g. (0.01 mole) of isopyridoxal (LXXV) in 10 ml. of hot (90°) water was treated with 0.76 g. (0.011 mole) of solid hydroxylamine hydrochloride. Upon cooling this solution to 40° and neutralizing it pH 6.5 with 10% aqueous NaOH, the product precipitated almost immediately. The yield was 1.8 g. (99%); m.p. 191-192° with decomposition. Mixed m.p. with substance obtained above showed no depression.

Anal. Calcd. for $C_8H_{10}N_2O_3$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.64; H, 5.53; N, 15.14.

4-(Aminomethyl)-3-hydroxy-5-(hydroxymethyl)-2-methylpyridine dihydrochloride (Pyridoxamine dihydrochloride) (XXVII) Following the procedure of Testa and Fava (79), 10 g. (0.055 mole) of pyridoxal oxime (XXXV) dissolved in 125 ml. of

glacial acetic acid and mechanically stirred was treated with 10.7 g. (0.26 g. at.) of zinc dust in three portions. The addition of the first portion (about 4 g.) caused the temperature of the mixture to raise spontaneously to about 70-72°, however, it was not allowed to exceed 75°. The temperature of the reaction mixture was allowed to fall to 40° and second portion of zinc dust was added. When the third portion of zinc dust was added (40°) only a moderate warming occurred. The resultant mass was filtered and the mixture of zinc dust and salts was washed with 75 ml. of glacial acetic acid. The combined filtrate and washings were evaporated under reduced pressure on a water-bath (60°) to an oily residue, which was dissolved in 80 ml. of water. The zinc salts in this solution were removed by treatment hydrogen sulfide, then mixed with 5 g. of "Celite" and filtered with suction. The zinc sulfide was washed with 50 ml. of water and the combined filtrate and washings were decolorized with "Norit-A", acidified with 25 ml. of concentrated hydrochloric acid and evaporated to dryness under reduced pressure. The crystalline residue was dissolved in approximately 100 ml. of boiling absolute methanol decolorized with "Norit-A" and concentrated under reduced pressure to a volume of 20 ml. then heated to boiling. To this hot methanolic solution there then was added 50 ml. of boiling n-propanol and upon cooling in an ice-bath the product separated in the form of shining white crystals. Further con-

centration of the mother-liquor afforded an additional small amount of the amine salt. The yield was 12 g. (90%); m.p. 128-129°.

Anal. Calcd. for $C_8H_{14}Cl_2N_2O_2$: C, 39.85; H, 5.85; N, 11.62. Found: C, 39.73; H, 5.89; N, 11.73.

5-(Aminomethyl)-3-hydroxy-4-(hydroxymethyl)-2-methylpyridine dihydrochloride (Isopyridoxamine dihydrochloride) (XXIV) In exactly the same manner as that described above for the preparation of pyridoxamine dihydrochloride (XXVIII), 3.7 g. (85%) of isopyridoxamine dihydrochloride, m.p. 198-199°, was obtained from 3.3 g. (0.018 mole) of isopyridoxal oxime (LXXVI) in 41 ml. of glacial acid and 5.5 g. (0.084 g. at.) of zinc dust. The product was recrystallized from a methanol-ethyl acetate mixture.

When 0.3 g. (0.0014 mole) if isopropylidene pyridoxamine (LXIII) was dissolved in 10 ml. of 1N HCl and on a steam-bath for 15 minutes, then evaporated to dryness. There was obtained 0.33 g. (97%) of material which was identical with the previously mentioned substance; m.p. 198-199°.

Anal. Calcd. for $C_8H_{14}Cl_2N_2O_2$: C, 39.85; H, 5.85; N, 11.62. Found: C, 40.06; H, 5.95; N, 11.96.

2,2,8-Trimethyl-5-(phenyliminomethyl)-4H-m-dioxino-/4,5-c/pyridine (Isopropylidene-5-pyridoxal anil) (LXXIX) A mixture of 2.07 g. (0.01 mole) of isopropylidene-5-pyridoxal (LXXIII) and 0.93 g. (0.01 mole) of aniline was heated on a

steam-bath for 15 minutes and cooled. To the resultant oily mass there was added 2 ml. of 95% ethanol and 5 drops of water. Crystallization of the product was induced by trituration of the ethanolic solution while cooling it in an ice-bath. After recrystallization from 70% ethanol there was obtained 2.67 g. (89%) of the pure product; m.p. 80-81°.

Anal. Calcd. for $C_{17}H_{18}N_2O_2$: C, 72.32; H, 6.42; N, 9.92. Found: C, 72.65; H, 6.48; N, 10.04.

5-(Anilinomethyl)-2,8,8-trimethyl-4H-m-dioxino[4,5-c]-pyridine (Isopropylidene-5-phenylaminomethylpyridoxine) (LXXX)

To a stirred solution of 3.1 g. (0.011 mole) of isopropylidene-5-pyridoxal anil (LXXIX) in 35 ml. of warm (50°) absolute methanol there was slowly added 0.57 g. (0.015 mole) of sodium borohydride. When the evolution of hydrogen had subsided the solution was refluxed for 15 minutes. The reaction mixture then was cooled in an ice-bath and treated with 10 ml. of 20% aqueous NaOH and 20 ml. of ice-water. Continued cooling with trituration caused the crystalline product to separate and an additional 50 ml. of ice-water then was added. The yield of the crude air-dried product was 3.05 g. (98%); m.p. 110-114°. After two recrystallizations from petroleum ether (Skelly B, b.p. 60-71.2°) 3 g. (96%) of the pure material was obtained; m.p. 114-115°.

Anal. Calcd. for $C_{17}H_{20}N_2O_2$: C, 71.80; H, 7.09; N, 9.85. Found: C, 72.15; H, 6.99; N, 9.92.

5-(Anilinomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol (5-N-phenylisopyridoxamine) (LXXXI) A solution of 2.5 g. (0.009 mole) of isopropylidene-5-phenylaminomethylpyridoxine (LXXX) in 15 ml. of 1N HCl was heated on a steam-bath for 15 minutes. The solution was cooled in an ice-bath and adjusted to pH 8 with 20% aqueous NaOH, wherewith, the crystalline product separated immediately. Subsequent to air-drying and recrystallization from absolute ethanol 2.0 g. (93%) of the desired substance was obtained; m.p. 182-183°.

Anal. Calcd. for $C_{14}H_{16}N_2O_2$: C, 68.83; H, 6.60; N, 11.47. Found: C, 68.91; H, 6.67; N, 11.31.

2,2,8-Trimethyl-5-(2-thiazolyliminomethyl)-4H-m-dioxino-/4,5-c/pyridine (Isopropylidene-5-pyridoxal 2-aminothiazole imine) (LXXXII) A solution of 1 g. (0.01 mole) of 2-aminothiazole and 2.07 g. (0.01 mole) of isopropylidene-5-pyridoxal (LXXIII) in 50 ml. of absolute ethanol was refluxed for 8 hours. The alcohol was removed by distillation under reduced pressure and the viscous oily residue was heated on a steam-bath for an additional hour, then cooled and allowed to stand at room temperature (25-30°) for 4 days. After this period of standing in an open flask the entire mass had solidified. Recrystallization from 40% ethanol afforded 2 g. (69%) of the pure, yellow crystalline product; m.p. 113-114°.

Anal. Calcd. for $C_{14}H_{15}N_3O_2S$: C, 58.11; H, 5.23; N, 14.52. Found: C, 58.19; H, 5.41; H, 14.74.

2,2,8-Trimethyl-5-(2-thiazolylaminomethyl)-4H-m-dioximo-/4,5-c/pyridine (Isopropylidene-5-(2-thiazolylaminomethyl)-pyridoxine) (LXXXIII) To a solution of 1.77 g. (0.006 mole) of isopropylidene-5-pyridoxal 2-aminothiazoleimine in 20 ml. of warm (45°) absolute methanol there was added slowly with gentle stirring 0.27 g. (0.007 mole) of sodium borohydride. When the evolution of hydrogen had subsided the mixture was refluxed for 15 minutes. After cooling in an ice-bath for several minutes the methanolic reaction mixture was treated with 20 ml. of ice-water and after the crystalline product began to separate an additional 50 ml. of ice-water was added drop-wise. There was obtained without further recrystallization 1.71 g. (96%) of the desired product; m.p. 118-119°.

Anal. Calcd. for $C_{14}H_{17}N_3O_2S$: C, 57.71; H, 5.88; N, 14.42. Found: C, 57.56; H, 6.17; N, 14.20.

3-Hydroxy-2-methyl-5-(2-thiazolylaminomethyl)-4-pyridine-methanol/5-N-(2-thiazolyl)-isopyridoxamine/ (LXXXIV) A solution of 1.6 g. (0.0055 mole) of isopropylidene-5-(2-thiazolylaminomethyl)-pyridoxine (LXXXIII) in 10 ml. of 1N HCl was heated on a steam-bath for 15 minutes, cooled and adjusted to pH 6 with 20% aqueous NaOH, wherewith, the crystalline product separated from solution. Recrystallization from absolute ethanol afforded 1.3 g. (95%) of the desired substance; m.p. 180-182°.

Anal. Calcd. for $C_{11}H_{13}N_3O_2S$: C, 52.57; H, 5.21; N, 16.72. Found: C, 52.80; H, 5.20; N, 16.67.

Oxidation of Pyridoxine Derivatives

Oxidation of 5-(anilinomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol (2,3-Dihydro-1-hydroxy-6-methyl-2-phenyl-7H-pyrrolo[4,5-c]pyridin-7-ol) (attempted) (LXXXV) To a stirred suspension of 1.2 g. (0.005 mole) of 5-(anilinomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol (LXXXI) in 50 ml. of chloroform there was added 5 g. of manganese dioxide "B" (102), and the mixture was heated under reflux for 15 minutes, cooled and stirring was continued at room temperature (25-30°) for 5 hours. Subsequent to suction filtration the manganese dioxide was washed with 600 ml. of boiling chloroform (until the washings were colorless). The combined red filtrate and washings were concentrated to a volume of 70 ml. under reduced pressure on a water-bath and by the slow addition (during a period of 2 days) of cold (4°) petroleum ether (Skelly B, b.p. 60-71.2°) to chloroform solution (4°), 0.75 g. (63%) of a very dark crystalline substance was obtained, which decomposes slowly above 170° without melting.

Anal. Calcd. for $C_{14}H_{14}N_2O_2$: C, 69.40; H, 5.82; N, 11.57. Found: C, 74.84, 74.60; H, 5.20, 5.26; N, 12.29, 12.32.

Oxidation of 3-hydroxy-2-methyl-5(2-thiazolylamino-methyl)-4-pyridinemethanol (2,3-dihydro-1-hydroxy-6-methyl-2-(2-thiazolyl)-7H-pyrrolo[3,4-c]pyridin-7-ol (attempted) (LXXXVI) To a stirred solution of 1 g. (0.004 mole) of 3-hydroxy-2-methyl-5-(2-thiazolylaminomethyl)-4-pyridine-methanol (LXXXIV) in 40 ml. of tetrahydrofuran (THF) there was added 4 g. of manganese dioxide "B" (10%), and the mixture was heated under reflux for 15 minutes, cooled and stirring was continued at room temperature (25-30°) for 5 hours. Subsequent to suction filtration the manganese dioxide was washed with 300 ml. of boiling THF (until the washings were colorless). The combined brownish-red filtrate and washings were evaporated to dryness under reduced pressure on a water-bath (45°). The brown solid residue was recrystallized by the slow addition of cold (4°) petroleum ether (Skelly B, b.p. 60-71.2°) to a cold (4°) solution of this substance in 25 ml. of chloroform. There was obtained 0.6 g. (60%) of a substance which decomposed slowly above 190° without melting.

Anal. Calcd. for $C_{11}H_{11}N_3O_2S$: C, 53.00; H, 4.45; N, 16.86. Found: C, 53.87, 53.71; H, 4.31, 4.07; N, 16.59, 16.70.

Concentration of the mother-liquor to a volume of 10 ml. followed by the addition of 50 ml. of Skelly B and cooling in a "Dry-Ice"-acetone bath afforded 0.15 g. (15%) of a substance which melted at 108-110° and the infrared spectrum of

which was identical with the substance initially isolated.

Anal. Calcd. for $C_{11}H_{11}N_3O_2S$: C, 53.00; H, 4.45; N, 16.86. Found: 52.55, 52.74; H, 4.20, 4.38; N, 15.34, 15.17.

Oxidation of 5-(2-aminoethyl)-3-hydroxy-4-(hydroxymethyl)-2-methylpyridine dihydrochloride (7,8-Dihydro-3-methyl-2,6-naphthyridin-4-ol) (attempted) (LXXXVII) To a stirred solution of 1 g. (0.0039 mole) of 5-(2-aminoethyl)-3-hydroxy-4-(hydroxymethyl)-2-methylpyridine dihydrochloride (LXVII) in 20 ml. of water there was added 20 ml. of tetrahydrofuran (THF), 0.16 g. (0.004 mole) of solid sodium hydroxide, and 3 g. of manganese dioxide "B" (10%) and the mixture was heated under reflux for 45 minutes. The manganese dioxide was removed by suction filtration and washed with 50 ml. of boiling 50% aqueous THF. The combined filtrate and washings were evaporated to dryness under reduced pressure on a water-bath (50°). The very dark green solid residue was recrystallized from 15 ml. of cold (4°) THF by the slow addition of cold (4°) petroleum ether (Skelly B, c.p. 60-70.1°). There was obtained 0.3 g. (48%) of a dark green solid which melts at 230-231° with decomposition.

Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.21; N, 17.27. Found: C, 51.89, 51.70; H, 5.29, 5.13; N, 13.48, 13.45.

Oxidation and hydrolysis of diethyl acetamido[5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridylmethyl]malonate (4-Formyl-5-hydroxy-6-methyl-3-pyridinealanine dihydrochloride (LXXXVIII)

To a stirred solution of 1.53 g. (0.004 mole) of diethyl acetamido [5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridyl-methyl]malonate (LXXI) in a mixture 20 ml. each of water and tetrahydrofuran (THF) there was added 5 g. of manganese dioxide "B" (10). The mixture was heated for 1 hour under reflux and filtered with suction. Subsequent to washing the manganese dioxide with 300 ml. (50 ml. portions) of boiling THF the combined filtrate and washings were evaporated under reduced pressure on a water-bath (50°) to a glassy yellow residue. The residue was dissolved in 20 ml. of concentrated hydrochloric acid and refluxed for 4.5 hours. The acid was removed by evaporation under reduced pressure on a water-bath (50°). The solid yellow residue was recrystallized from a mixture of n-propanol and ethyl acetate yielding 1.1 g. (88%) of the desired substance which melts at 128-130° with decomposition.

Anal. Calcd. for $C_{10}H_{14}Cl_2N_2O_4$: C, 40.42; H, 4.75; Cl, 23.86; N, 9.43. Found: C, 39.44, 39.91; H, 4.82, 4.96; Cl, 24.00; N, 9.56.

Derivatives of 3-Hydroxymethylpyridine

3-Chloromethylpyridine hydrochloride (LVI) To a stirred solution of 11.3 g. (0.095 mole) of thionyl chloride in 20 ml. of anhydrous tetrahydrofuran (THF) cooled to a temperature of -5° in an ice-salt mixture there was added all at once a solution of 10 g. (0.092 mole) of 3-hydroxymethylpyridine (LXXXIX) in 50 ml. of dry THF. The product separated almost immediately and mixture was kept in the ice-salt mixture for an additional 10 minutes, then while stirring heated to 60° on a steam-bath. The mixture then was evaporated to dryness under reduced pressure on a water-bath (50°) and solid residue was recrystallized from dry acetone containing 0.5 ml. of absolute ethanol to remove the excess thionyl chloride. There was obtained 14.9 g. (99%) of the very hygroscopic, vacuum dried product; m.p. 143-144°.

Anal. Calcd. for $C_6H_7Cl_2N$: C, 41.41; H, 4.05; N, 8.05. Found: C, 41.16; H, 4.44; N, 8.43.

3-Bromomethylpyridine hydrobromide (LXXVII) A solution of 10 g. (0.092 mole) of 3-hydroxymethylpyridine (LXXXIX) in 150 ml. of 48% hydrobromic acid was refluxed for 6 hours, then evaporated to dryness under reduced pressure on a water-bath. The solid residue was recrystallized from n-propanol and dried in vacuum over sulfuric acid. The yield of the pure material was 2- g. (86%); m.p. 151-152°.

Anal. Calcd. for $C_6H_7Br_2N$: C, 28.49; H, 2.79; N, 5.54.

mole) of 3-bromomethylpyridine hydrobromide and 1.71 g. (0.012 mole) of 4-methyl-5-(β-hydroxyethyl)thiazole in 10 ml. of absolute methanol was allowed to stand at room temperature (25-30°) in stoppered flask for 5 days after which the crystalline product had precipitated from the solution. The solvent was decanted and solid was recrystallized from 95% methanol solution by the slow addition of ether in a refrigerator over a period of 2 days. There was obtained 2.4 g. (51%) of the product (dried in vacuum over anhydrous calcium chloride) which decomposes slowly above 200° and melts with complete decomposition at 282-284°.

Anal. Calcd. for $C_{12}H_{16}Br_2N_2OS$: C, 36.38; H, 4.07; N, 7.07. Found: C, 37.40; H, 4.01; N, 7.39.

A similar attempt was made to prepare the analogous 3-(β-picolyl)-4-methyl-5-(β'-hydroxyethyl)-thiazolium chloride hydrochloride, however, all attempts to crystallize this substance failed.

3-Pyridylmethyl p-toluenesulfonate (attempted) (LXXV)

To a stirred solution of 1.75 g. (0.0092 mole) of p-toluenesulfonyl chloride in 10 ml. of anhydrous tetrahydrofuran cooled in an ice bath there was added dropwise during a period of 5 minutes, 1 g. (0.0092 mole) of 3-hydroxymethylpyridine (LXXXIX) and the reaction mixture was allowed to stand in the refrigerator for 24 hours. After removal of the solvent under reduced pressure all attempts to isolate a pure crystalline

product failed. Several other attempts were made using different solvents and in one instance no solvent was employed, however, the desired sulfonate ester was not obtained.

DISCUSSION

Synthesis of Pyridoxal and N-Substituted Pyridoxal Derivatives

The synthesis of pyridoxal hydrochloride (XXIV) (Figure 20) has been accomplished by an improved method, which has afforded a consistently higher yield of the product than previously described procedures. This method involves the oxidation of pyridoxine hydrochloride which has been neutralized with an equimolar amount of sodium hydroxide in a mixture of equal volumes of water and tetrahydrofuran. A quantity of manganese dioxide "B" (102) equal to twice the weight of pyridoxine hydrochloride is added to the mechanically stirred mixture, which then is heated under reflux for three-quarters of an hour. The manganese dioxide is removed and the aldehyde is isolated as the oxime (XXXV). The oxime is converted to pyridoxal hydrochloride by treatment with nitrous and hydrochloric acids. Before this procedure was worked out a number of attempts were made to find a suitable set of conditions which could be easily followed and repeated. This work is summarized in Table 1. Noteworthy is the fact that when the reactions were carried out over long periods of time the yields of the oxime (XXXV) were decidedly low. It is believed that the aldehyde is first formed and is then oxidized further to the acid. The only clue to this possibility is the blue fluorescence produced by the reaction mixture under an ultra-

violet lamp after removal of the manganese dioxide. Pyridoxic acid lactone (4-carboxy-3-hydroxy-5-hydroxymethyl-2-methylpyridine lactone) is known to fluoresce under these conditions, whereas, pyridoxine and pyridoxal do not. Another interesting observation made during the course of this particular phase of this work was that the use of a more active form of manganese dioxide (103), obtained from manganous sulfate and potassium permanganate, resulted in still lower yields of pyridoxal (isolated as the oxime). This factor tends to verify the notion that manganese dioxide is capable of oxidizing pyridoxine to pyridoxic acid.

The synthesis of three N-substituted pyridoxal derivatives was performed in each case by a method related to that of Heyl et al. (90) (Figure 20). Pyridoxal hydrochloride (XXIV) when refluxed with anhydrous methanol or anhydrous methanol containing hydrogen chloride gave the monomethylacetal of pyridoxal after treatment with solid sodium bicarbonate. During the course of this investigation the acetal intermediate was not isolated. The methanolic solution containing this material was, in one instance, treated with methyl iodide and after a period of heating under reflux the methiodide of the monomethylacetal (LVIII) was obtained. Treatment of an aqueous solution of the methiodide with freshly prepared silver chloride and then dilute hydrochloric acid produced the expected pyridoxal methochloride (LIX). In a similar

manner p-nitrobenzyl chloride when allowed to react with the monomethyl acetal of pyridoxal in methanolic solution gave the corresponding monomethyl acetal N-p-nitrobenzyl chloride (LX) which upon hydrolysis was converted to the desired pyridoxal derivative (LXI). The N-benzyl chloride derivative (LXII) was synthesized in an identical manner without isolating its monomethyl acetal intermediate.

Pyridoxine and Pyridoxamine Derivatives

In order to obtain a group of pyridoxine derivatives on which transformations and/or modifications have been made only on the 5-hydroxymethyl group, one important problem becomes immediately apparent. It is rather obvious that pyridoxine is a 4,5-dihydroxymethyl compound and practically any attempt to change one of these groups without simultaneously affecting the other seems almost impossible. However, oxidation of the 4-hydroxymethyl group to the 4-formyl, as in the synthesis of pyridoxal is an extremely facile matter. Another example of this kind is the more rapid hydrogenolysis of the 5-hydroxymethyl group or its acetate ester than that of the 4-hydroxymethyl group and/or its acetate ester (71, 72). Aside from one other reaction to be discussed later these two transformations appear to be the only two performed on this molecule wherein these groups are individually affected. It then is necessary either to investigate some other reactions of

alcohols or merely to find some method of blocking the one and leaving the other free.

Because this problem is not new other investigators have also dealt with it. Probably the most elegant solution in the area of vitamin B_6 chemistry was described by Baddiley and Mathias (89) in a report of an unambiguous synthesis of pyridoxal-5-phosphate (Figure 19). These workers were able to block the 4-hydroxymethyl group by merely forming the intramolecular ketal of acetone with the 3-hydroxy- and 4-hydroxymethyl groups of pyridoxine, thus leaving the 5-hydroxymethyl group free to be the only possible position that can be phosphorylated. The isopropylidene pyridoxine (LIV) being a ketal is hydrolyzed quite easily with dilute acid, whereby, restoration of the 4-hydroxymethyl and the 3-hydroxy groups are readily attained. Having considered these facts it was decided that this would be the proper approach to the synthesis of a series of pyridoxine derivatives in which the hydroxyl group of the 5-hydroxymethyl moiety had been replaced with certain other organic substituents.

In a manner very similar to that reported (89), pyridoxine was allowed to react with dry acetone in the presence of anhydrous zinc chloride (Figure 21). It was found to be more expedient, however, if the reaction mixture was made alkaline with aqueous sodium hydroxide rather than aqueous barium hydroxide, subsequent to the removal of the excess

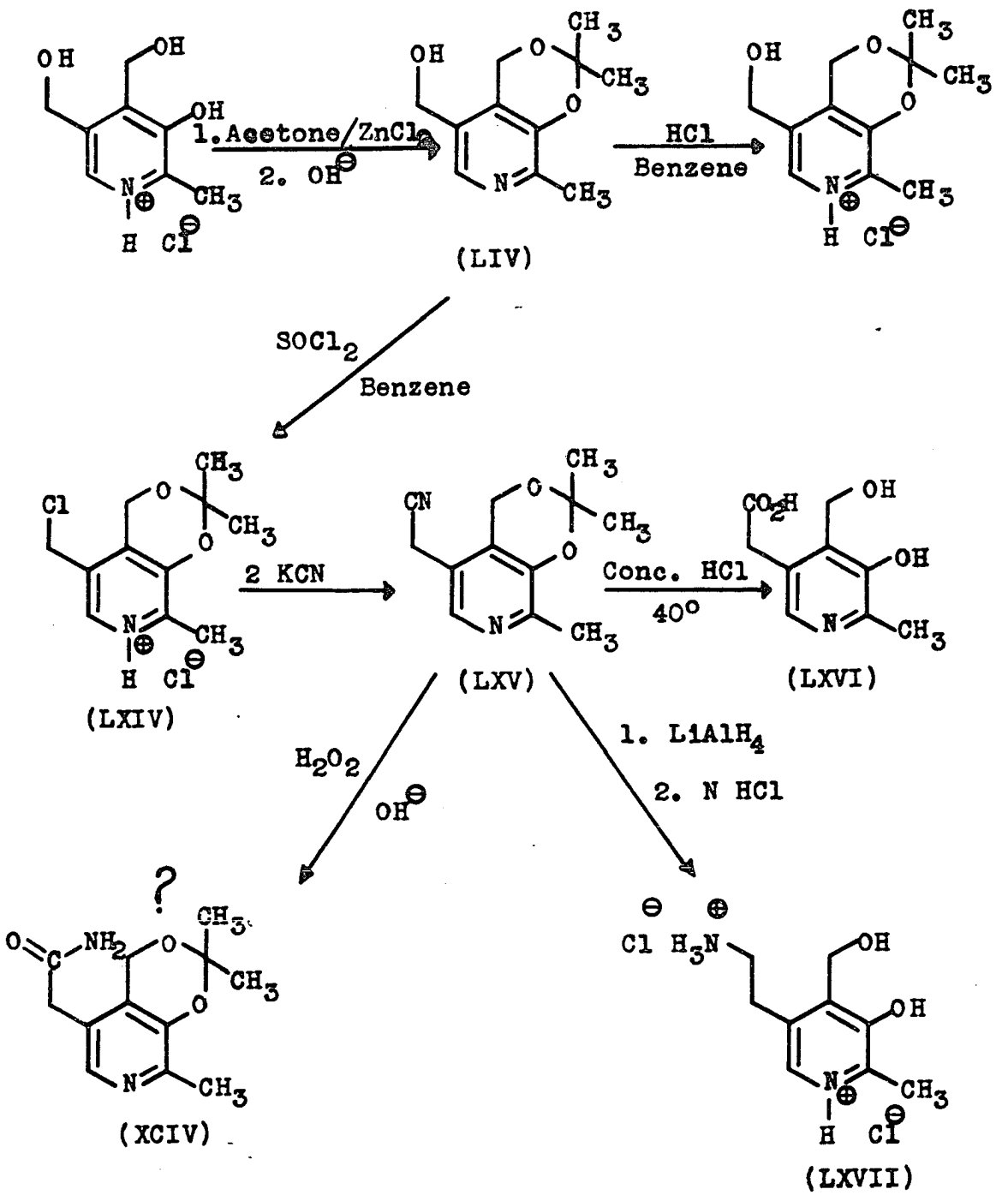


Figure 21. Pyridoxine and pyridoxamine derivatives

acetone. The application of sodium hydroxide permits the use of a smaller volume of water which simply renders the operation less cumbersome. Of greater significance is the fact that the product can be removed from the salts produced by merely washing them with boiling acetone, thus eliminating extraction with a Soxhlet apparatus. The crude product was also found to contain less impurities and the yields are generally 20% higher than those obtained by the method of Baddiley and Mathias (89).

The isopropylidene pyridoxine (LIV) was converted to the 5-chloromethyl hydrochloride derivative (LXIV) by treatment with thionyl chloride (Figure 21). The 5-chloromethyl compound (LXIV) was then employed as one of the essential intermediates in this investigation. By the reaction of this material (LIV) with sodium malonic ester and sodium acetamidomalonic ester the corresponding malonic ester derivatives (LXVIII) and (LXIX), respectively, were prepared (Figure 22). Hydrolysis of compound (LXVIII) with 1N HCl afforded compound (LXX) which contains the free phenolic and hydroxymethyl group, and treatment of both the parent compound (LXVIII) and the previously hydrolyzed substance (LXX) with boiling concentrated hydrochloric acid gave in both cases the propionic acid derivative (LXXII). The acetamidomalonic ester derivative (LXIX) was hydrolyzed with 1N HCl in order to remove the isopropylidene producing the expected compound (LXXI) which

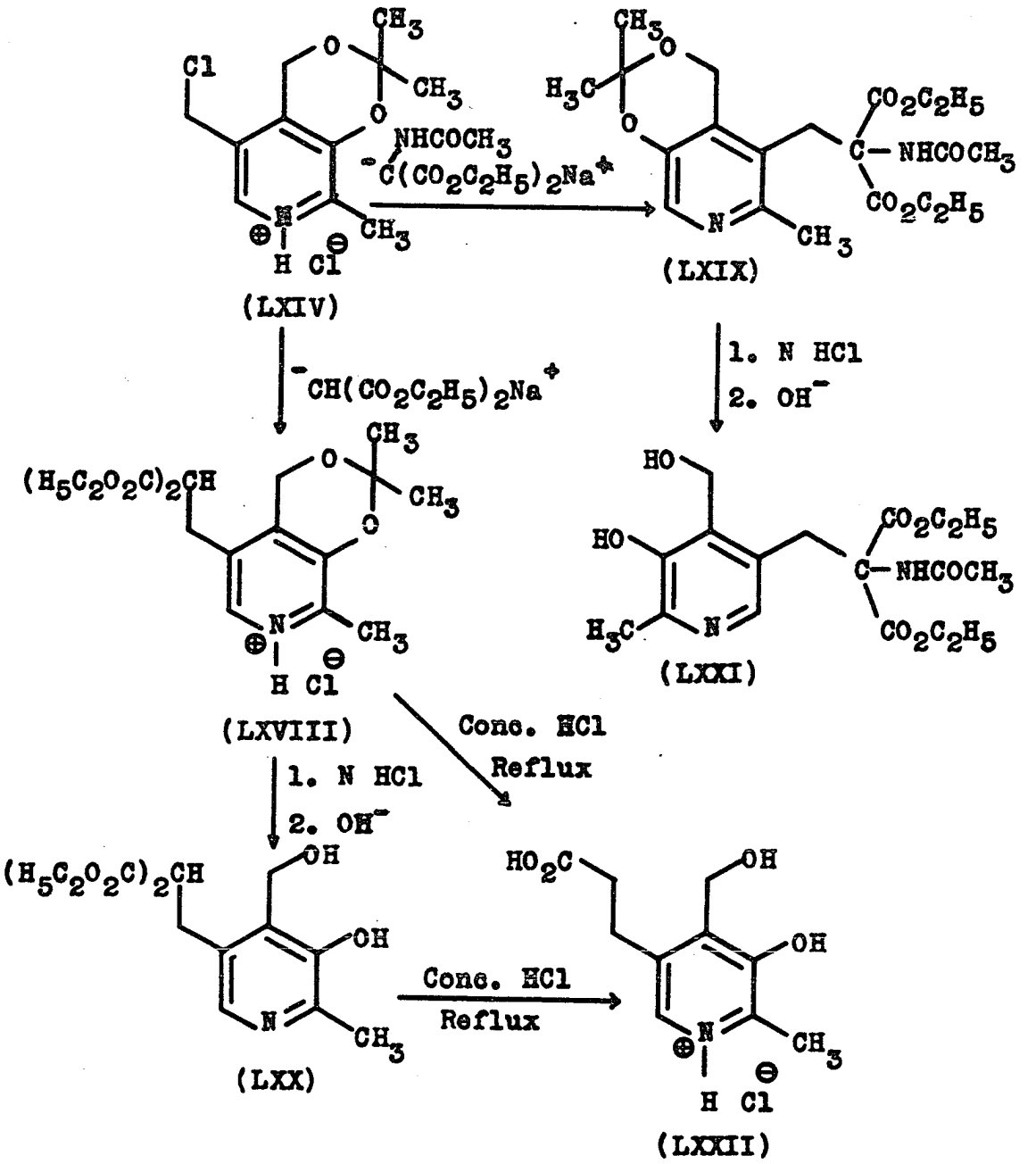


Figure 22. Pyridoxine and pyridoxamine derivatives

was treated in a different fashion to be discussed in a following section.

Reaction of the chloromethyl compound (LXIV) with two molecular equivalents of potassium cyanide in a mixture of water and acetone gave the desired nitrile (LXV) (Figure 21). The acetone was used along with water to minimize solvolysis or replacement of the chlorine atom by water and/or hydroxyl ions. This nitrile (LXIV) possesses the rare property of being recrystallizable from either hot water or petroleum ether (Skelly B, b.p. 60-71.2°). This was found to be a rather fortunate characteristic since an apparently polymeric impurity formed during the reaction is readily removed by recrystallization of the crude nitrile from water. This material could not be removed so simply in other solvent.

Several attempts were made to hydrolyze the nitrile (LXIV) to the amide (XCIV) by treatment with alkaline hydrogen peroxide (Figure 21). Whether or not this was accomplished is not known since the product of this reaction resisted numerous exhaustive attempts at purification as indicated by extremely inconsistent changes in its melting-point. Hydrolysis with concentrated hydrochloric acid at 40° gave only the acid (LXVI). Reduction of the nitrile (LXV) in ether with lithium aluminum hydride gave the 5-aminoethyl compound (LXVII) (Figure 21) isolated as the hydrochloride, along with a considerable amount of an intractable polymeric material.

It is believed that the hydride may have removed a proton forming a carbanion on the methylene group between the cyano group and the pyridine nucleus. The carbanion then might have attacked the intermediate imine produced by partial reduction of the cyano group forming the polymeric substance. There exist other possibilities, however, it is sufficient here to merely point out that the lithium aluminum hydride reduction of this compound (LXV) does not appear to proceed smoothly.

The oxidation of isopropylidene pyridoxine (LIV) by manganese dioxide "B" (102) in chloroform gave an excellent yield of isopropylidene-5-pyridoxal (LXXIII) (Figure 23). Hydrolysis of this aldehyde (LXXIII) with 1N HCl afforded isopyridoxal (XXXVIII) in very excellent over-all yield. Furthermore, the synthesis of isopyridoxl (XXXVII) was accomplished in essentially two steps (not including the hydrolysis), whereas, this substance (XXXVII) was previously synthesized in a total of five steps (Figure 14) (66).

Beginning with isopropylidene-5-pyridoxal (LXXIII), isopyridoxamine dihydrochloride (XXXIV) was obtained by two different routes (Figure 23). First, the aldehyde (LXXIII) was converted to the oxime (LXXIV) which upon reduction with lithium aluminum hydride gave isopropylidene-5-pyridoxamine (LXIII), the hydrolysis of which with 1N HCl afforded the desired isopyridoxamine dihydrochloride (XXXIV). When iso-

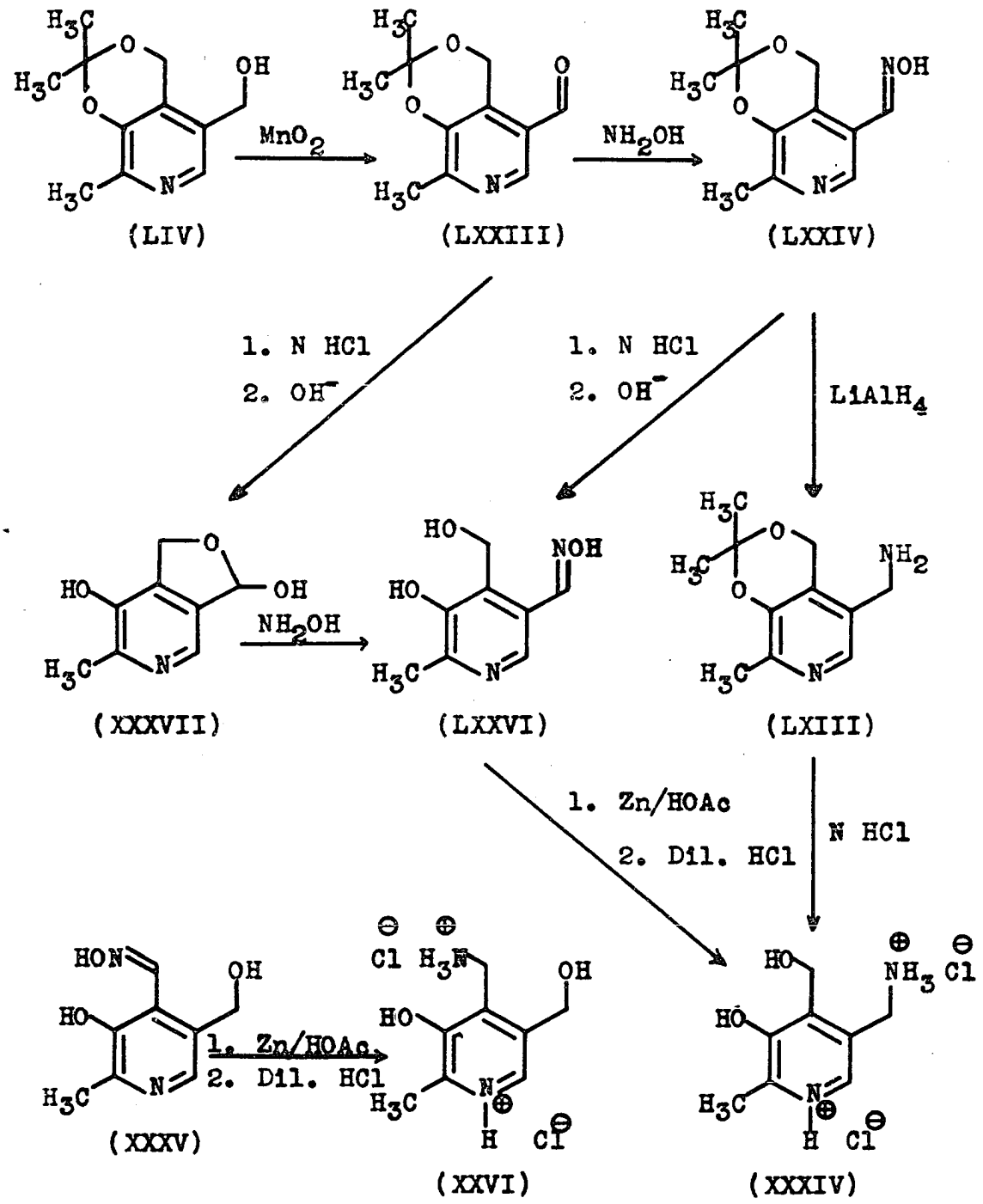


Figure 23. Pyridoxine and pyridoxamine derivatives

pyridoxal (XXXVII) was converted to the oxime (LXXVI) it was shown to be identical with the compound prepared by the hydrolysis of isopropylidene-5-pyridoxal oxime (LXXIV). Reduction of the oxime (LXXVI) with zinc and glacial acetic acid according to the procedure of Testa and Fava (79) gave also the isomer of pyridoxamine (XXXIV). Before this last reaction was undertaken a quantity of pyridoxal oxime (XXXV) was converted to pyridoxamine dihydrochloride (XXXIV) as previously described (79) in order to determine the general feasibility of the method (Figure 23).

Another series of compounds was prepared by first forming the imine (LXXXIX) of aniline and isopropylidene-5-pyridoxal (LXXIII) (Figure 24). Reduction of this imine (LXXIX) to the secondary amine (LXXX) was accomplished cleanly and smoothly with sodium borohydride in methanol. Hydrolysis of the secondary amine (LXXX) with 1N HCl gave the N-phenyl-aminomethyl derivative (LXXXI). By the reaction of 2-amino-thiazole with the same aldehyde (LXXIII), the 2-thiazolyl imine (LXXXII) was obtained, which through reduction in the same manner gave the desired amine (LXXXIII). Through the hydrolysis of the secondary amine (LXXXIII) with dilute hydrochloric acid the corresponding N-(2-thiazolyl)-aminomethyl compound (LXXXIV) was prepared.

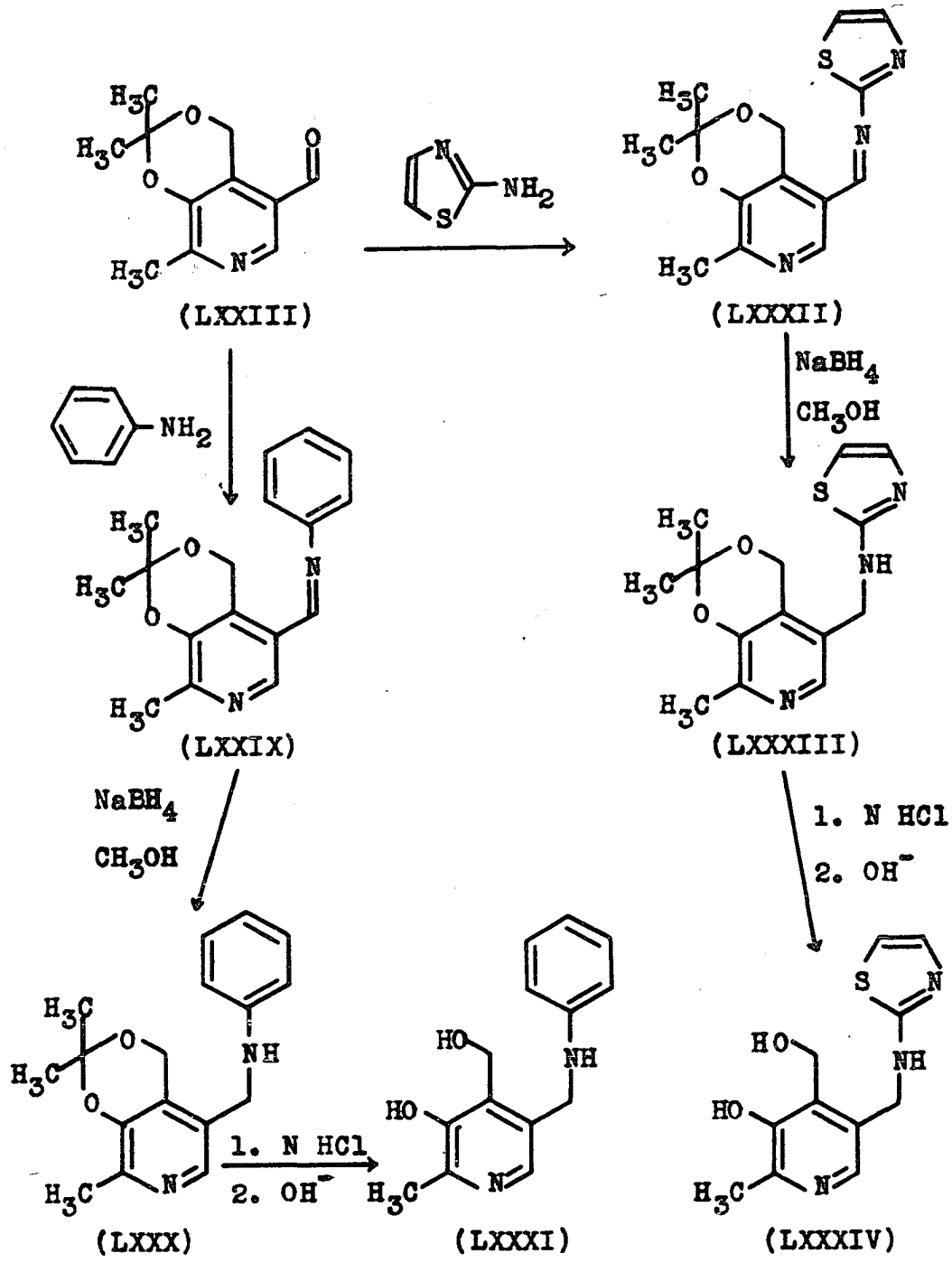


Figure 24. Pyridoxine and pyridoxamine derivatives

Oxidation of Pyridoxine Derivative

In order to possibly obtain a few of the desired model compounds sought by this investigation it was necessary to oxidize several of the pyridoxine derivatives previously discussed. By oxidation of these materials all of which contain a nitrogen atom in various positions of the 5-substituent it was hoped that possibly a series of internal (intramolecular) carbinol amines and imines could be produced. The oxidation was of course to be performed on the 4-hydroxymethyl groups of these compounds.

The first of these oxidations with manganese dioxide (Figure 25) was carried out on 5-(anilinomethyl)-3-hydroxy-2-methyl-4-pyridinemethanol (LXXXI) in chloroform. There was obtained from this reaction a very dark solid which when dissolved in chloroform forms a ruby red faintly irridescent solution. The infrared spectrum of this substance in potassium bromide shows the following peaks: 3400 $cm.^{-1}$, 3010 $cm.^{-1}$, 1640 $cm.^{-1}$, 1600 $cm.^{-1}$, 1515 $cm.^{-1}$, 1230 $cm.^{-1}$, 1055 $cm.^{-1}$, 758 $cm.^{-1}$, and 690 $cm.^{-1}$. The desired compound would be represented by structure LXXV, however, the analytical data seems to indicate that the material may possess structure XC or an isomer of this substance. The purity of this material may also be questionable since it does not melt and its infrared spectrum did not clear up after each of several attempts at purification.

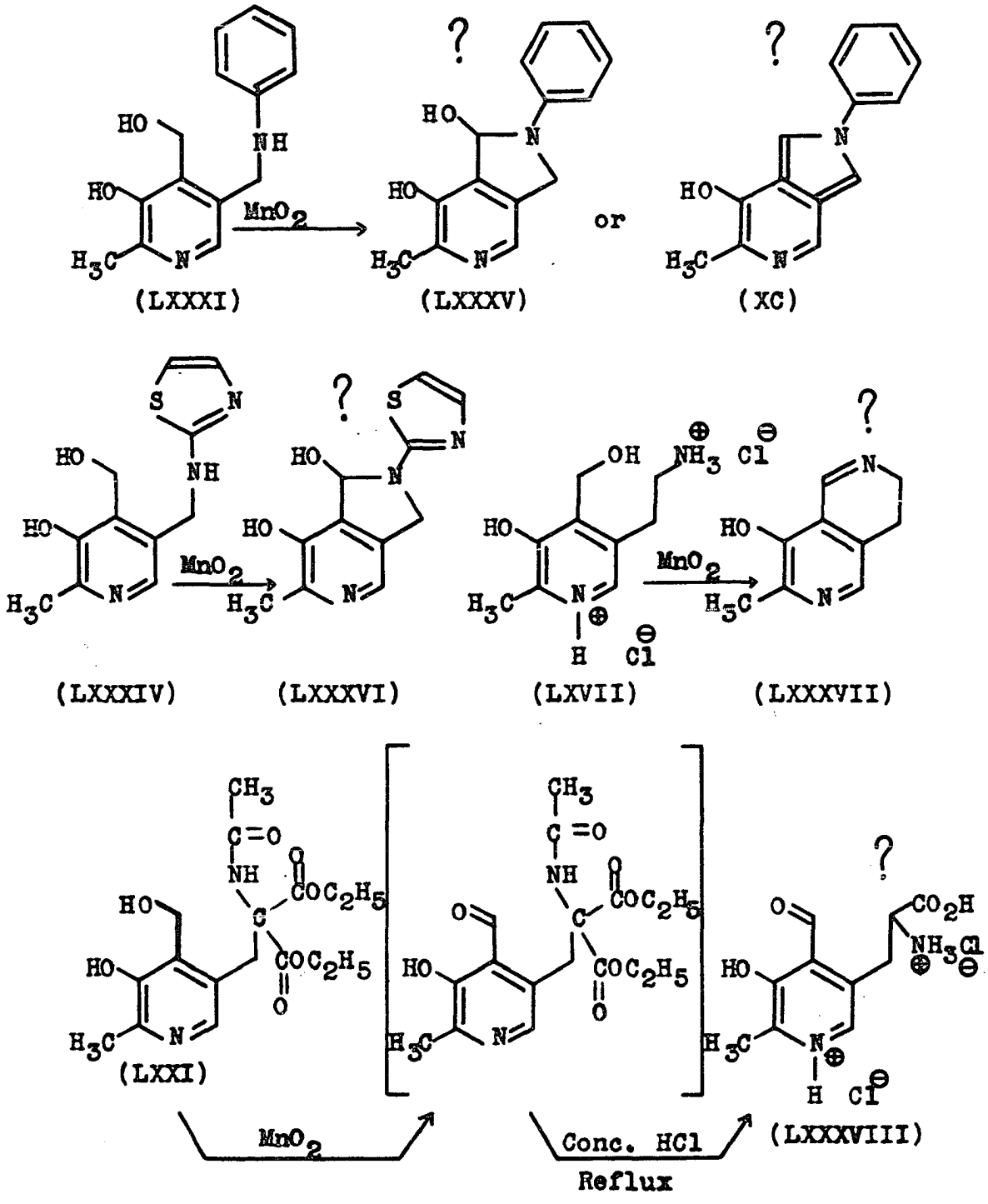


Figure 25. Oxidation of pyridoxine derivatives

Oxidation of 3-hydroxyl-2-methyl-5-(2-thiazolylaminomethyl)-4-pyridinemethanol (LXXXIV) in tetrahydrofuran with manganese dioxide produced a dark brown material whose infrared spectrum in potassium bromide exhibited the following peaks: 3400 cm.$^{-1}$, 3010 cm.$^{-1}$, 1510 cm.$^{-1}$, 1345 cm.$^{-1}$, 1150 cm.$^{-1}$, and 1050cm.$^{-1}$. The analytical data seems to indicate that this material at least has the same empirical formula as indicated by structure LXXXVI although the exact structure is doubtful.

The manganese dioxide oxidation of diethyl acetamido-[5-hydroxy-4-(hydroxymethyl)-6-methyl-3-pyridylmethyl]malonate (LXXI) in a mixture of tetrahydrofuran and water, and subsequent hydrolysis in refluxing concentrated hydrochloric acid gave a deep yellow compound. The ultraviolet absorption spectra of this material in 0.1N HCl exhibits a strong maximum at 289 mμ, a weaker band at 360 mμ, and a very weak band at 460 mμ. At pH 3.8 the bands at 289 mμ and 460 mμ diminish slightly, and concomitantly two new maxima appear at 320 mμ and 407 mμ. The bands at 290 mμ and 460 mμ almost disappear at pH 7, while the intensity of the maximum at 320 mμ increases, and the maximum at 407 mμ has shifted to 400 mμ with increased intensity. The band at 360 mμ has remained essentially constant. In 0.2 N NaOH there appears a strong band at 304 mμ shifted from 289 mμ with increased intensity. The intensity of the bands at 350 mμ and 400 mμ also increases

while the peak at 460 mμ has essentially disappeared.

This data apparently indicates the existence of 3-hydroxypyridine derivative, however, it does not support the presence of a pyridoxal imine so far as it is known. The spectra of this compound are not well understood, however, with that data which is ascertainable and the analytical data this material may be considered to be the desired substance (LXXXVII).

The final oxidation of these derivatives was undertaken on 5-(2-aminomethyl)-3-hydroxy-4-(hydroxymethyl)-2-methyl-pyridine dihydrochloride (LXVII). The product from this reaction was a very dark green substance. The ultraviolet absorption spectra of this substance in 0.1N HCl shows an intense peak at 291 mμ with a shoulder at 330 mμ and a broad band at 432 mμ of lower intensity. At pH 7 maxima appear at 320 mμ, 385 mμ and also at 432 mμ. In 0.02N NaOH bands occur at 303 mμ (possibly shifted from 291 mμ) and one other maximum at 365 mμ with the band at 432 mμ having nearly disappeared. In acidic solution the 432 mμ peak tails off at about 520 mu. Aside from the fact that this material is a 3-hydroxypyridine derivative nothing else is known about its structure. The analytical data did not fit any conceivable structure, however, the compound sought is represented by structure LXXXVII.

Derivatives of 3-Hydroxymethylpyridine

Interest in 3-hydroxymethylpyridine (LXXXIX) during the course of this work was mainly in the area of its relationship to pyridoxine in chemical reactivity. A few derivatives of this compound were prepared in order to determine practicability of similar transformations with vitamin B_6 (Figure 26). The reaction of thionyl chloride with 3-hydroxymethylpyridine (LXXXIX) tetrahydrofuran gave the 3-chloromethylpyridine hydrochloride (LVI) which upon treatment with sodium malonic ester afforded the diethyl 3-pyridylmethylmalonate (isolated as the hydrochloride) (LVII). By refluxing the pyridylcarbinol (LXXXIX) with 48% hydrobromic acid the 3-bromomethyl hydrobromide analog (LXXVII) was obtained.

The thimaine analog (LXXVII) of 3-hydroxymethylpyridine was prepared for use in another unrelated study. By allowing a methanol solution of 3-bromomethylpyridine hydrobromide (LXXVII) and 4-methyl-5-(β-hydroxyethyl)thiazole to stand 5 days the crystalline product (LXXVIII) separated from solution. A similar attempt to synthesize the chloride hydrochloride analog of this thiamine-type compound (LXXVIII) failed simply because the product could not be caused to crystallize. All attempts to prepare and isolate the p-toluenesulfonate ester of 3-hydroxymethylpyridine (LXXV) failed and although not necessarily an important compound in this work the failure to obtain it caused considerable concern.

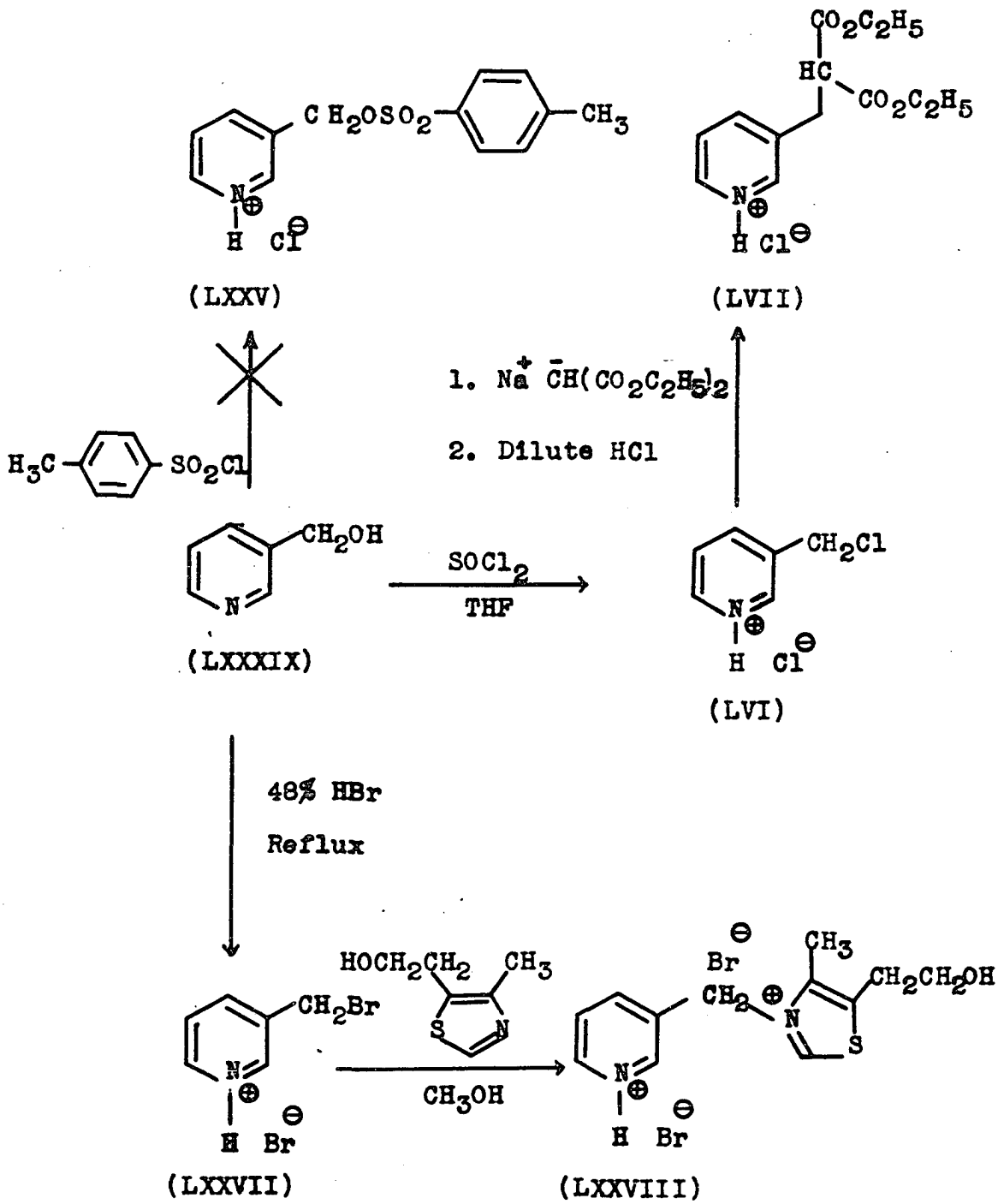


Figure 26. Derivatives of 3-hydroxypyridine

CONCLUSIONS

A search of the literature during the initial stages of this study was made in order to determine the scope of investigations dealing with vitamin B_6 and its derivatives. It was discovered that this area of research has been practically overlooked, except for those cases previously described. In view of these circumstances it was considered worthwhile to undertake the synthesis of several pyridoxine and pyridoxal derivatives which would serve as intermediates for the preparation of a wide variety of vitamin B_6-like compounds. The synthesis of isopropylidene-5-pyridoxal (LXXIII), isopropylidene-5-chloromethylpyridoxine hydrochloride (LXIV), and isopyridoxamine dihydrochloride (XXXIV) represents a partial attainment of this goal. The versatility of these substances as intermediates has been illustrated by their application in the synthesis of several imines, secondary amines, malonic ester derivatives and acids, to mention a few. As a result of this work it is now apparent that the way is clear for the synthesis of many other derivatives such as aldehydes, ketones, amino acids, α,β-unsaturated acids, and nitrostyrenes.

The improvement of the synthesis of pyridoxal (XXIV), isopyridoxal (XXXVII), and isopyridoxamine (XXXIV) will also be of value in facilitating the preparation of these compounds. The value of these improvements is based on a plan

to investigate the effect of all these derivatives as enzyme inhibitors, which may also give important information concerning the possible physiological properties of these substances. In event of a mass screening of these materials, it will then be necessary to produce the compounds in larger amounts which is obviously facilitated by good preparative methods.

The attempted oxidation of several pyridoxine derivatives (LXVII), (LXXI), (LXXXI) and (LXXXIV) with manganese dioxide to the corresponding pyridoxal analogs, although essentially unsuccessful is in no way indicative of the lack of applicability of this method. Only very little effort has been expended on this operation and the purification of the substances obtained. Therefore, because of the expediency of this procedure as a direct method of obtaining these pyridoxal analogs it should be given further study.

The primary objective of this work having been the synthesis of a group of vitamin B_6 derivatives, and this having been accomplished to a certain extent, it may be concluded that the information secured may point the way to other exciting areas of vitamin B_6 chemistry.

SUMMARY

Interest in vitamin B_6, its chemistry and physiology, has intensified continually since the inception of its existence. In recent years much attention has been focused on structures, requirements, and functions of hundreds of enzymes and enzyme systems. Among them the vitamin B_6 enzyme occupy a position of considerable prominence. Modern methods and techniques have made it possible to isolate and characterize many of these substances. Interest in the manner in which these materials accomplish their phenomenal physiological tasks has in part led to the present investigation, which had as its primary objective the synthesis a series of model compounds of vitamin B_6, to be studied later in the hope of better understanding in role in certain enzyme systems.

A brief historical review of the literature pertaining to the discovery, isolation, structure determination and chemistry of pyridoxine, pyridoxal, pyridoxamine, pyridoxal phosphate and pyridoxamine phosphate has been presented.

Improved syntheses of pyridoxal, isopyridoxal, and isopyridoxamine have been described. Several new derivatives of each of these compounds have also been presented and they encompass such substances as halogen compounds, acids, malonic and acetamidomalonic esters, amines, imines, benzyl and p-nitrobenzyl chloride quaternary salts, acetals, ketals and an amino acid.

Oxidation of several of these compounds with manganese dioxide has been attempted with little success since in several cases there exist considerable doubt as to the exact structure of the products.

The 3-pyridylmethyl analog of thiamine was also prepared for study in an unrelated investigation.

In conclusion it might be merely mentioned that in a very elementary manner so far, several of these derivatives appear to be more active catalysts, in the nonenzymic transamination of certain amino acids than pyridoxal.

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