

REACTIONS OF PYRIDOXINE

By

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TITLE: Reactions of Pyridoxine

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SCOPE AND CONTENTS: The chemistry of pyridoxine has been critically examined with a view to obtaining new intermediates suitable for the systematic degradation of the molecule. Use has been made of three new degradation schemes arising from this work to isolate atoms C-2, C-2', C-4, C-4' and C-5' of radioactive pyridoxine obtained from feeding experiments. The biosynthesis of pyridoxine is discussed in the light of the degradation results which demonstrate that pyridoxine is derived from three molecules of glycerol.

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(A) INTRODUCTION

1) DISCOVERY AND STRUCTURAL ELUCIDATION OF VITAMIN B₆ (PYRIDOXINE)

The existence of the substance known as vitamin B₆ (1,2) was originally inferred on the basis of nutritional experiments. Young rats, kept on a deficient diet, failed to grow normally and developed a type of dermatitis (1,2). The identification of the missing nutritional factor was achieved in 1938 when no fewer than five different laboratories announced the isolation, from rice bran or yeast (2,3), of the pure crystalline material which possessed B₆ activity, i.e. the ability of preventing the development of these deficiency conditions.

The vitamin, named pyridoxine by György (1), was isolated as the free base, C₈H₁₁NO₃, m.p. 160°, and as the hydrochloride, C₈H₁₂NO₃Cl, m.p. 204-206° (dec.) (4-8). The structure of the base was deduced independently by two groups of chemists: Stiller, Keresztesy and Stevens in the United States (9) and Kuhn, Wendt and Westphal in Germany (10-12).

The base was optically inactive, contained no alkoxy or N-alkyl residues and gave a deep red colour with aqueous ferric chloride, indicating the presence of a phenolic hydroxy group. The vitamin also contained one C-methyl group and three active hydrogens per molecule. The American group deduced that the

molecule was a 3-hydroxypyridine by comparison of the U.V. absorption spectra of the base in aqueous solutions at various pH values with those of variously substituted hydroxypyridines. Pyridoxine showed the greatest similarity to 2-methyl-3-hydroxypyridine, 2-methyl-5-hydroxypyridine and 3-hydroxypyridine. Similar displacements of the absorption maxima with change in pH were observed in the spectra of these compounds and those of pyridoxine.

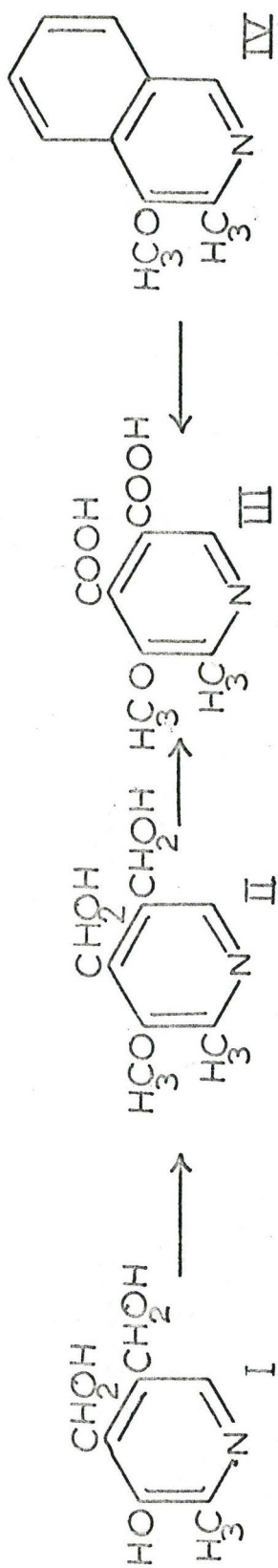
The German group noted that whereas the free base gave a deep blue colour with Folin-Dennis phenolreagent, the methyl ether gave a negative test. It was thus concluded that the compound was a 3-hydroxypyridine derivative.

Both groups used permanganate oxidations of the methyl ether, $C_9H_{13}NO_3$, m.p. $101-103^\circ$, derived from the vitamin by treatment with diazomethane, to obtain identifiable degradation products. Upon mild oxidation a dibasic acid which crystallised with one molecule of water, $C_9H_9NO_5$, m.p. $208-209^\circ$ (dec.) and a lactone, $C_9H_9NO_3$, m.p. $209-210^\circ$ were obtained. The simultaneous formation of the lactone in this oxidation led Kuhn to propose that the two acid groups were adjacently substituted in the pyridine nucleus.

More vigorous oxidation of the methyl ether with hot aqueous permanganate generated a tricarboxylic acid which possessed an acid function α to the nitrogen as demonstrated by

its positive colour reaction with ferrous sulphate. This tricarboxylic acid was readily decarboxylated to give a new carboxylic acid which now showed no reaction to the ferrous sulphate test. Hence the acid group α to the nitrogen had been lost. Since the original dicarboxylic acid also showed no reaction with ferrous sulphate the C-methyl residue was assumed to reside α to the nitrogen and to be oxidised under more vigorous conditions. Final proof of the structure of the dicarboxylic acid came when it was shown that oxidation of 3-methyl-4-methoxyisoquinoline (IV) gave a product which proved to be identical with the acid. Hence the diacid is 2-methyl-3-methoxypyridine-4,5-dicarboxylic acid (III).

The dibasic acid (III) was formed from the O-methyl vitamin (II) by addition of two oxygen atoms and with the loss of four hydrogen atoms, and since the vitamin has three active hydrogen atoms per molecule its structure was concluded to be I. The structure of the pyridoxine as 2-methyl-3-hydroxy-4,5-dihydroxy methylpyridine was confirmed by its synthesis (12,13).



2) BIOSYNTHESIS OF VITAMIN B₆

Recent work on the biosynthesis of pyridoxine involving administration of radioactive precursors to a mutant of Escherichia coli B, WG2, and partial degradation of the pyridoxine isolated from the organism using the Kuhn-Roth oxidation (Scheme I) led to the inference that pyridoxine is derived from three glycerol units. The biosynthetic scheme based on these results postulates that the C₂ unit, C-2,2', is derived from a two-carbon unit, which is in turn generated from glycerol via pyruvic acid. Two intact glycerol units account for the remaining carbon atoms (14) (figure I, p.7).

This hypothesis rests on the following evidence: Feeding experiments on the E. coli mutant with [3-¹⁴C] pyruvic acid yielded pyridoxine hydrochloride having 96[±]3% of the total specific activity associated with the acetate derived from the C₂ unit, C-2,2'. The C-2' atom was shown to possess 88[±]2% of the total pyridoxine activity by further degradation of the acetate.

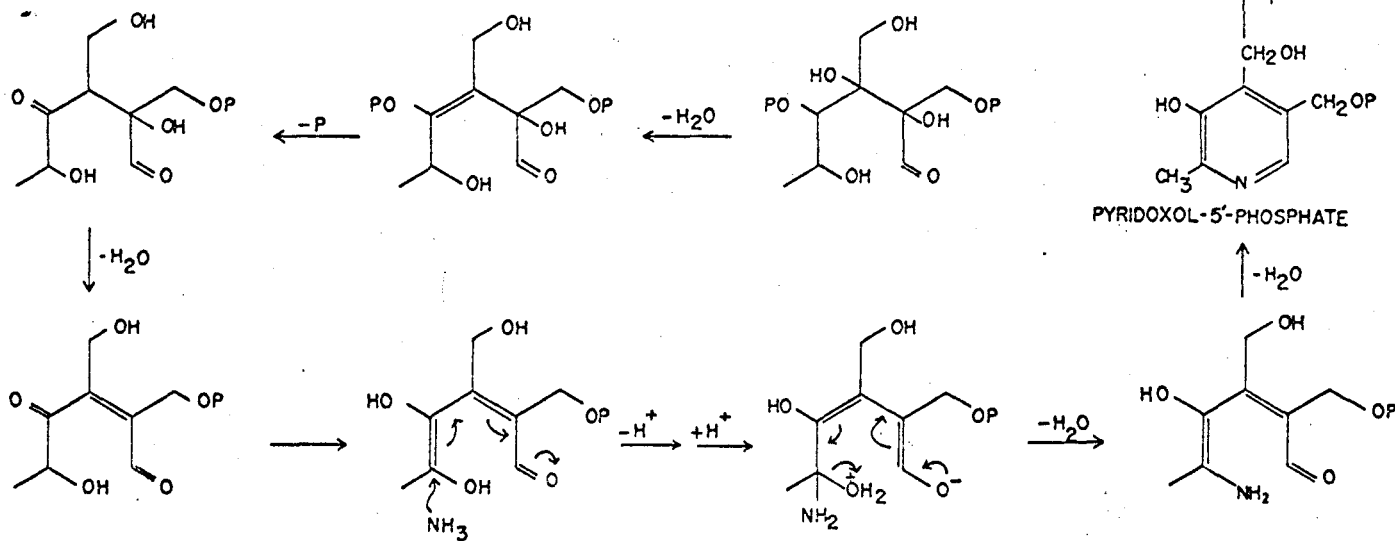
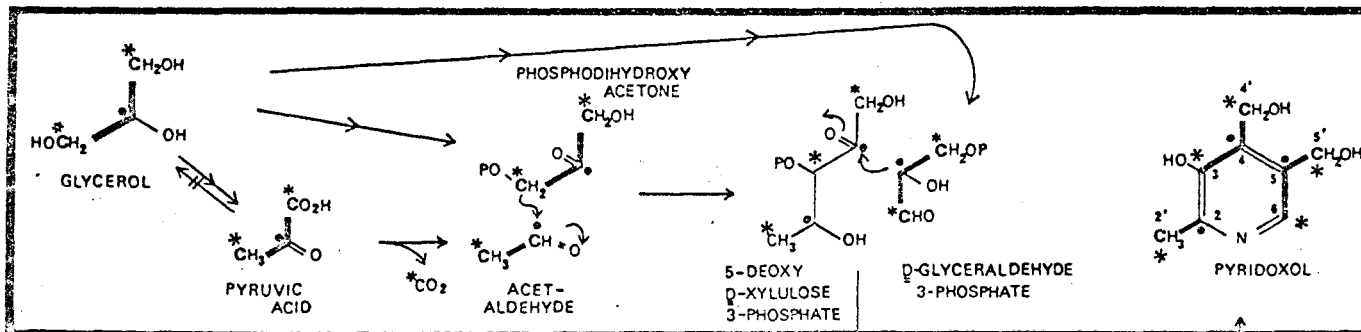
The corresponding experiment with the 2-¹⁴C radiomer of pyruvic acid yielded pyridoxine bearing nearly all of its activity in the Kuhn-Roth acetate (94[±]3%).

That the pyruvate was not incorporated as an intact C₃ unit was demonstrated from an incubation with 1,3-¹⁴C₂-

pyruvic acid equally labelled at C-1 and at C-3. Now, the Kuhn-Roth acetate accounted for $90 \pm 2\%$ of the total specific activity, while $84 \pm 2\%$ of the activity was located at the C-2' pyridoxine atom. Since intact incorporation of the C_3 unit should have yielded a Kuhn-Roth acetate bearing $\sim 50\%$ of the activity at C-2', the carboxyl carbon of the $1,3\text{-}^{14}C_2$ -pyruvate had been lost during the biosynthesis.

Thus pyruvic acid was shown to furnish the C-2' and C-2 atoms of pyridoxine from positions C-3, and C-2, respectively of the acid. Feeding experiments with $1\text{-}^{14}C$ -glycerol also yielded active pyridoxine, and Kuhn-Roth oxidation showed the acetate to account for $21 \pm 1\%$ of the total specific activity. Furthermore, the activity was located at the C-2' position ($18 \pm 1\%$). Thus the glycerol incorporation was non-random.

Figure I shows the scheme for the biosynthesis of pyridoxine proposed on the basis of the results obtained from pyruvate and glycerol feeding experiments. It is postulated that glycerol acts as a precursor of pyruvic acid which decarboxylates to form acetaldehyde. This condenses with phosphodihydroxyacetone, derived from glycerol, via an aldol type condensation to yield 5-desoxy-D-xylulose-3-phosphate. This in turn reacts with



POSTULATED SCHEME FOR THE BIOSYNTHESIS OF PYRIDOXINE

D-glyceraldehyde-3-phosphate derived from a third glycerol molecule to yield an intermediate which after a series of dehydrations, dephosphorylations, proton transfers and attack by ammonia generates pyridoxine.

The scheme requires that incorporation of 1-¹⁴C-glycerol into pyridoxine should yield product labelled equally at C-2', C-3, C-4', C-5' and C-6. The Kuhn-Roth acetate should be labelled at the C-2' position and should contain 20% of the total activity, as found by experiment.

Further verification of this scheme for the biosynthesis requires isolation of the other predicted sites of activity from pyridoxine derived from 1-¹⁴C-glycerol and 2-¹⁴C-glycerol and demonstration that each site has associated with it the relative specific activity predicted by the scheme.

It was the objective of this work to develop degradative methods to separate other carbon atoms of the pyridoxine molecule so that the distribution of activity from glycerol could be more fully investigated, and the hypothesis subjected to critical tests.

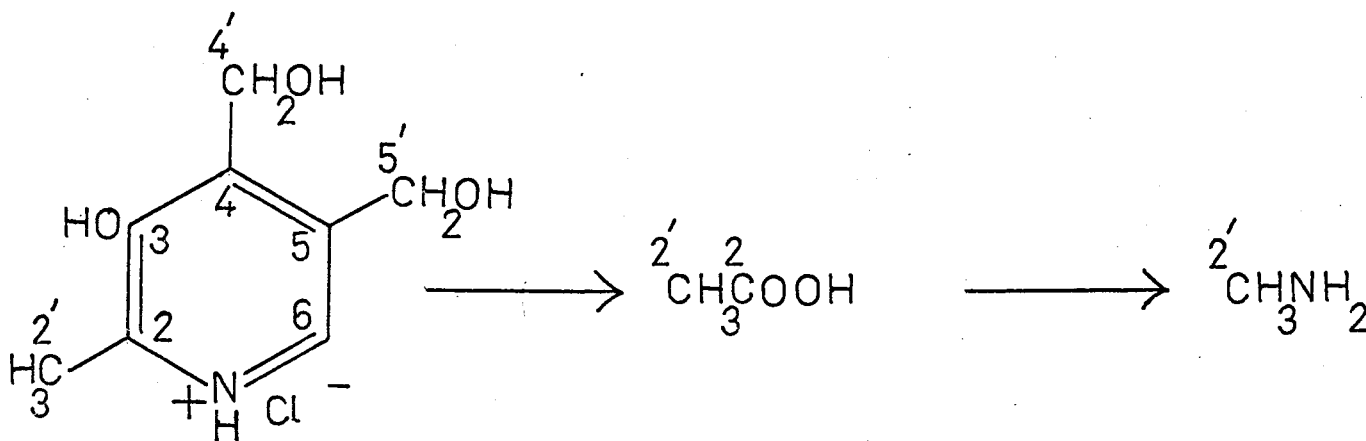
(B) CHEMISTRY OF PYRIDOXINE

1) The oxidation of pyridoxine and degradations based upon it.

The study of the oxidation of pyridoxine, one of the first reactions of the vitamin to be investigated, contributed to its structural elucidation. Generally, chromic acid oxidations lead to a breakdown of the pyridoxine nucleus and under Kuhn-Roth conditions acetic acid is generated from the C₂ unit, C-2, C-2', and can be collected. This reaction serves as an excellent means of isolating these two carbon atoms. The overall yield of the reaction is excellent and the acetate obtained can be further degraded via a Schmidt reaction to liberate C-2' as methylamine (Scheme I).

Permanganate oxidations of pyridoxine give rise to a variety of products depending upon the reaction conditions. Mild oxidation of the O-methyl ether of B₆ with aqueous permanganate at room temperature gives two products (9), a lactone (V), and a diacid (III). The structure of the lactone (V) was proven by its total synthesis from acyclic precursors (15).

The analogous oxidation of free pyridoxine leads to a lactone (VI), bearing the carboxyl function at C-4' (16). On treatment with diazomethane this lactone gives a new methyl ether. Other reports of this oxidation state that the major products under similar conditions are the lactone (VI) and the 4-carboxylic acid (VII) plus a trace of the diacid (VIII) (17).



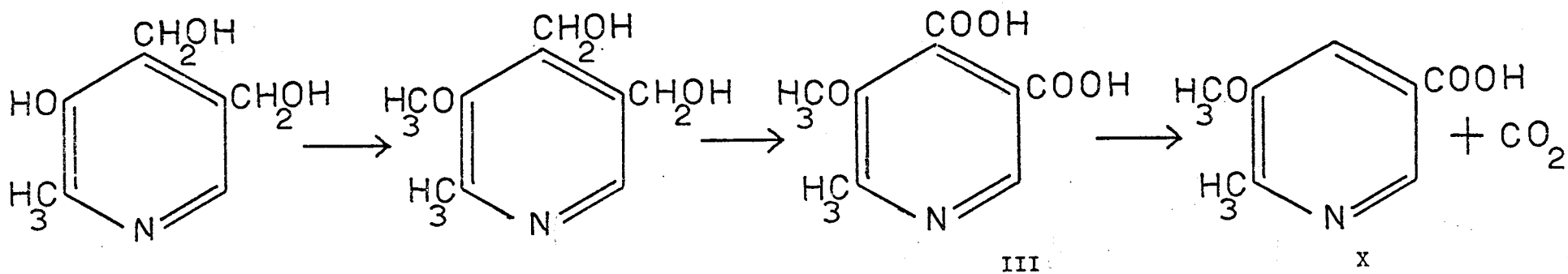
DEGRADATION SCHEME I: THE ISOLATION OF C-2' AND C-2 OF PYRIDOXINE

Permanganate oxidations under more vigorous conditions lead to the oxidation of the C-methyl group (C-2'). Thus hot permanganate oxidation of the O-methyl ether of pyridoxine (II) generates the tricarboxylic acid (IX), as described earlier (11). This reaction can be terminated before the oxidation of the C-2' methyl group and the resulting diacid (III) may be isolated in a yield of 40% (17).

A degradation scheme based on the specific decarboxylation of the C-4' acid group to give the 5-carboxylic acid (X), has been proposed (17) (Scheme II, p.12). Thus (III) is preferentially decarboxylated at the 4-position when it is heated in nitrobenzene at 180-200° for 0.5 to 1 hour. A drawback to this reaction scheme is the low yield during the O-methylation step (45%) (17).

The double decarboxylation of the O-methyl dicarboxylic acid (III) has also been reported to occur when (III) is heated in phenylmethane in the presence of a copper chromite catalyst (18). The 2-methyl-3-hydroxypyridine, (XI), was formed and isolated as the picrate.

Oxidation of the 5'-hydroxymethylene group to the aldehyde has been described when the 3 and 4' positions of pyridoxine are blocked via the cyclic acetonide, as in (XII) (3,4'-O-isopropylidene-pyridoxine) (19).



DEGRADATION SCHEME II: THE ISOLATION OF C-4 OF PYRIDOXINE

Compound (XII) is obtained in nearly quantitative yield by passing dry hydrogen chloride in excess through a suspension of pyridoxine hydrochloride in anhydrous acetone (20). Oxidation to (XIII) is achieved in excellent yield (83%) by treatment of the acetone (XII) with dry chromium trioxide in anhydrous pyridine, the reaction mixture being warmed to reflux temperature during two hours and refluxing continued for ninety minutes (19).

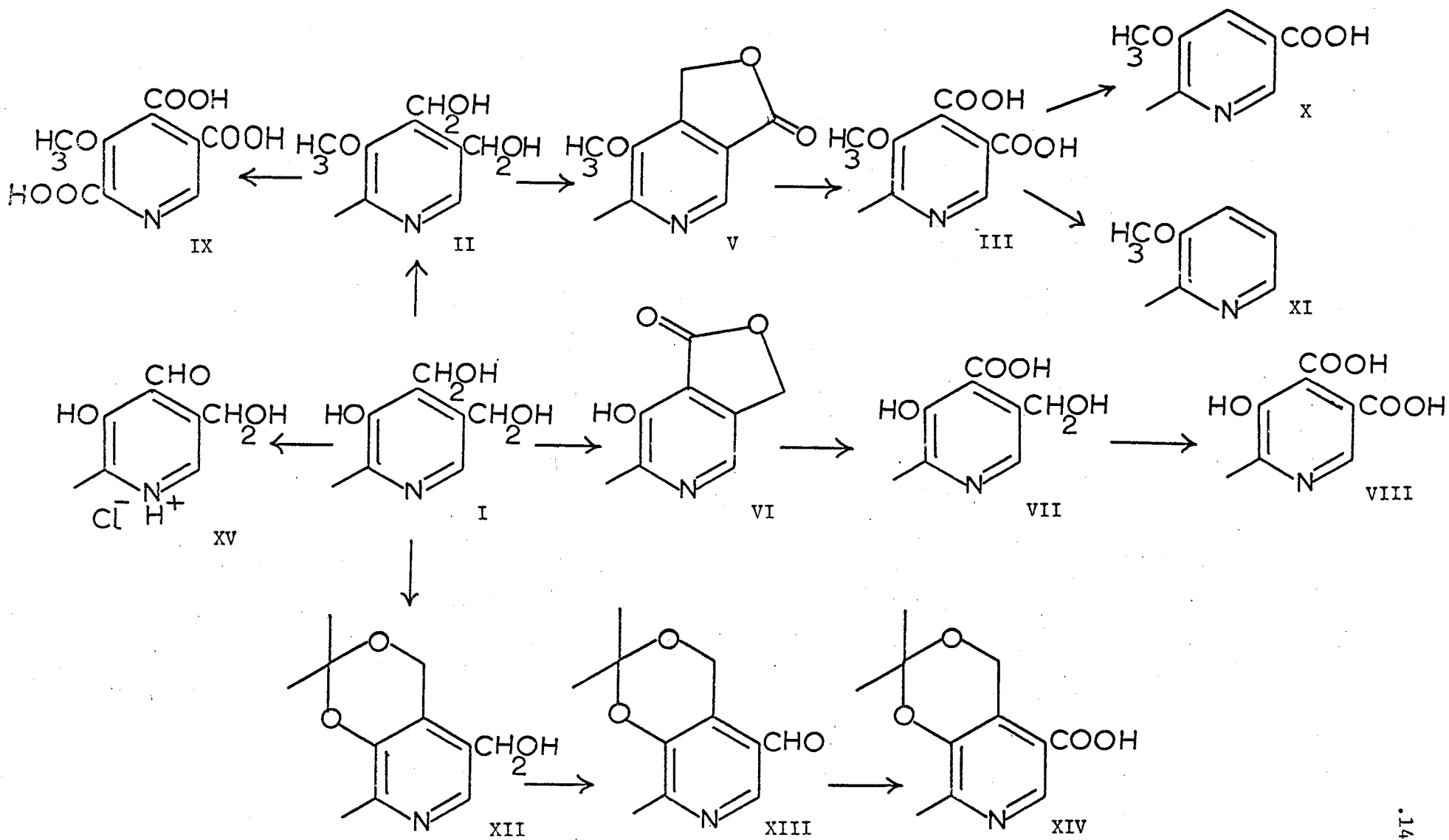
The 5 -monoacid (XIV), can also be obtained from the acetone (XII) either directly by oxidation with saturated aqueous permanganate at 100° for two to five minutes (yield of 44%), or from the 5 -aldehyde (XIII), using identical conditions, giving the product in 68% yield based on the 5 -hydroxymethyl acetone (18).

Mild oxidation of pyridoxine hydrochloride with active manganese dioxide oxidises the 4 -hydroxymethylene group exclusively, yielding pyridoxal hydrochloride (XV), as the sole product (21,22). These reactions might be used for the isolation of the C-2', C-4' and C-5' atoms. Their applicability depends upon the yields of the reactions and on the simplicity of isolating pure product. Of the reactions described, the Kuhn-Roth oxidation is the simplest for the isolation of C-2' and C-2.

2) The reduction of pyridoxine.

a) Catalytic reduction.

3-Hydroxypyridines, substituted 3-hydroxypyridines and quaternary salts of 3-hydroxypyridines are reported to undergo reduction



SOME OXIDATION REACTIONS OF PYRIDOXINE AND ITS DERIVATIVES

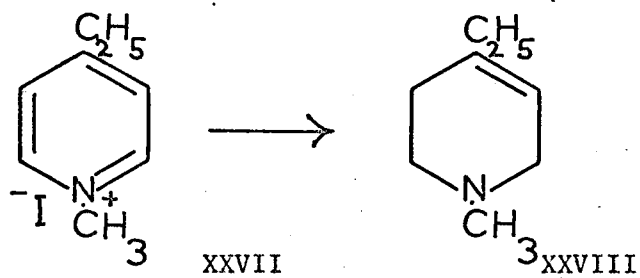
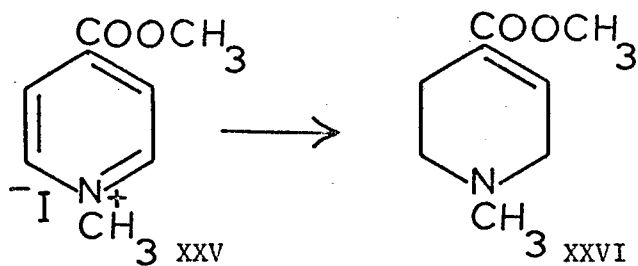
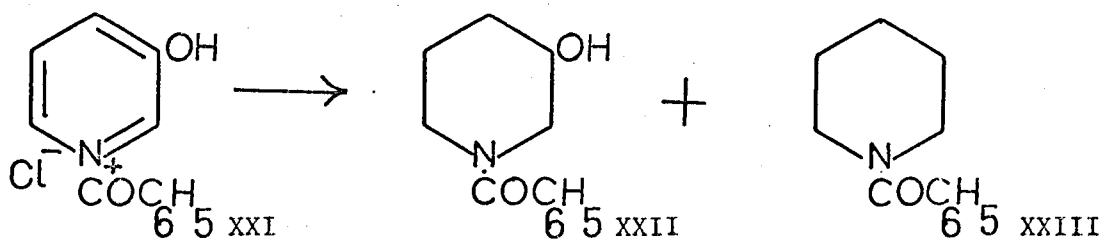
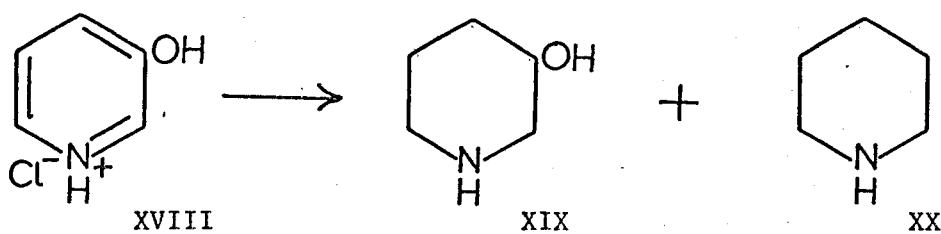
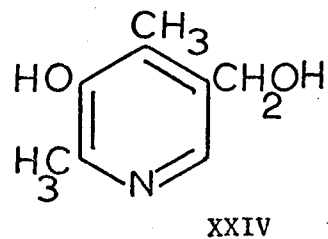
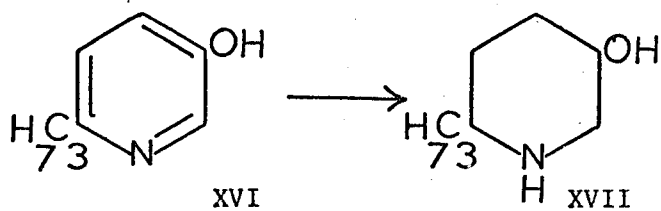
with noble metal catalysts in the presence of hydrogen (22-25). Thus 2-n-propyl-5-hydroxypyridine (XVI), is reduced to the 2-n-propyl-5-hydroxypiperidine, (XVII), using Adam's catalyst in glacial acetic acid and a hydrogen pressure of fifty pounds per square inch (23). Hydrogenolysis of the C-OH bond of 3-hydroxypyridine to give the piperidine has also been reported. Thus both the 3-hydroxypiperidine, (XIX) and the unsubstituted piperidine, (XX), are formed during the platinum reduction of 3-hydroxypyridine hydrochloride, (XVIII), in absolute ethanol at sixty pounds hydrogen pressure (25).

The reduction of quaternary 3-hydroxypyridinium salts is also a well documented reaction (24,26). Reduction of N-benzoyl-3-hydroxypyridinium chloride, (XXI), for example, by platinum in ethanol, occurs comparatively rapidly at one atmosphere hydrogen pressure to give the N-benzoyl-3-hydroxypiperidine, (XXII), (31%), plus the N-benzoylpiperidine, (XXIII), (34%) (24). Reduction of the free base in this case leads to the formation of 3-hydroxypiperidine only.

Considering the above examples, it comes as a surprise to discover that the pyridoxine nucleus is stable to catalytic hydrogenation. However, the 4-hydroxymethyl group is reduced under these conditions and the only new product is 4'-desoxypyridoxine, (XXIV), formed in low yields (27).

b) Hydride reductions.

Pyridoxine and its hydrochloride are stable to metal hydride reducing agents even though substituted pyridines have been reportedly reduced by such reagents (28).



Borohydride reduction of quaternary pyridinium salts generally gives the Δ^3 product. Thus N-methyl-4-carboxymethylpyridinium iodide, (XXV) gives N-methyl-4-carboxymethyl- Δ^3 -piperidine (XXVI) (29). Similarly N-methyl-4-ethylpyridinium iodide (XXVII) is reduced in aqueous sodium hydroxide to N-methyl-4-ethyl- Δ^3 -piperidine (XXVIII), in over 82% yield (30).

No analogous metal hydride reductions of quaternary salts of pyridoxine have been reported.

c) Metal-alcohol reductions.

No metal alcohol reductions of pyridoxine or its derivatives have been reported. However, sodium-alcohol reductions of pyridines are well documented and give a variety of products depending upon the alcohol used. Thus sodium in n-butyl alcohol is reported to give better yields of tetrahydropyridines than does sodium in ethyl alcohol (30). The products have been identified in some cases as Δ^3 -piperidines (31,32). Other products formed during these reductions include piperidines and ring opened products arising from opening of 1,4-dihydropyridines (33,34).

d) Hydrazine reduction.

Pyridoxine hydrochloride is converted to 4'-desoxypyridoxine, (XXIV), in nearly quantitative yield when it is refluxed with anhydrous hydrazine for 18 hours. The hydroxymethyl group at C-5' remains unchanged. Under the same conditions the 4'-methyl ether is also converted into 4'-desoxypyridoxine (35).

3) Nucleophilic and electrophilic substitution reactions of pyridoxine.

No nucleophilic or electrophilic substitution reactions of pyridoxine or its derivatives have been reported. Substitution reactions which give rise to intermediates suitable for further degradation reactions in the

pyridine system include phenylation, nitration and attack by hydroxide ion on quaternary pyridinium systems as in Decker's reaction.

a) Phenylation of pyridine and its derivatives.

Direct phenylation of the pyridine nucleus can be achieved using phenyl magnesium halides on the free base, but only in low yields (36). The reaction of phenyl and alkyl Grignard reagents with pyridine-1-oxides or pyridine-1-alkoxides proceeds readily to give the 2-substituted pyridines in good yields (37,38). Also, quaternary pyridinium salts react with alkyl Grignard reagents (39) and benzyl Grignard reagents (40) to form unstable 1,2-dihydropyridines which can be easily oxidised to the 2-substituted aromatic system.

The most generally used reagent for phenylation of the pyridine nucleus is phenyl lithium. Again an unstable 1,2-dihydro compound is initially generated and the phenylated pyridine can be formed from this adduct by heating above 100° (41), by use of a high boiling point solvent such as xylene or toluene (42,43), by oxidation with nitrobenzene (44), by oxidation, either by passage of a stream of dry air through the reaction mixture or by air oxidation on workup (45).

The advantage of a phenylated intermediate lies in its ease of oxidation to benzoic acid using aqueous permanganate. The carboxyl carbon of this benzoic acid is derived from the carbon atom to which the phenyl group is attached in the intermediate.

b) 2 - Pyridone formation.

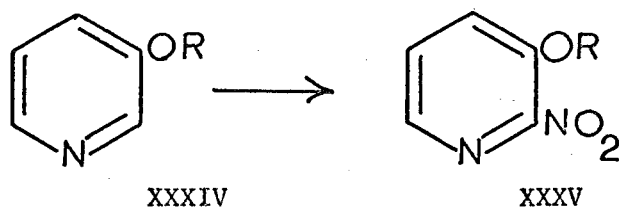
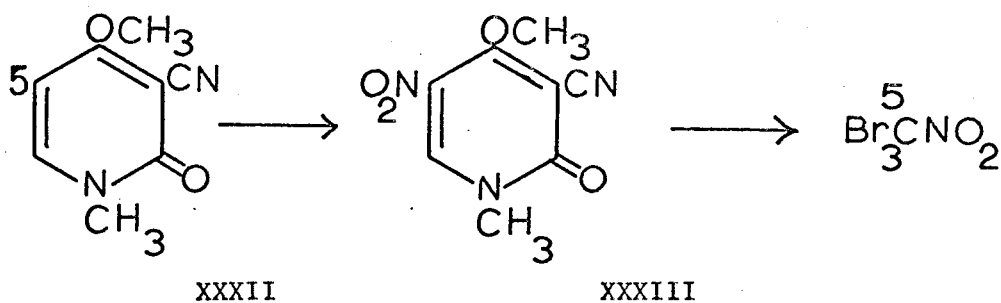
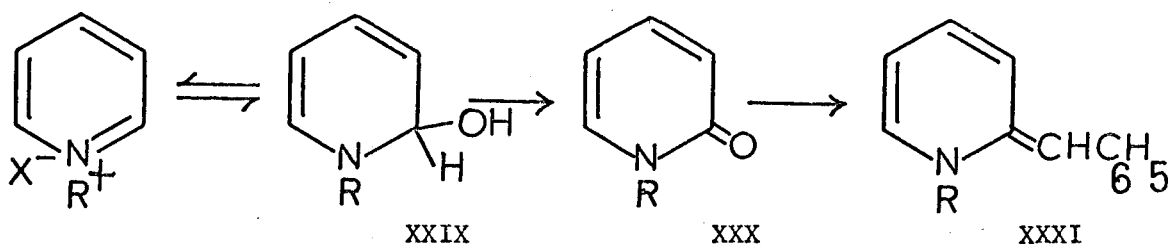
Generally 2-pyridones are readily synthesised from quaternary pyridinium salts in aqueous alkaline ferricyanide solution (Decker's reaction).

The reaction involves initial nucleophilic attack by hydroxide ion to form the 2-hydroxy-1, 2-dihydropyridine, (XXIX). This reaction is reversible but the equilibrium is shifted to the right as the dihydropyridine is oxidised by the ferricyanide to the 2-pyridone (XXX) (46). An N-alkylpyridone, once formed, is susceptible to direct alkylation either with alkyl Grignard or alkyl lithium reagents. An example of this type of reaction is that between N-methylpyrid-2-one, (XXX) ($R=CH_3$) and benzyl magnesium bromide which gives N-methyl-2-benzylidene-dihydropyridine (XXXI) (47).

c) Nitration.

A nitropyridine is an attractive intermediate since on treatment with calcium hypobromite it yields bromopicrin (Br_3CNO_2) bearing the carbon atom to which the nitro group was attached in the nitropyridine (48). The utilisation of such a series of reactions leading to the isolation of bromopicrin bearing the C-5 atom of ricinine (XXXII) has been described (49). In this case ricinine readily underwent nitration with concentrated sulphuric acid and 90% nitric acid at room temperature for two hours. Nitration was assumed to have occurred at C-5 since this position is activated by the C-4 methoxy group towards electrophilic substitution while the nitrile group at C-3 deactivates the 6 position for electrophilic substitution. The 5-nitroderivative, (XXXIII), yielded bromopicrin on treatment with calcium hypobromite and subsequent heating. Although the nitration of pyridoxine has not been reported, the nitration of a 3-hydroxypyridine (XXXIV) is documented (50,51). The product is 2-nitro-3-hydroxypyridine (XXXV, $R=H$). The 3-methyl ether of 3-hydroxypyridine,

under similar conditions (concentrated sulphuric acid and fuming nitric acid), gave 2-nitro-3-methoxypyridine (XXXV, R=CH₃) (52).



(C) ROUTES FOR THE SYSTEMATIC DEGRADATION OF PYRIDOXINE

Prior to the investigations described in this section, the only practical degradation of the pyridoxine molecule was that involving the direct Kuhn-Roth oxidation, which isolated the C-2 and C-2' atoms (Scheme I, p.10).

The reaction sequence shown in scheme II (P.12) has been proposed as suitable for the isolation of C-4'. Its usefulness is severely limited because of the low yield during the O-methylation step.

The following describes attempts to apply the reactions of pyridoxine and of similarly substituted pyridine compounds, described in Section B, to obtain new intermediates suitable for the isolation of the remaining five carbon atoms of pyridoxine.

1) Isolation of C-2 and C-2' and of C-2 plus C-4 and C-2' plus C-4'

Carbon atoms C-2 and C-2' are readily obtained as acetic acid, when pyridoxine is oxidised with chromium trioxide in 10% sulphuric acid. C-2' is then separated from C-2 by a Schmidt reaction and isolated as methylamine (Scheme I). As an extension of this Kuhn-Roth oxidation, the 4'-desoxypyridoxine hydrochloride (XXIV), was oxidised under the same conditions. It was hoped that two acetic acid molecules would be generated from the C₂ units, C-2, C-2' and C-4, C-4' from each molecule of the 4'-desoxy compound, as shown in scheme III, p.24.

It was found that steam distillation of the reaction mixture after chromic acid oxidation of a known weight of the 4'-desoxypyridoxine hydrochloride yielded a steam distillate which, on titration with sodium

hydroxide, was found to require more than one equivalent of base. The range of results was 1.1 to 1.3 gram equivalents, the average value for six runs being 1.2 gram equivalents of base.

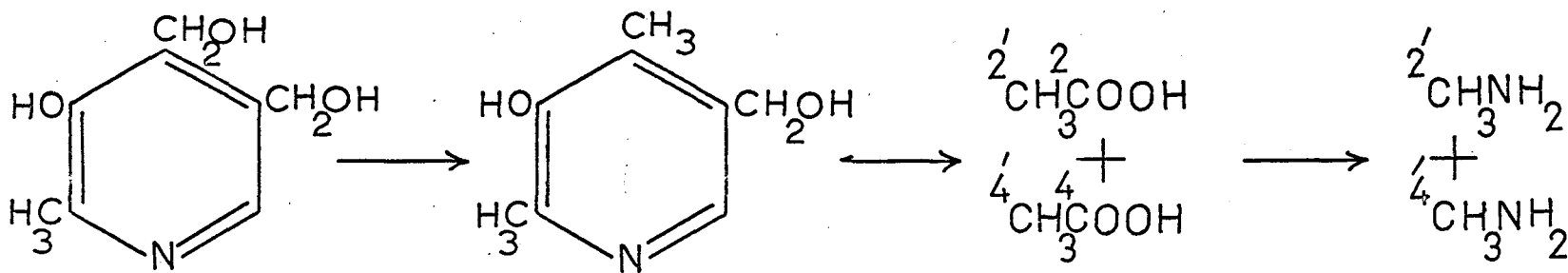
Similarly, oxidation and subsequent titration of the steam distillate from a series of oxidations of the free base of 4'-desoxypridoxine showed that more than one gram equivalent of acetic acid was generated per gram equivalent of the 4'-desoxy base. Thus no hydrogen chloride was distilled over with the acetic acid during the workup of the product mixture from the 4'-desoxypridoxine hydrochloride oxidations. This is possibly due to the hydrochloric acid being oxidised to perchloric acid during the oxidation thus producing an acid which does not form an azeotrope with water and hence is not steam distillable from the reaction mixture.

The yields of acetic acid obtained by direct Kuhn-Roth oxidation of free B₆ base and B₆ hydrochloride were 0.81 and 0.73 equivalents, respectively. Thus it appears that an appreciable fraction of the acetic acid obtained during the oxidation of 4'-desoxypridoxine is indeed derived from the C-4 and C-4' positions of the vitamin. Further evidence substantiating this conclusion will be presented in Section D.

The mixture of acetates derived from the C-2', C-2 and C-4', C-4 positions of pyridoxine was further degraded by a Schmidt reaction to give methylamine derived from the C-2' and C-4' positions only.

2) Isolation of C-4'

As noted above, the Kuhn-Roth oxidation of the 4'-desoxypridoxine hydrochloride yields acetic acid apparently derived from the C-2, C-2' and C-4, C-4' positions of pyridoxine. A Schmidt reaction on this acetate



DEGRADATION SCHEME III; THE ISOLATION OF C-2, C-2', C-4 AND C-4' OF PYRIDOXINE

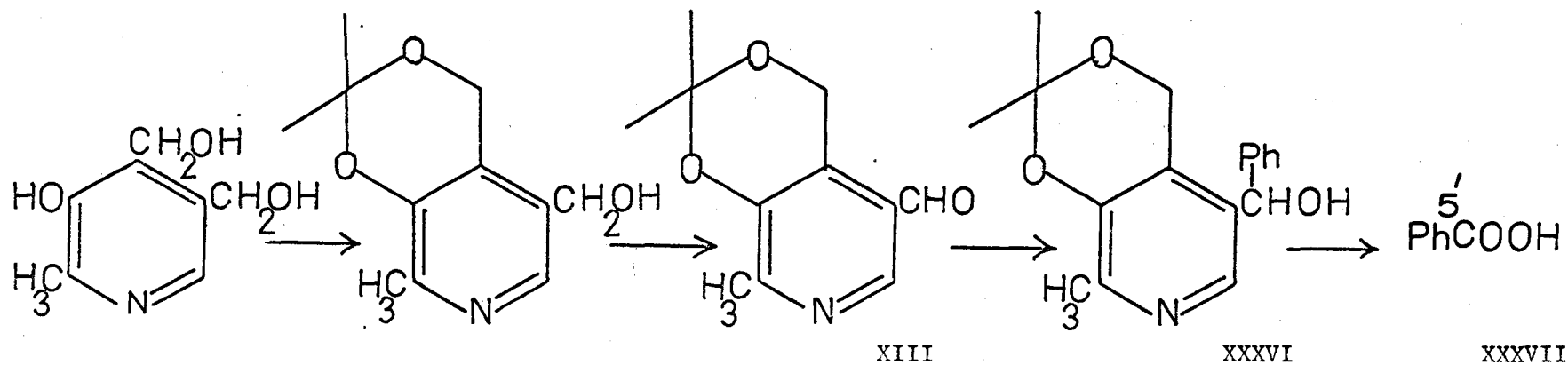
liberates methylamine bearing the C-2' and C-4' atoms of pyridoxine.

C-4' can also be isolated by decarboxylation of the methoxypyridine dicarboxylic acid (III), as shown in scheme (II), p.12. As noted, the drawback to this scheme was the reported low yield in the O-methylation step (17). This methylation has now been re-examined and the yield in this step has been raised from 45% to 60%, making the scheme practicable starting with 120 mg quantities of pyridoxine hydrochloride.

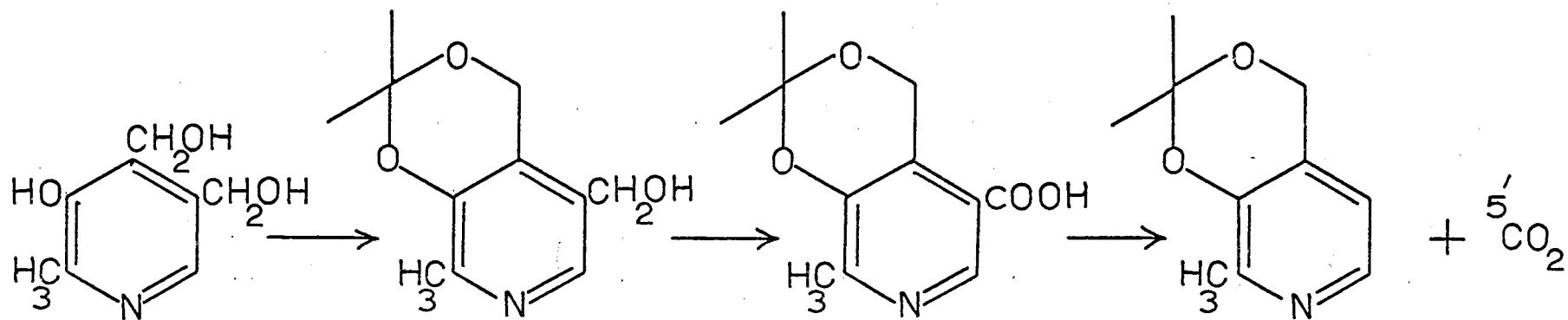
3) Isolation of C-5'

Oxidation of 3,4-O-isopropylidene-pyridoxine: It was noted earlier that treatment of pyridoxine hydrochloride with dry hydrogen chloride in acetone yields the cyclic acetonide, (XII), having the 3 and 4' positions blocked. This product can be easily oxidised to the 5 - aldehyde, (XIII), or the 5 - acid, (XIV) (19). The 5 -aldehyde is an attractive compound since on phenylation the 5 -phenylcarbinol (XXXVI) should be formed, which on oxidation with potassium permanganate should generate benzoic acid (XXXVII), bearing the 5'-carbon atom of pyridoxine. It was possible to isolate C-5' by this reaction sequence. Phenylation of the aldehyde proceeded readily with phenyl Grignard reagent in 84% yield and benzoic acid was indeed obtained from the phenylcarbinol in 70% yield.

The degradation scheme based on these reactions is shown as scheme IV, p. 26. Although the scheme involves four steps, each step gives over 70% yield and it has been found practical to perform the degradation on less than 50 mg of pyridoxine hydrochloride and obtain 9 mg of benzoic acid (32% yield based on B₆ hydrochloride).



DEGRADATION SCHEME IV: THE ISOLATION OF C-5' OF PYRIDOXINE



DEGRADATION SCHEME V: THE ISOLATION OF C-5' OF PYRIDOXINE.

The 3,4'-acetonide - carboxylic acid (XIV), could also serve as an intermediate in a degradation scheme aimed at the isolation of C-5' since its decarboxylation should yield C-5' as carbon dioxide which can be collected as barium carbonate. The acid (XIV) has now been obtained in 90% yield by direct oxidation of the acetonide, (XII), with potassium permanganate in aqueous pyridine. However, attempts to decarboxylate the acid (XIV) by heating in nitrobenzene at 180-200°, with or without a copper chromite catalyst, conditions which were successful in bringing about the decarboxylation of another pyridine - 3-carboxylic acid, nicotinic acid (53), failed to yield the desired product. When temperatures above 215° were maintained, some carbon dioxide evolution was observed and on workup, after evolution had ceased, two nonacidic products were isolated. The major product exhibited infrared bands at 1780 and 1720 cm^{-1} , indicating that the anhydride of the acid had formed. The experiment was repeated using a more dilute solution of the 5'- acid in nitrobenzene, but again decarboxylation occurred in less than 10% yield. Although this decarboxylation step gave a poor yield of carbon dioxide, degradation scheme (V), p. 27. is practically feasible, but in the light of the higher yields in degradation scheme (IV) and also because of the greater ease of counting active benzoic acid as opposed to barium carbonate, scheme (IV) is to be preferred to scheme (V) for the isolation of C-5'.

4) Attempted isolation of C-3 and C-5

In an attempt to gain access to C-3 and C-5 of pyridoxine various attempts were made to reduce the aromatic nucleus. The general

objective of these experiments was to convert pyridoxine into a 3-ketopiperidine derivative, whose keto group would be amenable to further degradation.

a) Catalytic reduction.

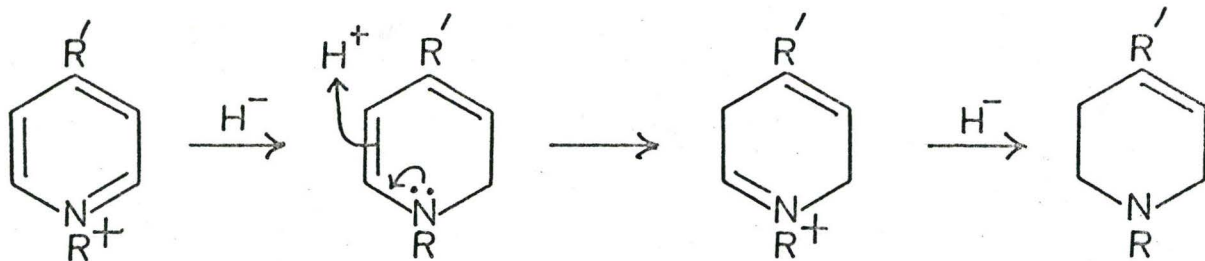
The only reported reductions of pyridoxine involve reduction of the C-4' hydroxymethyl group to a methyl group with either hydrazine (35) or catalytically (27).

Catalytic reduction of pyridoxine was attempted using a variety of solvents, ranging from acetic acid to ethanol, with platinum oxide catalyst at 60° under fifty pounds hydrogen pressure for over sixty hours. Only a trace of new product was observed which was identical with 4'-desoxypyridoxine. Reduction of the acetonide, (XII), under these conditions gave no new product.

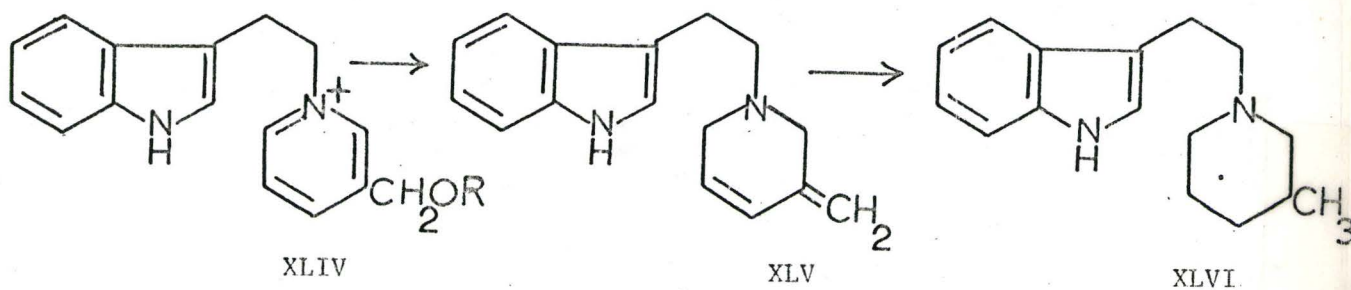
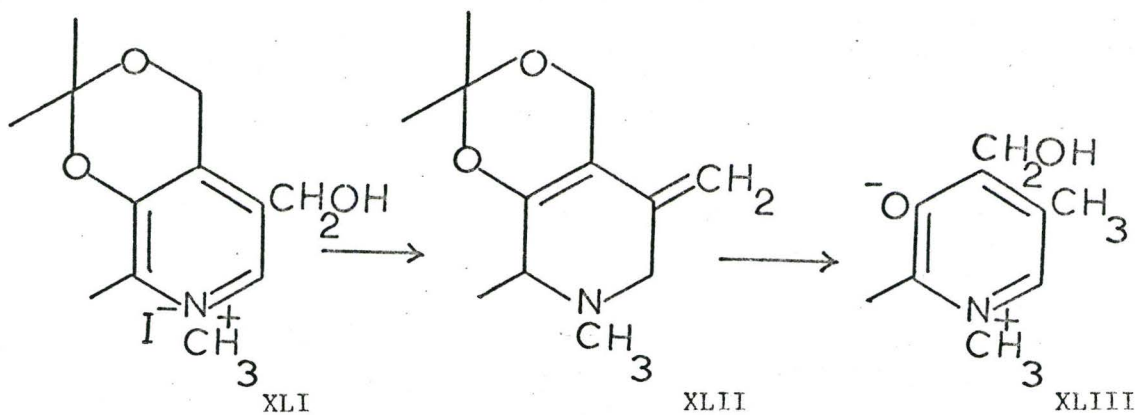
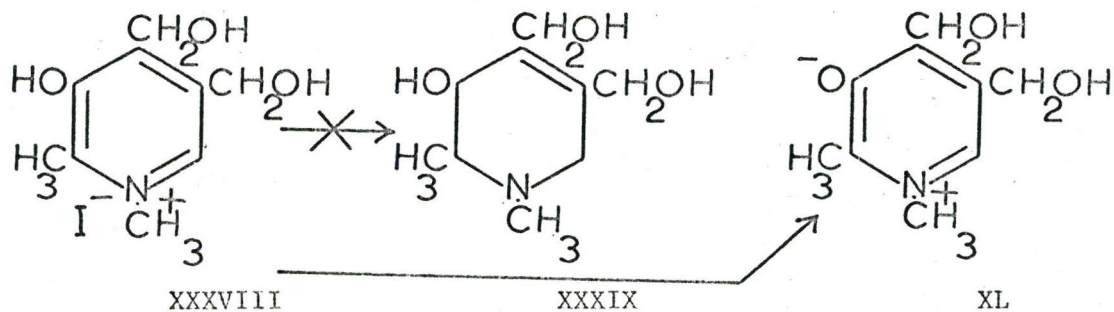
b) Hydride reduction.

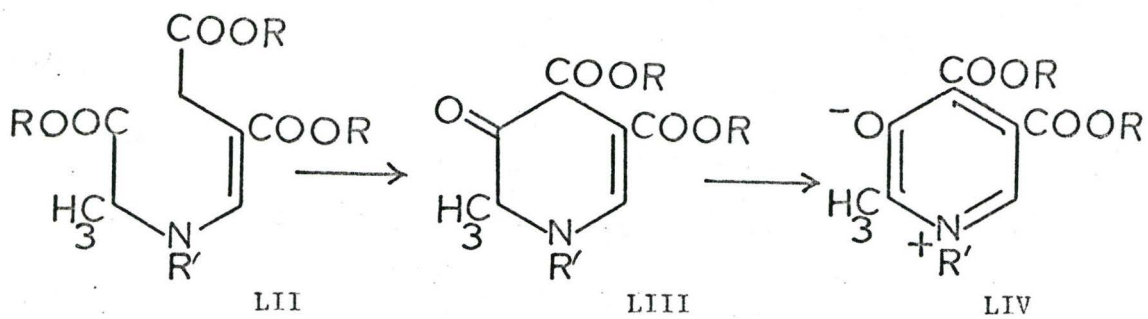
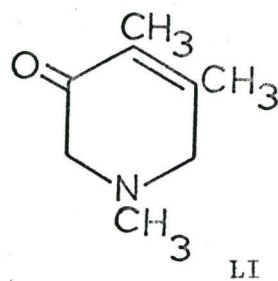
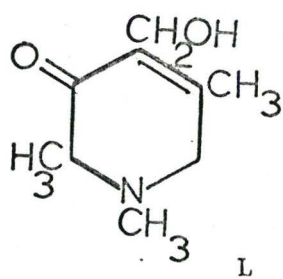
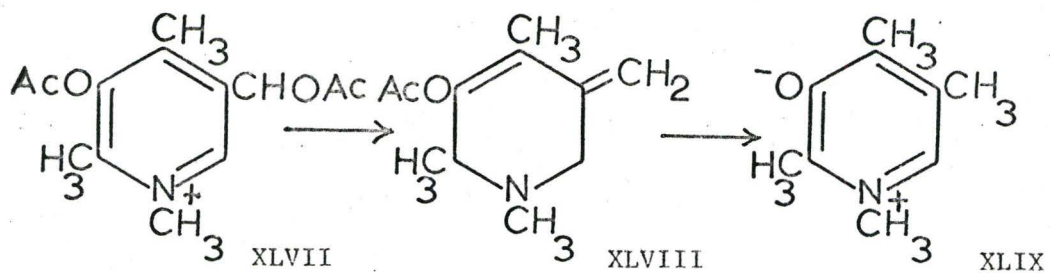
Hydride reduction of quaternarypyridinium systems generally gives the Δ^3 -piperidine via the mechanism shown (p.30) (54). If such a mechanism occurred in the hydride reduction of N-methylpyridinium iodide, (XXXVIII), then the resulting product should be the Δ^3 -piperidine, (XXXIX), assuming initial hydride attack at the unsubstituted centre adjacent to the nitrogen. This product could be useful for the isolation of C-3 and C-5 through oxidation to an α,β unsaturated ketone and phenylation via either 1,2 or 1,4 addition, respectively, followed by oxidation of the phenylated product to benzoic acid in the usual manner.

The methiodide of pyridoxine, (XXXVIII), was found to be stable to sodium borohydride in methanol, the betaine, (XL), being isolated on workup. When the reduction of the 3,4- acetonide of pyridoxine methiodide,



PROPOSED MECHANISM FOR THE HYDRIDE REDUCTION OF A PYRIDINIUM SYSTEM





(XLI), was investigated, reduction proceeded smoothly with borohydride in methanol or with lithium aluminium hydride in tetrahydrofuran (but not in ether). A mixture of products was obtained in 75% yield. The main component was identified from its spectral and mass spectral characteristics as 1,2-dimethyl-3,4'-O-isopropylidene-4-hydroxymethyl-5-methylene- Δ^3 -piperidine (XLII). The infra red spectrum of this product showed the loss of the 5'-hydroxyl group, and the bands at 3080 and 1660 cm^{-1} indicated the presence of a vinyl type group. This conclusion is supported by the P.M.R. spectrum which showed olefinic singlets at 4.56 δ and 4.60 δ . The overall structure is further supported by the P.M.R. spectrum which showed a methyl doublet at 1.50 δ , assigned to the C-2' group which is split by the proton at C-2. The N-methyl protons were observed at 2.42 δ as a singlet peak. The acetonide methyl peaks were seen as a six proton singlet at 1.52 δ and the 4'-methylene protons as a two proton doublet at 4.38 δ . The three protons α to the nitrogen were observed as a multiplet centred at 3.38 δ . The mass spectrum gave a molecular ion at 209 mass units which is in agreement with the assigned structure.

An analogous reductive elimination has been observed in the hydride reductions of the N-[β -(3-indolyl)ethyl] pyridinium salt, (XLIV), (R=H), which likewise underwent dehydration, giving the exocyclic diene (XLV), whose structure was proven by catalytic reduction using platinum on carbon, to (XLVI), identical with a synthetic sample (55).

Since it was reported that XLV was obtained in very high yield in the borohydride reduction of the indolyethylpyridinium salt (XLIV, $R=COCH_3$), the analogous reaction conditions were used in the pyridoxine series. It was found that the reduction of the 3,5'-diacetate of 4'-desoxypyridoxine methiodide, (XLVII), prepared by acetylation of the 4'-desoxypyridoxine hydrochloride, (XXIV), and subsequent treatment with methyl iodide, proceeded in over 90% yield and gave one single product, XLVIII. This product also exhibited I.R. bands at 3098 and 1670 cm^{-1} as well as acetate bands at 1765 and 1210 cm^{-1} . Olefinic protons were observed in the P.M.R. as two proton singlets at 5.14 δ and 5.18 δ . Other P.M.R. peaks were a three proton doublet at 1.30 δ due to the 2'-methyl protons, the methyl-acetate protons at 1.68 δ , the 4'-methyl protons at 2.22 δ , and the N-methyl protons at 2.48 δ . The three protons α to the nitrogen were again observed as a multiplet centred at 3.36 δ . A molecular ion at 195 mass units was observed, in agreement with the assigned structure.

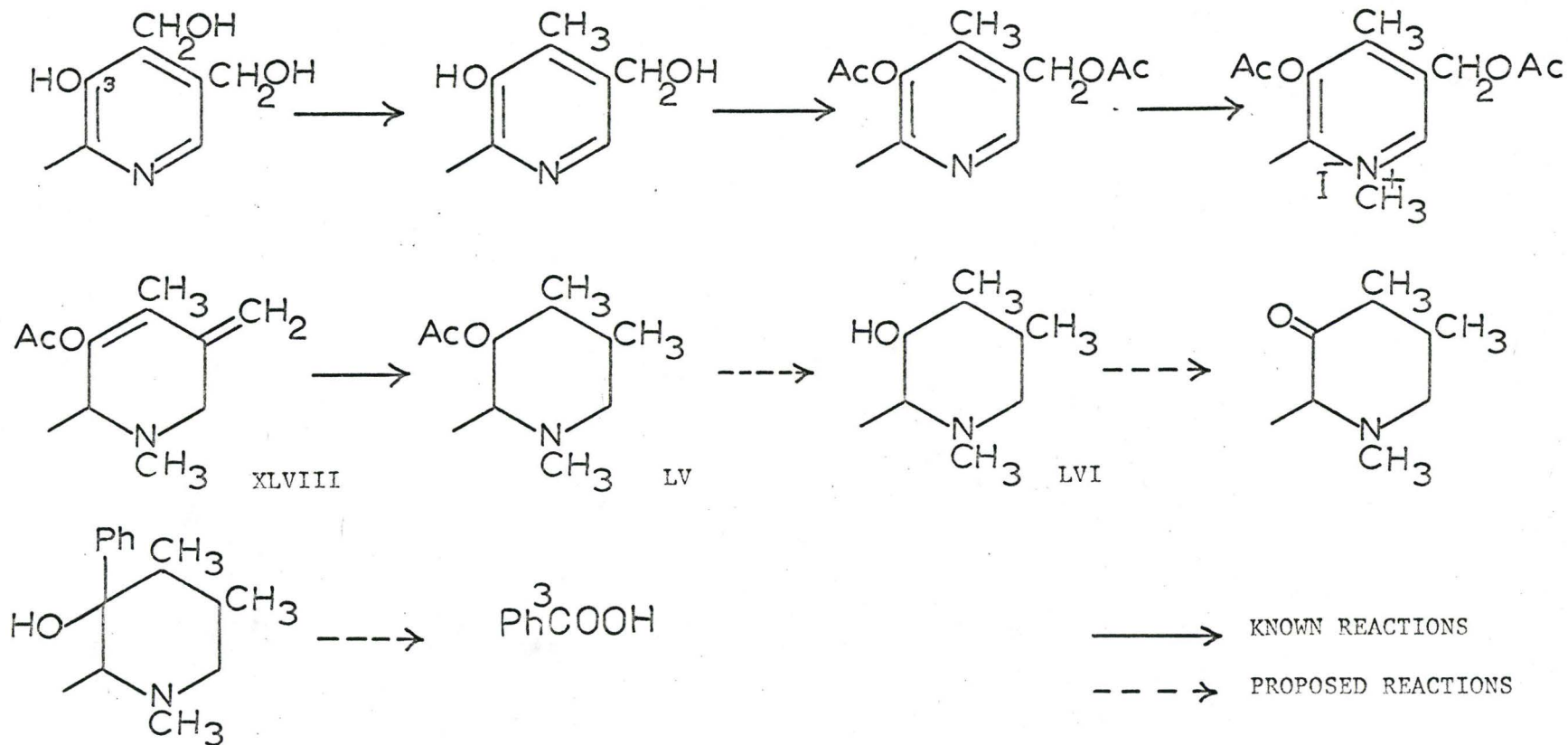
Hydrolysis of either reduction product XLII or XLVIII would generate an unstable en-3-ol. It was hoped that these enols would rearrange to form the corresponding α,β unsaturated ketones, L, or LI respectively, which could be phenylated either by 1,2 addition or 1,4 addition to the α,β unsaturated ketones, permitting isolation of either the C-3 or C-5 position as benzoic acid. Unfortunately the reduced monoacetate (XLVIII), on standing in dry air or on alkaline hydrolysis gave rise to a crystalline product having a U.V. absorption spectrum identical to the starting product XLVII, indicating that the reduction

product had reoxidised to a pyridinium system. The structure of this reoxidised pyridinium salt is postulated as the betaine XLIX, since the molecular ion in the mass spectrum had a value of 151 mass units. Also, the P.M.R. spectrum showed four three-proton singlet peaks at 2.07 δ , 2.17 δ , 2.43 δ and 3.98 δ assigned to the 5'-4'-2' and N-methyl groups respectively. A final one proton singlet peak was observed at 7.24 δ due to the C-6 aromatic proton.

Similarly, acid hydrolysis of the acetonide group of the hydride reduction product XLII afforded a pyridinium betaine tentatively assigned the structure XLIII by analogy with the structure of the betaine XLIX and from its mass spectral and I.R. data.

The instability of such partially reduced N-substituted-3-ketopyridine systems has been observed before. Thus the cyclic ketoester LIII, formed by a Dieckmann condensation of ethyl N-1-carbethoxyethyl-N-methylaminomethylenesuccinate, LII (R=Et, R'=Me), was found to dehydrogenate on exposure to dry air or on repeated recrystallisations from alcohol-ether, to form the betaine LIV (54).

Because of the propensity of the partially reduced acetate, (XLVIII), to revert to a pyridinium system, it was further reduced catalytically to the 3-acetoxypiperidine (LV). This on basic hydrolysis should generate the free alcohol, (LVI). Oxidation of the alcohol to the ketone, followed by phenylation and oxidation to liberate benzoic acid bearing C-3 of pyridoxine is possible and is shown in degradation scheme VI. This scheme involves seven reactions and although the first four are known to take place in good yields, the practicability of this scheme when dealing with milligram quantities of starting product is in doubt.



PROPOSED DEGRADATION SCHEME VI: ISOLATION OF C-3 OF PYRIDOXINE

Pyridoxine hydrochloride was found to react with sodium in isopropyl alcohol in only very low yields, possibly due to the formation of stable sodium salts. The 3,4-acetonide (XII), however, was reduced smoothly under these conditions, yielding a complex mixture of products. Thin layer chromatographic analysis indicated the presence of four major components and an I.R. spectrum showed OH and C=C bands. It was hoped that two of these products would be the Δ^3 -piperidine, (LVII) and the 1,4-dihydropyridine, (LVIII). Hydrolysis of these compounds would furnish the 3-keto system required for the isolation of the C-3 position. However, acid hydrolysis of the reaction mixture led to a single major product which exhibited a U.V. absorption spectrum typical of a 3-hydroxypyridine. It also gave a blue colour with Gibbs' reagent, indicating that the unsaturated 3-keto-piperidine systems had been reoxidised to a 3-hydroxypyridine. (This 3-hydroxypyridine has not yet been characterised).

5) Attempted isolation of C-6

a) Attempted phenylation of pyridoxine.

Attempts to phenylate the 6-position of pyridoxine directly with phenyl lithium or phenyl magnesium bromide failed. The 3,4'-acetonide, XII, also failed to react. The reason for this failure probably lies in the acidity of the C-2 methyl group which forms a lithium salt in the presence of phenyl lithium.

Some success was had when the methiodide of the 3,4'-acetonide of B₆ (LIX) was treated with phenyl Grignard. A new product was formed,

as indicated by T.L.C. analysis, but the oxidation of the 1,2-dihydro intermediate, (LX), in boiling toluene led to tar formation instead of the expected 6-phenylpyridinium salt, (LXI).

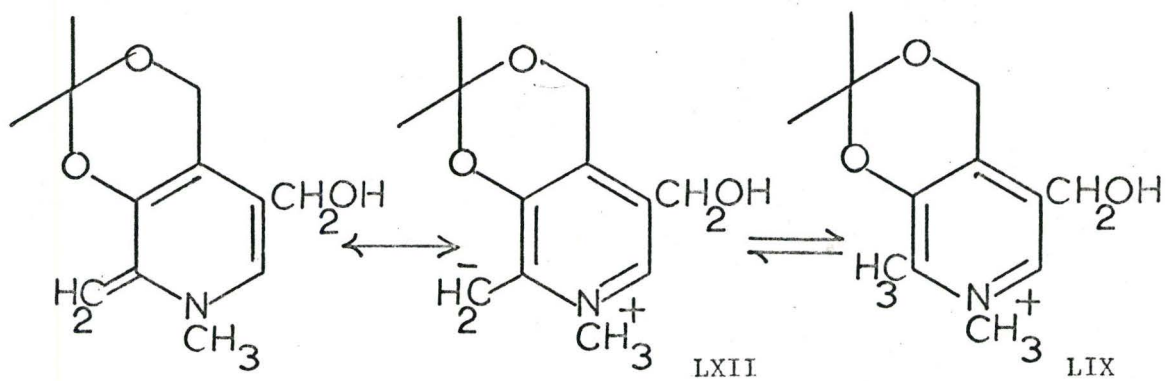
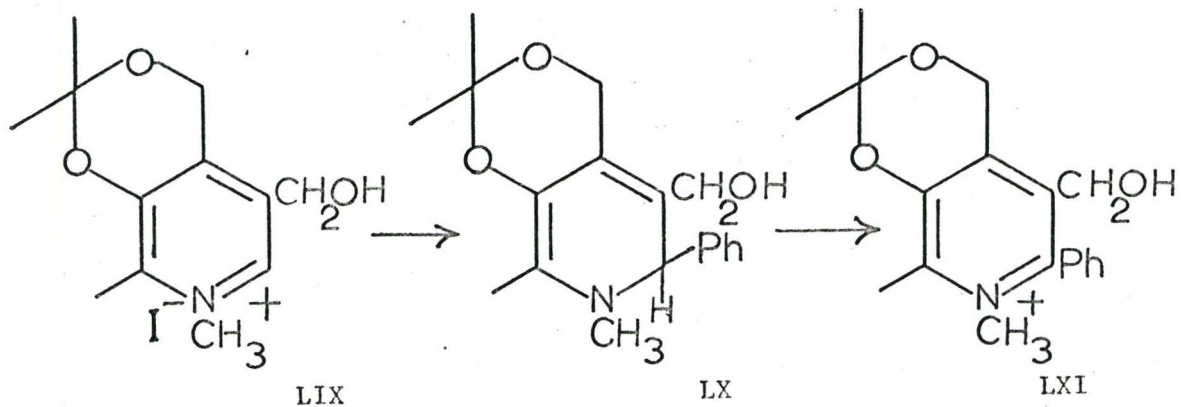
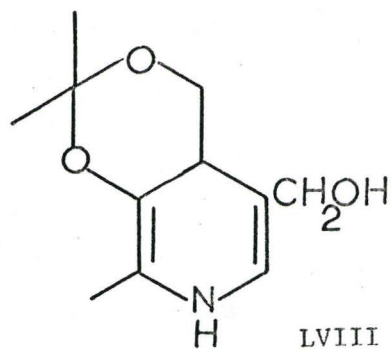
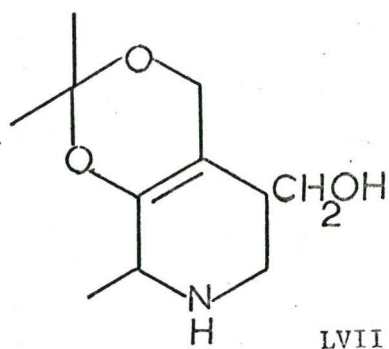
b) Decker's reaction.

It was hoped to generate a pyridone by treatment of the methiodide of 3,4'-acetonide of pyridoxine, (LIX), with alkaline potassium ferricyanide solution. However, virtually no reaction was observed. This may again be due to the acidity of the 2-methyl group. Under the strongly basic reaction conditions a pyridone methide (LXII) is formed which inhibits attack by hydroxide ion to form a 1,2-dihydro intermediate, (LXIII), which would be oxidised to the required pyridone, (LXIV).

The failure of Decker's reaction with a simple 2-methylpyridinium salt has been noted before (55).

c) Nitration.

Two attempts were made to nitrate pyridoxine and its O-methyl ether under the conditions described for the nitration of 3-hydroxypyridines. In the case of pyridoxine hydrochloride, a non polar product was obtained in low yield which was an oxidation product and inactive to Gibbs' reagent. In the case of the methyl ether no reaction was observed.



(D) THE DISTRIBUTION OF ^{14}C IN PYRIDOXINE DERIVED FROM 1,3- ^{14}C -GLYCEROL AND 2- ^{14}C -GLYCEROL

The hypothesis of pyridoxine biosynthesis, which was put forward (14) on the basis of the incorporation of activity from labelled pyruvate and glycerol into C-2 and C-2' of pyridoxine was outlined in Section A of this thesis.

It is apparent from figure I that, if this hypothesis is correct, incorporation of activity from 1,3- ^{14}C -glycerol via the proposed biosynthetic scheme should generate pyridoxine bearing ^{14}C at positions C-2', C-3, C-4', C-5' and C-6. If 1,3- ^{14}C -glycerol is the sole carbon source during such an experiment, then each of these positions must be equally labelled, i.e. each should carry one fifth of the activity of the intact molecule. Similarly, incorporation of activity from 2- ^{14}C -glycerol should generate pyridoxine bearing labelled carbon atoms at C-2, C-4 and C-5, each of which should carry one third of the total activity of the intact pyridoxine. Application of the degradation schemes, I, II, III and IV, developed in this work, to the active samples obtained from such feeding experiments should yield results on the basis of which it should be possible to determine whether or not the pyridoxine contained the predicted pattern of activity.

It had been shown earlier (14) that direct Kuhn-Roth oxidation of pyridoxine hydrochloride obtained from 1,3- ^{14}C -glycerol gave a sample of acetate bearing $22 \pm 1\%$ of the total specific activity of the pyridoxine from which it was obtained. Furthermore, over 90% of the activity associated with the acetate was located at its methyl group, i.e.

at the C-2' position of pyridoxine. This was shown by the activity of the methylamine derived from this acetate by a Schmidt degradation. The postulated biosynthetic scheme predicts that one fifth (i.e. 20%) of the total specific activity of pyridoxine resides at the C-2' position and that the C-2 position should contain no activity.

Additional data on the distribution of activity within pyridoxine derived from 1,3-¹⁴C-glycerol have now been obtained (Table I, p 44). Degradation scheme II isolated the decarboxylated product bearing 78⁺²% of the total specific activity. This indicates that C-4' carries \approx 20% of the total activity. C-5' of pyridoxine, isolated as benzoic acid by degradation scheme IV, contained 22⁺¹% of the total activity. Thus both C-4' and C-5' carry one fifth of the total activity, as predicted by the hypothesis.

Information on the distribution of activity from pyridoxine derived from 2-¹⁴C-glycerol has also been obtained (Table I, p 44). The 2-¹⁴C-glycerol experiment would be predicted to generate pyridoxine carrying ¹⁴C at C-2, C-4 and C-5. Direct Kuhn-Roth oxidation of pyridoxine isolated from this feeding experiment gave acetate carrying 35⁺¹% of the specific activity of the parent molecule, in agreement with the value of one third (33%) predicted by the scheme. The methylamine derived from this acetate now carried only 2.4^{+0.4}% of the total activity, showing that the activity of the acetate is located at C-2 of pyridoxine as predicted.

Degradation via scheme II yielded the monocarboxylic acid which possessed all the specific activity associated with the pyridoxine. The only carbon atom, which is lost in this reaction sequence, C-4', therefore

does not contain activity. Finally, the benzoic acid isolated from the C-5' position via scheme IV, was also inactive. The lack of label in both the C-4' and the C-5' position of pyridoxine is in agreement with the proposed scheme of biosynthesis.

These results are unambiguous and are clearly in agreement with the predictions of the hypothesis (scheme I) which proposes that pyridoxine is biosynthesised in E. coli B, WG2 from three glycerol molecules and indicates a possible mechanism for the biosynthetic pathway. The hypothesis is strongly supported by the results. For further verification of the mechanism, intact incorporation of postulated intermediates must be tested and their natural occurrence in the system must be demonstrated.

The validity of degradation scheme III is open to question, since it has not been unambiguously demonstrated that the C₂ unit, C-4', C-4, contributes significantly to the acetate derived from the oxidation of 4'-desoxypyridoxine hydrochloride. The yield of acetate on Kuhn-Roth oxidations of 4'-desoxypyridoxine hydrochloride was found to be 1.2 - 1.4 equiv. per mole. as determined by titration. It follows that at least a small fraction of this acetate must be derived from the C₂ fragment, C-4', 4. The actual contribution made by the fragment C-4', 4, to the isolated acetate can be deduced from Table 2: Since virtually all activity of serine-derived pyridoxine resides in the C₂ unit, C-2', 2, (as shown by Kuhn-Roth degradation of this pyridoxine), the C₂ unit, C-4', 4, of this sample contains little, if any, activity. Since the specific activity of the acetate isolated by degradation of the 4'-desoxypyridoxine obtained

from serine-derived pyridoxine, was less than 70% of that of the intact vitamin (Table 2), at least one-third of the acetate, obtained in this way, must have been derived from the C_2 units, C-4', 4. It is evident that if the labelling pattern in the C_2 unit, C-4', 4, of a given pyridoxine sample is significantly different from that of the C_2 unit, C-2', 2, the specific activities of the samples of acetate and methylamine obtained from it by Scheme I and Scheme III must differ. Conversely, if these specific activities do not differ significantly, the labelling patterns of the C_2 units, C-4', 4 and C-2', 2, must be similar. Since degradation Schemes I and II permit individual assay of activity at C-2', C-2 and C-4' respectively, it follows that the activity at C-4 can be deduced.

Referring to Table I it can be seen that the results bearing on the labelling of C-4 obtained in the Kuhn-Roth oxidation, are in complete agreement with prediction. Thus in the 1,3- ^{14}C -glycerol experiment, the acetate, derived from the C_2 units C-2', 2 and C-4', 4, carries $22 \pm 1\%$ of the total specific activity. Furthermore all this activity is located at the methyl group (C-2', C-4'), as shown by the activity of the methylamine derivative, which carries $19 \pm 1\%$ of the total activity. This agrees with the value for activity at C-2', 2, and C-4', discussed above. Thus it may be inferred that C-4 is inactive as required by the hypothesis.

Similarly the acetate, obtained from the 4'-desoxypyridoxine derivative in the 2- ^{14}C -glycerol experiment, carries $32 \pm 1\%$ of the total specific activity of the pyridoxine from which it was derived. Since direct Kuhn-Roth oxidation of this pyridoxine had located 33% of the activity at C-2, and C-4' had been shown to be inactive by direct de-

gradation, it may be inferred that C-4 carries \sim 33% of the activity, as required by the hypothesis. Thus application of degradation scheme III gives results for the activity of C-4 of pyridoxine which add to the evidence supporting the validity of the scheme for the biosynthesis of pyridoxine.

TABLE I

Observed and predicted distribution of activity in pyridoxine derived from ^{14}C -glycerol.Relative specific activity (percent) (pyridoxine=100)
of the product derived from

Product (carbon atoms of pyridoxine)	1,3- ^{14}C -glycerol			2- ^{14}C -glycerol		
	Observed	Predicted	S.A. ^a	Observed	Predicted	S.A. ^a
Pyridoxine (all)	100 ± 3	100	See Table 3	100 ± 3	100	See Table 3
Scheme I						
Acetic acid (C-2',2)	22 ± 1	20	0.51 ± 0.01	35 ± 1	33	0.38 ± 0.001
Methylamine (C-2')	18 ± 1	20	(0.20 ± 0.004)	2.4 ± 0.4	0	0.03 ± 0.004
Scheme III						
4'-Desoxypyridoxine (all)	98 ± 1	100	1.63 ± 0.04	101 ± 4	100	1.10 ± 0.03
Acetic acid (C-2',4',2,4)	22 ± 1	20	0.36 ± 0.01	32 ± 1	33	0.35 ± 0.01
Methylamine (C-2',4')	19 ± 1	20	0.31 ± 0.01			
Scheme II						
3-O-Methylpyridoxine (all)	99 ± 2	100	0.94 ± 0.02			
4,5-Dicarboxylic acid (III) (all)	100 ± 3	100	0.93 ± 0.03			
5-Monocarboxylic acid (X) (all but C-4')	78 ± 2	80	0.73 ± 0.02	103 ± 6	100	0.79 ± 0.03
∴ C-4'	22 ± 2	20		0	0	
Scheme IV						
5'-Phenylcarbinol (XXXVI) (all)	98 ± 2	100	1.64 ± 0.02	99 ± 5	100	1.08 ± 0.05
Benzoic acid (C-5')	22 ± 1	20	0.37 ± 0.01	0.5 ± 0.2	0	0.005 ± 0.002

a Specific Activity (counts min.⁻¹ mmole⁻¹) × 10⁻⁴.

TABLE 2

Partial degradation of pyridoxine derived from 3-¹⁴C-Serine.

Product (carbon atoms of pyridoxine)	Relative specific activity	S.A. ^a
Pyridoxine (all)	100 ± 3	0.29 ± 0.01
		(diluted) 0.12 ± 0.005
Scheme I		
Acetic acid (C-2',2)	92 ± 4	0.27 ± 0.01
Scheme III		
4'-Desoxypyridoxine (all)	100 ± 5	0.12 ± 0.005
Acetic acid (C-2',4',2,4)	63 ± 3	0.074 ± 0.002

^a Specific Activity (counts min⁻¹. m mole⁻¹) X 10⁻⁴.

TABLE 3Specific activities of pyridoxine hydrochloride ^a.

Degradation Scheme	1,3- ¹⁴ C-Glycerol	2- ¹⁴ C-Glycerol
I	2.39 \pm 0.06 (1.12 \pm 0.03)	1.09 \pm 0.03
III	1.67 \pm 0.03	1.09 \pm 0.03
II	0.94 \pm 0.01	0.76 \pm 0.04
IV	1.67 \pm 0.03	1.09 \pm 0.03

^a Specific activity (counts min⁻¹ · m mole⁻¹) X 10⁻⁴.

(E) EXPERIMENTAL

Degradation scheme I.

1) 'Direct' Kuhn-Roth oxidation of pyridoxine hydrochloride.

Pyridoxine hydrochloride (60 mg) of known specific activity (14) was placed in a flask fitted with a reflux condenser. Dilute sulphuric acid (10 ml, 10% v/v) was added, followed by chromium trioxide (2g). The solution was refluxed overnight. The cold solution was transferred to a steam distillation apparatus and the solution was heated while a slow stream of nitrogen was passing through the apparatus. The condensed distillate was collected over about 4 hours when 80 ml of distillate had been collected. The volume of the solution in the heated flask was maintained at a constant level by periodic addition of 5 ml portions of water. The acetic acid-water distillate was titrated against sodium hydroxide of known normality to pH 7, using a pH meter. The neutral solution was evaporated to dryness in an oven at 90° to yield sodium acetate, 24 mg (72%).

2) Acetyl- α -naphthylamide.

A portion of the sodium acetate (~5 mg.) was dissolved in water (1 ml). To this solution was added a solution of α -naphthylamine hydrochloride (15 mg) in water (1 ml). To the mixture was added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (~40 mg). On stirring, the acetyl- α -naphthylamide separated. This was filtered off, washed with water, recrystallised from benzene-hexane and sublimed in vacuo below 100° to yield acetyl- α -naphthylamide (5 mg). This was plated using 2 drops of dimethylformamide (D.M.F.) containing 1% collodion.

3) 2,4-Dinitrofluorobenzene derivative of methylamine.

A portion of the sodium acetate from (1) (~ 15 mg) was heated with sodium azide (50 mg.) and concentrated sulphuric acid (1 ml) at about 70° . The carbon dioxide evolved was passed in a slow stream of nitrogen through a trap containing a 10% solution of potassium hydroxide. When carbon dioxide evolution had ceased, the reaction flask was cooled in ice and the trapped carbonate precipitated by addition of barium chloride solution. The precipitated barium carbonate was filtered off. The traps on the apparatus were now refilled with methanol containing one drop of saturated sodium bicarbonate solution and four drops of 2,4-dinitrofluorobenzene. The cooled acid reaction solution was basified ($\text{pH} > 12$ to pH paper) by addition of excess potassium hydroxide solution. The reaction flask was reheated to $\sim 70^\circ$ for three hours and the apparatus flushed with nitrogen. The methanol solution from the traps was evaporated to dryness. The residue was dissolved in ether (5 ml) and extracted with water (2 x 1 ml). The ether solution was evaporated to a small volume when 2,4-dinitro-N-methylaniline crystallised on standing. The product was filtered off and sublimed at 3.0×10^{-3} mm. and $100-120^\circ$ to yield 8 mg of yellow solid mp. 177° . The 2,4-dinitro-N-methylaniline was plated in 2 drops of dimethyl formamide containing 1% collodion.

Degradation scheme III

4) 4'-Desoxypyridoxine hydrochloride, XXIV. (35).

A mixture of pyridoxine hydrochloride (60 mg) of known specific activity and anhydrous hydrazine (95%, 5 ml) was refluxed for 18 hours with exclusion of moisture. The solution was then evaporated to dryness under vacuum at 100°. The residue was dissolved in a mixture of tetrahydrofuran and methanol from which hydrazine hydrochloride crystallised. The filtrate was evaporated to dryness and dissolved in anhydrous tetrahydrofuran. Etherial hydrochloric acid solution was added to the tetrahydrofuran solution and the precipitated 4'-desoxypyridoxine hydrochloride was filtered off and recrystallised from methanol/ether to yield 4'-desoxypyridoxine hydrochloride. (45 mg.81%). The specific activity of this intermediate was determined by plating a sample on an aluminium planchette using a 10% sucrose solution, and counting.

5) 'Indirect' Kuhn-Roth oxidation of 4'-desoxypyridoxine hydrochloride, XXIV. 4'-Desoxypyridoxine hydrochloride (42 mg) of known specific activity was oxidised as described in (1) to yield sodium acetate, 22 mg (60%). The acetyl- α -naphthylamide and 2,4-dinitro-N-methylaniline derivatives were prepared as described in (2) and (3) respectively.

Degradation scheme II (17)

(6) O-Methylpyridoxine, II.

Pyridoxine hydrochloride (120 mg) of known specific activity was dissolved in a solution of diazomethane in methanol. After 12 hours the solvent was evaporated to ~5 ml and ether (5 ml) was added to precipitate pyridoxine betaine which was filtered off. The filtrate was

evaporated to dryness to yield O-methylpyridoxine. This was sublimed in vacuo at 3.0×10^{-3} mm. pressure and $100-120^\circ$ to yield O-methylpyridoxine (65 mg) (65%) (57).

7) 2-Methyl-3-methoxypyridine-4,5-dicarboxylic acid, III.

O-Methylpyridoxine (50 mg) was stirred with potassium permanganate solution (184 mg. in 10 ml of water) at room temperature for ten hours. The excess permanganate was destroyed with methanol at $60-70^\circ$ and the solution was filtered. The combined filtrate and washings were concentrated under vacuum to ~ 5 ml and the solution applied to a column (10 cm) of Dowex-1 in the formate form. The column was washed with aqueous formic acid (0.25M, 40ml) to elute 3-O-methyl-4-pyridoxic acid lactone, which was discarded. The column was then eluted with 5M formic acid until the U.V. absorption at 280 μ reached blank values. The eluate was evaporated to dryness under vacuum and the residue recrystallised from water. The yield of 2-methyl-3-methoxy-pyridine-4,5-dicarboxylic acid mp. $221-223^\circ$ (dec) was 43.5 mg (61%).

8) 2-Methyl-3-methoxypyridine-5-carboxylic acid, X.

A suspension of 2-methyl-3-methoxy-pyridine-4,5-dicarboxylic acid, III, (40 mg) in nitrobenzene (4 ml) was heated under nitrogen in a flask fitted with a nitrogen inlet tube and reflux condenser, with a carbon dioxide absorber (barium hydroxide solution) connected to the condenser. The temperature was maintained between $180-190^\circ$ for 0.5-1 hour until all evolution ceased. The compound dissolved completely. The decarboxylation product, which crystallised on cooling, was washed with benzene and ether, then sublimed at 180° and 3×10^{-3} mm. to give

2-methyl-3-methoxy-pyridine-5-carboxylic acid as a colourless solid mp. 224°. (molecular ion m/e 167). The P.M.R. spectrum of an inactive sample in perdeuterodimethylsulphoxide showed peaks at 2.40 δ s (3H), 3.86 δ s (3H), 7.62 δ d (1H) (J=0.5HZ), and 8.52 δ d (1H) (J=0.5HZ).

The intermediates II and III and the final decarboxylation product X were plated in a 1% solution of collodion in D.M.F.

Degradation scheme IV

9) 3,4'-O-Isopropylidenepyridoxine, XII.

Pyridoxine hydrochloride (50 mg.) of known specific activity was suspended in dry acetone (5 ml) at 0°C. Anhydrous hydrogen chloride was passed through the cold stirred solution until the solid dissolved (~ 5 minutes). The solution was stored at -10° for three hours, excess anhydrous ether (10 ml) was added and the mixture kept at -10° for a further 30 minutes. The product which had separated was filtered off and washed with ether, to give 3,4'-O-isopropylidenepyridoxine hydrochloride (55 mg.). It was dissolved in water and added to a saturated sodium bicarbonate solution (5 ml.). The mixture was cooled for 30 minutes and the free base removed to yield 3,4'-O-isopropylidenepyridoxine (44 mg) (87%).

10) 2-Methyl-3,4'-O-isopropylidenepyridoxine-4-hydroxymethyl-5-formyl-pyridine, XIII (19).

The 3,4'-O-isopropylidenepyridoxine (44 mg) was dried under vacuum, dissolved in anhydrous pyridine (5 ml) containing chromium trioxide (vacuum dried, 34 mg.) and the mixture was heated for thirty minutes with exclusion of moisture to bring the solution to reflux temperature and

was then refluxed for a further 90 minutes. The cold solution was filtered and evaporated to dryness under vacuum. The residue was dissolved in saturated sodium bicarbonate (15 ml) and the product was extracted into chloroform (3 x 15 ml). The chloroform extract was dried (K_2CO_3), evaporated to dryness and the residue was subjected to vacuum distillation at 3.0×10^{-3} mm. below 80° when a colourless liquid which crystallised on standing was obtained (29 mg) (70%). This was shown by comparison of its melting point, I.R. spectrum and T.L.C. properties to be identical to 2-methyl-3,4'-O-isopropylidene-4-hydroxymethyl-5-formylpyridine obtained from oxidising 'cold' 2-methyl-3,4'-O-isopropylidene-pyridoxine under identical conditions.

11) 2-Methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-phenyl-carbinol, XXXVI.

2-Methyl-3,4'-O-isopropylidene-4-hydroxymethyl-5-formylpyridine from (9) (29 mg) was dissolved in anhydrous tetrahydrofuran (2 ml) and phenyl magnesium bromide (60 mg) in tetrahydrofuran (1 ml) was added. The solution was refluxed for 2 hours and was treated with dilute hydrochloric acid (0.1M, 5 ml). The acid solution was extracted with chloroform (3 x 25 ml) and was then carefully basified with excess saturated sodium bicarbonate solution. The basic solution was extracted with chloroform (3 x 25 ml) and the chloroform extract was dried (K_2CO_3), and evaporated to dryness. The residue was sublimed in vacuo at 3.0×10^{-3} mm. and $100-120^\circ$ to give a white solid m.p. $159-160^\circ$ (30 mg) (79%). A sample of the 2-methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-phenylcarbinol was plated using D.M.F. + 1%

collodion and its specific activity determined. An inactive sample showed a molecular ion at 285 mass units, $\nu_{\text{CHCl}_3}^{\text{Max}}$, 3600, 3010, 872 cm^{-1} . The P.M.R. in CDCl_3 showed peaks at 1.41 δ s(6H), 2.32 δ s(3H), 4.62 δ m(2H), 5.69 δ (1H), 7.24 δ s(6H), and 7.84 δ s(1H) p.p.m. downfield from an internal T.M.S. standard. Anal. Found: C, 71.80; H, 6.72; N, 4.76. Calculated for $\text{C}_{17}\text{H}_{19}\text{NO}_3$ C, 71.56; H, 6.71; N, 4.91%.

12) Permanganate oxidation of 2-methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-phenylcarbinol to benzoic acid.

The 5-phenylcarbinol, XXXVI, (30 mg) of known specific activity was heated with stirring at 100° with potassium permanganate (250 mg) in water (10 ml) for twelve hours. Sodium bisulphite was added to the solution which was then acidified with 10% (v/v) sulphuric acid until excess permanganate had been destroyed. The colourless acid solution was then extracted with ether (3 x 25 ml). The ether solution was washed with water (10 ml), dried (Na_2SO_4) and evaporated to dryness to yield a white solid. This solid on vacuum sublimation at 3.0×10^{-3} mm. and below 70° yielded benzoic acid mp $123-124^\circ$, 9 mg (70%). The benzoic acid was plated using methanolic sodium hydroxide solution (2 drops of 1% solution).

Degradation scheme V

13) 2-Methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-carboxylic acid, XIV. 3,4'-O-Isopropylidene-pyridoxine, XII, (150 mg) was dissolved in pyridine (1 ml). A suspension of finely powdered potassium permanganate (250 mg) in pyridine (1.5 ml), and aqueous sodium hydroxide (2M, 0.6 ml) was added. The undissolved permanganate

was transferred to the reaction vessel in the minimum volume of water. The mixture was heated on a waterbath for 5 minutes. The cold solution was filtered and carefully acidified with dilute hydrochloric acid to pH \sim 5 when the acid was extracted into ethyl acetate. The ethyl acetate solution was dried (Na_2SO_4) and evaporated to dryness to yield a white solid mp 220-222° (dec.) (140 mg) (93%) identical to 2-methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-carboxylic acid obtained by oxidation of 2-methyl-3,4'-O-isopropylidene-4-hydroxymethyl-5-formylpyridine with aqueous permanganate (19).

14) Attempted decarboxylation of 2-methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-5-carboxylic acid, XIV.

2-Methyl-3,4'-O-isopropylidene-4-hydroxymethylpyridine-carboxylic acid, XIV (52 mg) was suspended in nitrobenzene (5 ml) in a flask fitted with a nitrogen inlet tube, a reflux condenser and a carbon dioxide absorber ($\text{Ba}(\text{OH})_2$ solution in a trap). The flask was heated and a slow stream of nitrogen was passed through the apparatus. Gaseous evolution was observed above 215° and the oil bath temperature was maintained in the range 215-225° until gaseous evolution ceased (\sim 1 hour). During this time the barium hydroxide solution became cloudy. On cooling the nitrobenzene was extracted with ammonia solution (1M) in the presence of a little carbon tetrachloride to aid separation of the phases. The nitrobenzene phase was extracted with hydrochloric acid (0.1M) and the acid extract basified with sodium bicarbonate and extracted with chloroform (3 x 25 ml). The chloroform extract was dried (Na_2SO_4) and evaporated to dryness to yield a white solid (28 mg). T.L.C. analysis showed the product to consist of two components, and an I.R. spectrum of the mixture

showed anhydride bands at 1780 and 1720 cm^{-1} . The yield of barium carbonate was 5 mg (10%).

15) Catalytic reduction of pyridoxine and of 3,4'-O-isopropylidene-pyridoxine.

Pyridoxine hydrochloride (200 mg) was dissolved in glacial acetic acid (10 ml) and platinum oxide catalyst (70 mg) was added. The mixture was shaken at $\sim 60^\circ$ under hydrogen (50 p.s.i.) for 70 hours. After cooling, the mixture was filtered and the filtrate evaporated to dryness. A T.L.C. analysis of the product showed two spots, one due to a major, more polar, product having the same R_f value as pyridoxine hydrochloride, the other due to a minor product having an R_f value identical with that of 4'-desoxypyridoxine hydrochloride. Both spots gave a blue colour with Gibb's reagent. The experiment was repeated using identical conditions but using methyl alcohol (5 ml) as solvent. Again, unchanged pyridoxine and 4'-desoxypyridoxine were detected as the sole products. 3,4'-O-Isopropylidene-pyridoxine, XII, (200 mg) in absolute methyl alcohol (5 ml) was subjected to identical reduction conditions to those described above. In this case T.L.C. analysis showed only unchanged starting material and the acetonide, mp 111-112°, was isolated on evaporation of the filtrate.

16) Pyridoxine methiodide.

Pyridoxine base (200 mg) was added to a mixture of benzene (100 ml) and methanol (10 ml). Methyl iodide (4 ml) was added and the solution was refluxed for 12 hours. The solution was evaporated to ~ 30 ml and the crystals which separated were filtered off and washed with benzene and ether. Yield of pyridoxine methiodide was 366 mg (92.4%), mp 178-180° rising to 188-189° after recrystallisation from methanol.

17) 3,4'-O-Isopropylidenepyridoxine methiodide.

3,4'-O-Isopropylidenepyridoxine, XII, (100 mg) (19), was dissolved in a mixture of benzene (40 ml) and methanol (4 ml). Methyl iodide (1 ml) was added and the solution refluxed for 12 hours. The cold solution was evaporated to \sim 5 ml under vacuum below 60° and the crystals which separated were filtered off and washed with benzene. The yield of 3,4'-O-isopropylidenepyridoxine methiodide was 151 mg (91%) mp. 167-168° (dec). The P.M.R. spectrum, in perdeuterodimethylsulphoxide, showed peaks at 1.58 δ s (6H), 2.56 δ s (3H), 4.24 δ s (3H), 4.60 δ d (2H), 5.12 δ s (2H), 5.78 δ t (1H), and 8.51 δ s (1H) p.p.m. from T.M.S. Anal. Found: C, 40.89; H, 5.01; I, 35.89. Calculated for $C_{12}H_{18}INO_3$: C, 41.04; H, 5.17; I, 36.14%.

18) Sodium borohydride reduction of pyridoxine methiodide.

Pyridoxine methiodide (100 mg) was dissolved in absolute methanol (10 ml) and excess sodium borohydride (50 mg) was added to the ice-cooled, stirred solution. After 30 minutes the solution was evaporated to dryness under vacuum below 60° and the solution added to sodium bicarbonate solution (10%, 10 ml). The basic solution was extracted with ethyl acetate (2 x 25 ml). The ethyl acetate solution was dried, (Na_2SO_4), and evaporated to dryness to yield 2 mg of gum.

The sodium bicarbonate solution was carefully acidified with dilute hydrochloric acid and applied to a Dowex-50 column (10 cm) in the hydrogen form. The column was washed with water until the washings were neutral. The column was then eluted with ammonia solution (5% v/v) and the eluate evaporated to dryness to yield a white solid, mp 188 °C, which was identical to pyridoxine betaine obtained as a side-product in

the diazomethane treatment of pyridoxine hydrochloride (reaction 6). The yield of pyridoxine betaine was 43 mg. The P.M.R. in D_2O showed peaks at 2.48 δ s (3H), 4.04 δ s (3H) and 7.60 δ s (1H) p.p.m. from T.M.S.

19) Sodium borohydride reduction of 3,4'-O-Isopropylidenepyridoxine methiodide.

3,4'-O-Isopropylidenepyridoxine methiodide (200 mg) was dissolved in absolute methanol (20 ml). Sodium borohydride (200 mg) was added to the stirred solution at $0^\circ C$. After 30 minutes at $0^\circ C$ the solution was evaporated to dryness and the residue was dissolved in chloroform (50 ml). The chloroform solution was extracted with saturated sodium bicarbonate solution, dried (K_2CO_3) and evaporated to dryness to yield a pale yellow liquid (62 mg). Analysis by thin layer chromatography (T.L.C.) showed one major product and three more polar minor products. The residue was dissolved in hexane and applied to a neutral alumina column (10g, activity I) in hexane. The column was eluted with 25 ml portions of eluant in 10% (v/v.) increments from hexane to benzene and benzene to chloroform. Fractions 9-12, which contained the major product, were combined and evaporated to dryness to yield colourless, liquid (25 mg). The mass spectrum showed a molecular ion at m/e 209. $\overset{CHCl_3}{\underset{MAX}{\curvearrowright}}$ 3080 and 1660 cm^{-1} (olefinic C-H stretching bands). The P.M.R. spectrum in $CDCl_3$ showed peaks at 1.50 δ d (3H), 1.52 δ s (6H), 2.42 δ s (3H), 3.38 δ m (3H), 4.38 δ d (2H), and 4.56 δ s (1H) and 4.60 δ s (1H) p.p.m. from T.M.S.

20) Hydrolysis of the product from the borohydride reduction of 3,4'-0-isopropylidenepyridoxine methiodide, XLII.

3,4'-0-Isopropylidenepyridoxine methiodide (200 mg) was reduced with sodium borohydride in methanol in the usual manner (see above). The residue from the chloroform extract was dissolved in tetrahydrofuran (15 ml) and aqueous perchloric acid was added (60% w/v., 10 drops). The mixture was left at room temperature for twelve hours. The solution was made basic with sodium hydroxide solution (0.1N) and extracted with chloroform. The chloroform extract was dried (K_2CO_3) and evaporated to dryness to yield a pale yellow liquid (5 mg). T.L.C. analysis showed it to consist of two products and an I.R. spectrum of the mixture showed bands at 1730 and 1670 cm^{-1} indicating the presence of a carbonyl containing compound. The mixture was separated on silica T.L.C. plate in benzene. The least polar product (3 mg) exhibited saturated C-H bands only in its I.R. spectrum. The more polar product (1 mg) was unstable and on standing became a more polar product, m/e 151, exhibiting an OH peak at 3450 cm^{-1} in nujol.

The basic aqueous solution contained an identical product to the product m/e 167 into which the more polar product isolated from the chloroform extract decomposed, as was demonstrated by comparison of their T.L.C. properties.

21) 4'-Desoxy-3,5'-diacetylpyridoxine methiodide.

4'-Desoxypyridoxine hydrochloride (500 mg) (56) was dissolved in pyridine (20 ml) and acetic anhydride (5 ml) was added. The solution was refluxed overnight when no reaction to Gibb's reagent was observed.

The solution was evaporated to dryness under vacuum at 100°. The residue was dissolved in chloroform (25 ml), and extracted with sodium bicarbonate solution (2 x 25 ml). The chloroform layer was dried (K_2CO_3) and evaporated to dryness to yield a pale yellow oil (566 mg). This liquid was dissolved in a mixture of benzene (20 ml) and methanol (5 ml) and refluxed for 12 hours with methyl iodide (5 ml). On evaporation to a small volume, crystals separated which were filtered to yield a yellow solid (645 mg) mp = 175-177°. A sample, recrystallised from methanol/ethyl acetate, had mp. 178-179° (dec). The mass spectrum showed a peak at 237 mass units (molecular ion - N-methyl group). The P.M.R. spectrum in perdeuterodimethylsulphoxide showed peaks at 2.14 δ_s (3H), 2.38 δ_s (3H), 2.44 δ_s (3H), 2.58 δ_s (3H), 4.32 δ_s (3H), 5.36 δ_s (2H) and 9.08 δ_s (1H), p.p.m. from T.M.S. ν_{max} in nujol showed acetate peaks at 1720 and 1220 cm^{-1} .

22) Sodium borohydride reduction of 4'-desoxy-3,5'-diacetylpyridoxinemethiodide.

4'-Desoxy-3,5'-diacetylpyridoxine methiodide (200 mg) was dissolved in absolute methanol (10 ml) and sodium borohydride (66 mg) was added to the stirred solution at 0°C. After 60 minutes the solution was evaporated to dryness and the residue dissolved in chloroform (50 ml). The chloroform solution was extracted with saturated sodium bicarbonate solution (50 ml), washed with water (50 ml), dried (K_2CO_3) and evaporated to dryness to yield a colourless liquid (90 mg) (92%). The mass spectrum showed a molecular ion at 195 mass units. ν_{max} 3098, 1670 cm^{-1} (olefinic C-H stretching bands) and 1715 and 1210 cm^{-1} (acetate).

The P.M.R. in CDCl_3 showed 1.30 δ d (3H), 1.68 δ s (3H), 2.22 δ s (3H), 2.48 δ s (3H), 3.36 δ m (3H), 5.14 δ s (1H), 5.18 δ s (1H) p.p.m. from T.M.S.

23) Hydrolysis of the reduction product from the borohydride reduction of 4'-desoxy-3,5'-diacetylpyridoxine methiodide.

The borohydride reduction product (62 mg) was dissolved in sodium hydroxide solution (10% w/v., 20 ml) and the solution was refluxed for 10 hours, and was then extracted with chloroform. The chloroform solution was dried (K_2CO_3) and evaporated to dryness to yield a brown gum (6 mg) consisting of a complex mixture as demonstrated by T.L.C. analysis. The aqueous layer was acidified with concentrated hydrochloric acid and applied to a column (1 x 10 cm) of Dowex 50 W-X 8 in the protonated state. The column was washed with distilled water until the washings were neutral. The column was then eluted with dilute ammonia solution (5% v/v, 100 ml). The ammoniacal solution was evaporated to dryness to yield a white solid (43 mg) (89%). An analytical sample had m.p. 217-218° (dec.). The P.M.R. spectrum in perdeutero-dimethylsulphoxide showed peaks at 2.07 δ s (3H), 2.17 δ s (3H), 2.43 δ s (3H), 3.98 δ s (3H), and 7.24 δ s (1H). The mass spectrum showed a molecular ion at m/e 151. Anal. Found: C, 63.32; H, 9.17; N, 8.36. Calculated for $\text{C}_9\text{H}_{15}\text{NO}_2$: C, 63.90; H, 8.94; N, 8.28%.

24) Catalytic reduction of the 4'-desoxy-3,5'-diacetylpyridoxine methiodide borohydride reduction product.

The borohydride reduction product (104 mg) was dissolved in methanol (10 ml) and platinum oxide (60 mg) was added. The container was shaken for 12 hours at 60 p.s.i. hydrogen pressure. The solution

was filtered and evaporated to dryness, dissolved in chloroform and extracted with saturated sodium bicarbonate solution. The chloroform extract was dried (K_2CO_3) and evaporated to dryness. The residue was dissolved in benzene and the solution applied to a column (1 X 10 cm) of neutral alumina in benzene. Elution with benzene and evaporation of the eluate yield a colourless liquid (25 mg) (22 %). ν_{max} 1730, 1280, 1125 and 1074 cm^{-1} (acetate). The mass spectrum showed a molecular ion at m/e 199.

25) Sodium-¹propyl alcohol reduction (Bouveault Blanc reduction) of 3,4'-0-isopropylidenepyridoxine.

3,4'-0-Isopropylidenepyridoxine (200 mg) was dissolved in isopropyl alcohol (5 ml). Small pieces of sodium (0.5g) were added to the refluxing solution over 30 minutes. The solution was refluxed for a further 90 minutes when all the sodium had dissolved. The cold solution was extracted with chloroform after addition of water. The chloroform extract was dried (K_2CO_3) and evaporated to dryness to yield 137 mg of pale yellow oil. T.L.C. analysis showed the reduction mixture to consist of four major components. The mixture of products was dissolved in dilute hydrochloric acid (1M, 20 ml) and the solution was refluxed for four hours. The solution now gave a positive reaction with Gibb's reagent, showing that a 3-hydroxypyridinium system had been regenerated. The solution was basified with excess sodium bicarbonate and extracted with chloroform (2 X 50 ml). The chloroform extract was dried (K_2CO_3) and evaporated to dryness to yield a brown gum (15 mg)

which was shown to consist of a mixture of compounds by T.L.C. analysis. An infra red spectrum of the gum showed no carbonyl bands in the $1700 - 1800 \text{ cm}^{-1}$ region.

The base aqueous sodium bicarbonate soluble product was not further investigated. The analogous reduction of pyridoxine base (100 mg) in isopropyl alcohol (10 ml) with sodium (200 mg) gave mainly pyridoxine on workup which entered the aqueous phase during the chloroform extraction.

26) Attempted oxidation of 3,4'-O-isopropylidene-pyridoxine methiodide with alkaline potassium ferricyanide (Decker's reaction).

A mixture of 3,4'-O-isopropylidene-pyridoxine methiodide (60 mg) and potassium ferricyanide (300 mg) was dissolved in water (5 ml) in a small separatory funnel. Ethyl acetate (10 ml) was added followed by a potassium hydroxide solution (10% w/v., 5 ml). The aqueous solution immediately turned red. The funnel was shaken vigorously and the ethyl acetate phase removed and a fresh portion of ethyl acetate (10 ml) was added to the funnel which was reshaken. Five ethyl acetate extracts were removed from the funnel over a period of 3 days. The combined ethyl acetate solutions were evaporated to dryness under vacuum below 60° to yield an oil (4 mg). T.L.C. analysis of this product showed the presence of two components; one very mobile and another more polar product. An I.R. spectrum of the mixture in chloroform showed the absence of OH groups and the presence of a carbonyl group from the bands at $1730, 1670$ and 1620 cm^{-1} . These bands compare with those at $1720,$

1660 and 1600 cm^{-1} in the I.R. spectrum of 1-methyl-8-methoxy-quinol-2-one. The two components of the oil were separated on a preparative T.L.C. silica plate which was developed in acetone, but the mass spectra of the components failed to give identifiable fragmentation patterns.

27) Attempted phenylation of pyridoxine and 3,4-O-Isopropylidenepyridoxine.

3,4'-O-Isopropylidenepyridoxine (54 mg) was dissolved in dry tetrahydrofuran (5 ml). This solution was added to an ethereal solution of phenyl lithium (2.5 ml) contained in a flask fitted with a nitrogen inlet tube and a reflux condenser. The apparatus was flushed with nitrogen and refluxed overnight (12 hours). The cold solution was then added to a saturated solution of sodium bicarbonate (15 ml) which was extracted with ether (3 X 25 ml). The combined ether extracts were dried (K_2CO_3) and evaporated to dryness to yield starting product plus a very nonpolar product. The mixture was dissolved in dilute hydrochloric acid (0.1M, 10 ml) and the solution was extracted with ether (3 x 25 ml). The ether extract now contained only the nonpolar product (biphenyl?). The acid solution was carefully basified with sodium bicarbonate and extracted with chloroform. The chloroform extract was dried (K_2CO_3) and evaporated to dryness to give starting material (30 mg).

The analogous reaction was attempted with pyridoxine (100 mg), which was dissolved in tetrahydrofuran (15 ml) and treated with ethereal phenyl lithium solution (2.5 ml). Unchanged pyridoxine was obtained on workup after refluxing the reaction mixture for twelve hours.

28) Attempted phenylation of 3,4'-O-Isopropylidene-pyridoxine methiodide., LIX.

3,4'-O-Isopropylidene-pyridoxine methiodide (100 mg) was dissolved in anhydrous tetrahydrofuran (25 ml). Excess phenyl magnesium bromide in tetrahydrofuran (60 mg in 1 ml) was added to the refluxing solution. The solution was refluxed for 4 hours with exclusion of moisture when a T.L.C. analysis of the solution showed that a new less polar product had been formed with no trace of starting product. The tetrahydrofuran was gradually displaced from the reaction flask by the addition of toluene to the refluxing solution. After 25 ml of toluene had been added, the solution was refluxed for a further 3 hours. On evaporation of the dark brown solution to dryness under vacuum, a black tar was obtained which was insoluble in dilute hydrochloric acid and was not further investigated.

29) Attempted nitration of (a) 3-O-methylpyridoxine and
(b) pyridoxine hydrochloride.

(a) 3-O-Methylpyridoxine (50 mg) was dissolved in concentrated sulphuric acid (5 ml). The solution was stirred vigorously at 0° and fuming nitric acid (2 ml) was added dropwise. The reaction mixture was allowed to warm to room temperature over 2 hours and was then heated on a steam bath for 2 hours. The solution was added to crushed ice (10 g) and the solution was basified to pH8 with aqueous sodium hydroxide (10% w/v.). The solution was extracted with ether (3 x 20 ml) and the ether extract dried (Na_2SO_4) and evaporated to dryness to yield unchanged starting material, mp. 101°.

(b) Pyridoxine hydrochloride (300 mg) was dissolved in concentrated sulphuric acid (5 ml). To the stirred solution at 0° was added concentrated nitric acid (1 ml). The solution was stirred at room temperature for 1 hour and was then added to crushed ice (10 g.). The acid solution was applied to a Dowex-50W column (1 x 10 cm) in its acid form. The column was washed with water until the washings were neutral and was then eluted with dilute ammonia solution (5% v/v; 100 ml). The ammoniacal solution was evaporated to dryness to yield a yellow solid, 5 mg, which was nonpolar and inactive to Gibb's reagent.

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