VITAMIN B_6 BIOSYNTHESIS

IN

ESCHERICHIA COLI

By

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ABSTRACT

The biosynthesis of vitamin B_6 (pyridoxal-5'-phosphate) is investigated by tracer methodology in two pyridoxineless mutant strains of Escherichia coli B, WG2 and WG3. Pyridoxol from mutant WG2 and pyridoxal, which was converted to pyridoxol, from mutant WG3 were isolated from the culture fluid and cells, respectively, and degraded to determine the distribution of activity derived from several radioactively labelled compounds.

Competition experiments employing ^{14}C-labelled samples of glycerol and glycolaldehyde indicate that two pathways leading to C-5',-5,-6 of pyridoxol can occur in E. coli. In mutant WG2, a mutant which closely resembles the wild-type strain, the major pathway utilizes glycerol and related trioses as the carbon source for the structural units of the pyridoxol carbon skeleton: C-1,-3 of glycerol yields C-2', -3,-4',-5' and -6; C-2 of glycerol yields C-2,-4 and -5 of the vitamin. In mutant WG3, the minor pathway utilizes glycolaldehyde which supplies C-5 and -5' of pyridoxal and spares the incorporation of glycerol into this two-carbon unit. Glycerol is the source of the other six carbon atoms. The major pathway is blocked in mutant WG3 and the "glycolaldehyde pathway" becomes the sole source of vitamin B_6.

Competition experiments employing [2-^{14}C]glycerol in the presence of either non-labelled pyruvate, acetate, or hydroxypyruvate in mutant WG2, demonstrate that the two-carbon unit, C-2,-2', required for pyridoxol biosynthesis, is derived from pyruvate. Incorporation of radioactivity derived from [2-^{14}C]pyruvaldehyde, [1-^{14}C]ribose, and [1-^{14}C]-acetate into C-2,-2' of pyridoxol can be rationalized by way of pyruvate.

The identity of the pyruvate-derived C_2 unit remains to be established. The normal end-products of pyruvate catabolism have been eliminated as precursors of the two-carbon unit, C-2,-2' of pyridoxol. On this basis, it is inferred that acylating agents associated with the thiamin-dependent multienzyme complex, pyruvate dehydrogenase, may be involved in the biosynthesis of the vitamin.

A hypothesis, consistent with the results obtained from the tracer experiments, is proposed for the biosynthesis of vitamin B_6. The first steps consist of acyl transfer to dihydroxyacetone-1-phosphate followed by transamination yielding an aminosugar, 4-amino-4,5-dideoxypentulose. Condensation of the aminosugar with glyceraldehyde-3-phosphate or a closely related compound derivable also from glycolaldehyde by the minor route, followed by dehydration yields pyridoxol (Scheme 27).

Radioactive pyridoxol derived from [1-^{3}H,2-^{14}C]glycerol indicates the loss of one tritium atom relative to ^{14}C during the course of biosynthesis of the vitamin.

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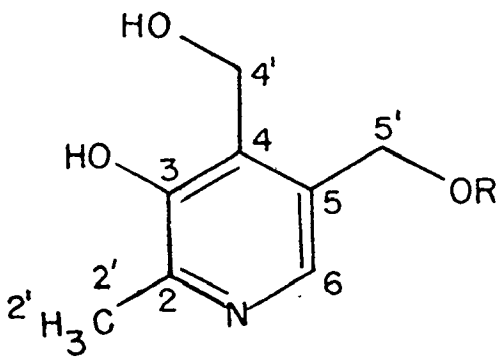
INTRODUCTION

The naturally occurring substances which are structurally related to 3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine (1) (Fig. 1) constitute a family of compounds collectively referred to as vitamin B_6. Three known forms of the vitamin, pyridoxine (1), pyridoxal (2), and pyridoxamine (3), are present in cell tissues predominantly as the 5'-phosphate esters rather than as the unphosphorylated substances. It is generally accepted that two of the vitamin B_6 compounds, pyridoxal-(5) and pyridoxamine-5'-phosphate (6), are intimately associated with the metabolism of α-amino acids. Hence, vitamin B_6 is a vital requirement for all living organisms.

1. HISTORICAL

The existence of the vitamin in nature was recognized in the course of the early work in animal nutrition. A substance which was considered to be a physiologically active antidermatitis factor was termed vitamin B_6 by György in 1934.[1,2] Shortly thereafter, reports from several laboratories claimed the isolation of a compound, from rice bran[3-5] and from yeast,[6,7] which exhibited vitamin B_6 activity in animal experiments. Preliminary reports of the structure of this substance appeared shortly after its isolation.[8,9] In 1939, the identity of the vitamin with pyridoxine, 3-hydroxy-4,5-



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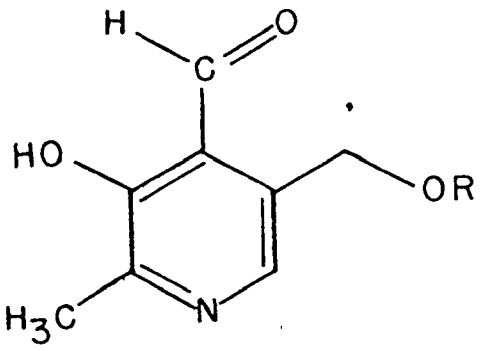

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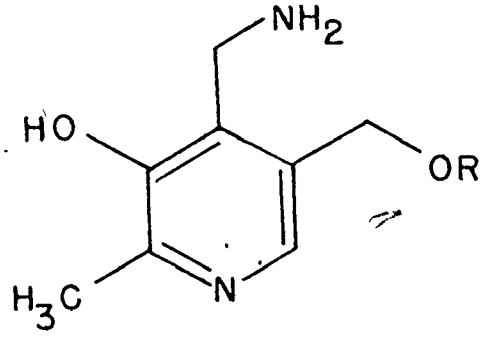

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Figure 1: Members of Vitamin B_6 Family.

R = H; A) Pyridoxol (1), B) Pyridoxal (2), C) Pyridoxamine (3).

R = $PO_3^{=}$; A) Pyridoxol-5'-Phosphate (4), B) Pyridoxal-5'-Phosphate (5), C) Pyridoxamine-5'-Phosphate (6).

bis(hydroxymethyl)-2-methylpyridine (1) was confirmed by synthesis.[10-12] The synthetic compound demonstrated the same biological activity as that isolated from nature.

The existence of pyridoxine-like substances which were biologically more active than pyridoxine was first recognized during a study of growth promotion.[13,14] These substances were later identified as two pyridoxine analogues: An oxidation product, pyridoxal (2) and an amination product, pyridoxamine (3).[15-17]

Subsequent studies demonstrated that pyridoxal, pyridoxamine and pyridoxol could be converted to their 5'-phosphate esters[18] and that these substances were interconvertible.[18] Furthermore, it was demonstrated that the enzymes catalyzing these transformations were present in microorganisms as well as mammalian tissues.[19,20] On this basis, the suggestion that the enzyme catalyzed interconversions of the various forms of the vitamin might be associated with a metabolic function of vitamin B_6 was advanced.[18,19]

In an attempt to define the metabolic role of vitamin B_6, Gunsalus designated pyridoxal phosphate as a coenzyme for the decarboxylation of the amino acids tyrosine[21,22] and arginine in Streptococcus faecalis.[23,24] It is now known that pyridoxal-5'-phosphate (5) aids in non-oxidative transformations such as decarboxylations, transaminations, racemizations and condensations of the amino acids.

Other enzymic processes involving vitamin B_6 as a cofactor were elucidated in the fifties and comprehensive compilations of these reactions have been assembled.[25,26] Information concerning the biochemistry of vitamin B_6 is now available and well understood.[27-31] However, one aspect which still warrants investigation is its biosynthesis.

Vitamin B_6 is synthesized by higher plants, some fungi, yeast and microorganisms. However, the vitamin is a nutritional requirement for all higher animals investigated to-date. The biosynthetic sequence leading from primary metabolites to vitamin B_6 is still not understood: As a consequence, no definitive information concerning the nature of any precursor on route to the vitamin is available. Several published articles[32-39] have dealt with this aspect of vitamin B_6 and these contain all the available information regarding the biosynthetic pathway of the vitamin. This information has been inconsistent and, at times, contradictory. It is the intention of this thesis to clarify some of these inconsistencies and to expand on the existing hypothesis for the biosynthesis of vitamin B_6.

CHAPTER I

BIOGENESIS OF VITAMIN B_6

1.1 Introduction

Only within the last decade has any significant progress been made in attempts to elucidate the biosynthetic sequence leading from acyclic amphibolic precursors to the substituted pyridine ring derivatives collectively known as vitamin B_6. Investigators involved with this topic have divided the problem into two segments: The reactions leading from acyclic precursors to the first cyclic intermediate on route to vitamin B_6 and the interconversions of the various forms of the vitamin in the terminal stages of the biosynthesis.[18] The techniques used to investigate the biosynthesis of the vitamin in microorganisms catered to the two areas. Early enzymic and nutritional studies with mutated organisms were largely confined to the later stages of vitamin B_6 biosynthesis whereas studies which employed isotopic tracers (depending on the availability of the tracer or a method for its synthesis) gathered information about the early metabolic transformations of a particular chemical substance and thereby allowed its fate in the organism to be followed. This division is anticipated to converge at a point where the first cyclic intermediate leading to vitamin B_6 is identified.

Several obstacles have impeded the progress of establishing the sequence of biosynthesis of vitamin B_6. As a consequence of the interconvertibility of the various forms of the vitamin, the identity of the physiologically active substance is not known with any degree of certainty. However, the mutant work of Dempsey[40,41] has placed pyridoxal-5'-phosphate (5) as the probable biologically active compound. Secondly, the lack of organisms which produce workable quantities of the vitamin or of any intermediates leading to pyridoxal-5'-phosphate (5) had hindered the investigation of the early biochemical transformations of putative precursors of the vitamin.

These obstacles can be overcome and the biosynthetic sequence leading to vitamin B_6 can be investigated adequately provided mutant, enzymic and isotopic studies are carried out in the same microorganism, and the information obtained by the different methods is complementary and consistent.

1.2 Methods Employed to Investigate the Biosynthesis of Vitamin B_6

1.2.1 The Use of Mutant Organisms

The study of congenital metabolic abnormalities, the "inborn errors of metabolism" in humans, has yielded much information about genetics and metabolic processes.[42] In microorganisms, metabolism can be investigated by employing the microbial mutant or auxotroph, which can be produced

by irradiation or by treatment of a culture with a chemical mutagen followed by appropriate selection techniques. Mutants containing a single genetic lesion can be selected.

The single mutation usually manifests itself in the organism as a metabolic block, hence the organism is unable to synthesize a particular compound. This mutated organism has a nutritional requirement which can be satisfied by addition, to the culture medium, of any compound which lies beyond this metabolic block. Otherwise, no growth of the organism occurs. Compounds which are suspected to lie between the metabolic block and a target molecule may be tested by administering these substances to cultures of this mutant and observing a growth response of the culture. Intermediates preceding the metabolic block, on the other hand, may accumulate in the culture fluid and may be isolated and identified.

The identification of intermediates on route to the target molecule becomes much more difficult if no prior knowledge concerning these substances as putative precursors to the target molecule is available. By methodically testing the culture fluid of each mutant auxotrophic for the target compound, with every other available mutant, which is also auxotrophic for the same compound, and observing a growth response, the mutants may be arranged in an order which corresponds to the sequence leading to the target molecule. This method of testing is called cross-feeding or syntropism.

The most comprehensive mutant study of the biosynthesis of vitamin B_6 employing the bacterium Escherichia coli B has been carried out by Dempsey and coworkers. Their investigations include both genetic and nutritional (crossfeeding) studies.[39]

Using a modified penicillin enrichment technique, several hundred mutants of E. coli B auxotrophic for vitamin B_6, have been isolated[40] and were shown by their ability to crossfeed one another and by genetic techniques to be blocked at the pdx gene, loci coding for the enzymes required for the biosynthesis of vitamin B_6.[43,44] However, not all mutants, isolated in this manner, which demonstrate a dependence on vitamin B_6 as a growth factor, can be regarded as true vitamin B_6 auxotrophs. One such type of mutant is the "pyridoxine responsive" or "Km mutant". "Km" mutants synthesize normal amounts of vitamin B_6 but as a result of the genetic lesion, produce a defective apoenzyme which cannot bind its cofactor adequately. The reduced affinity of the apoenzyme (presumably an aminotransferase for pyridoxal-5'-phosphate) for the enzyme hinders the biosynthesis of an amino acid. These mutants will grow normally either when elevated levels of vitamin B_6 within the cell are achieved or when the amino acid is added as a supplement. Several mutants of this type have been described,[45-49] and a rapid means of screening for these mutants has also been reported.[50] A second class of mutant which must also be excluded as a true vitamin B_6

auxotroph is the "pseudopyridoxineless" mutant. This type of mutant neither synthesizes vitamin B_6 nor grows when pyridoxol is added to the culture medium. However, these mutants respond with full growth and pyridoxine synthesis when a single nutrient, such as another cofactor, is added to the culture medium. Dempsey has described several such mutants.[51]

The earlier nutritional studies with mutants which were not well characterized led to many contradictory opinions regarding the biosynthesis of vitamin B_6 and as a consequence, hindered the elucidation of this metabolic pathway. Thus, for a valid investigation of the biosynthesis of vitamin B_6 employing mutants, certain criteria must be met in order to define a mutant as a true pyridoxine auxotroph. On this basis, Dempsey proposed that a true pyridoxineless mutant of E. coli can be defined as one which does not synthesize any pyridoxol when removed from exponential growth and suspended in a glucose salts medium and which also required pyridoxol or pyridoxal at a final concentration of no more than 6×10^{-7} M (the final concentration of vitamin B_6 in E. coli cultures at full growth), for full growth in a defined medium.[39]

The quantity of vitamin B_6 in cultures of E. coli at full growth is included in the definition of a true pyridoxineless mutant because normally, the biosynthesis of vitamin B_6 in E. coli is subject to special mechanisms for its regulation.[52,53] Only a small amount of the vitamin is

required to function as a cofactor.[54] Furthermore, this stipulation excludes the "Km" or "pyridoxine responsive" mutants which require elevated levels of the vitamin for growth of the cultures.

The mutants of E. coli B[39] and E. coli K12[50,55] which have been isolated and comply with the definition of pyridoxine-less mutants, have been arranged into five separate chromosomal groups. Each of the five groups or genotypes represents a malfunctioning or deficient enzyme which is normally required for pyridoxol biosynthesis. Three other distinct loci which also code for enzymes associated with vitamin B_6 biosynthesis, have been described.[43] Thus, the information obtained from genetic studies, together with the knowledge of pyridoxol kinase, an enzyme known from biochemical studies,[56,57] implies that at least five enzymes are necessary to biosynthesize vitamin B_6 from a common metabolite (Fig. 2).

The existence of pyridoxol kinase and oxidase (genotype H, Fig. (2)), in E. coli was demonstrated in a separate study using E. coli B mutant WG2, a group IV mutant which grows on pyridoxal and pyridoxamine but not pyridoxol.[43,57]

The characteristics of each of the five unlinked genetic groups and how they pertain to vitamin B_6 biosynthesis have been recently described by Dempsey.[39] This summary details the enzyme deficiencies, which are known, and the nutritional requirement of mutants in each group. Only the mutants which pertain to the context of this thesis will be

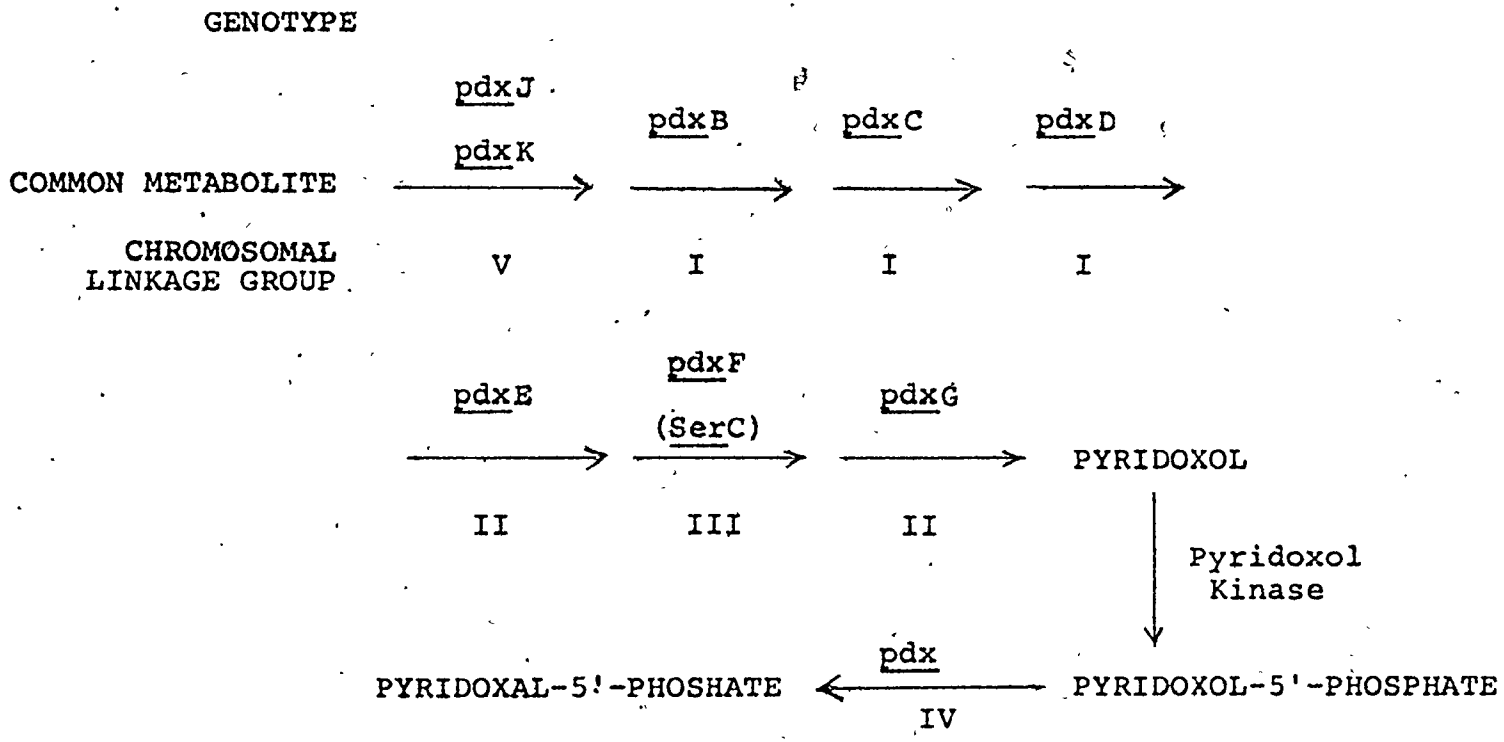


Figure 2: Number of Enzymes Postulated in the Biosynthesis of Vitamin B_6.[41]

described in the appropriate sections.

1.2.2 The Use of Tracers

The application of tracer techniques to investigate the metabolic fate of a substrate "labelled" with an isotope has proved to be a very powerful method to elucidate biosynthetic sequences *in vivo* with minimum perturbation of the test organism. The tracers normally used for such studies include the radioisotopes (^{14}C, ^{3}H, ^{35}S, ^{32}P) and the stable isotopes (^{2}H, ^{13}C, ^{15}N). Since individual tracers have their own means of detection, each can be adequately quantified.

After administration of a substrate, labelled in a known position, to a living organism and provided that a suitable incubation time has elapsed, the compound under study is isolated, purified and the isotopic content measured. In the event that the product contains the tracer derived from the substrate, the position(s) and relative quantity of the tracer within the product must be determined in an unambiguous manner.

Target molecules labelled with radioisotopes derived from appropriately labelled substrates, must be chemically degraded, by selective degradation methods of known mechanisms, to yield products representing fragments of the carbon skeleton of the compound being investigated. Isolation and purification of these degradation products followed by radioassay, determines the quantity and location of tracer which may then

be related back to the initial target compound. It is often difficult to locate, within the target compound, the position in which tracer has entered. This is primarily due to the incorporation of tracer into positions which are unreactive to mild degradation conditions. Furthermore, to be unaware that the incorporation of tracer into a target molecule which upon degradation may yield the degradation product from two segments of the molecule, may lead to an erroneous conclusion. Hence, only partial analysis is obtained since the recovery of tracer which has entered the target compound is incomplete.

When stable isotopes are used in biosynthetic studies, it is not necessary to use degradative methods to determine position and quantity of tracer which has been incorporated into the compound under study. Non-destructive nuclear magnetic resonance techniques are applied for analysis and the position of label can be determined directly from the spectrum. This expedient method employed to study biosynthesis is restricted to test organisms which demonstrate high levels of incorporation of labelled precursor into the target compounds.[58-63]

In most experiments, the choice of the appropriately labelled substrates to be tested as precursors for a compound under investigation is dependent on prior knowledge of the incorporation pattern of certain related substrates already tested in an organism, together with some intuition. This choice is also limited by the commercial or synthetic availability of the isotopically labelled compound.

If meaningful results are to be extracted from a tracer experiment, the labelled substrate must reach the site of biosynthesis and should be incorporated into the target compound being investigated. Its mode of incorporation into the target molecule is a reflection of the metabolic transformation that the substrate has undergone. However, the mere incorporation of tracer does not constitute a precursor-product relationship; only if a non-random entry of tracer is observed (i.e., if activity is confined to a single or several specific sites), can any conclusion be made regarding the intermediacy of the substrate as a precursor of the target compound. Other criteria may also be employed to assess precursor efficiency.[64,65]

The specific incorporation of an isotopically labelled substrate into a product demonstrates that this substrate is a precursor or intermediate on route to the product. Other techniques, which have been used to gain more information about the biochemical fate of the precursor, employ multi-labelled substrates, trapping, and isotope competition methods.

The introduction of two or more isotopes, of the same or different nuclei, into more than one position of the substrate yields either a doubly or multiply labelled compound. If employed in a tracer experiment, a compound yields useful information only if the position and quantity of each isotope is known. The use of a doubly or multiply labelled compound

in an experiment permits the detection of entry of the precursor into a product as an intact unit, provided all the isotopes present in the labelled precursor are found in the product whose quantitative isotope distribution corresponds to that of the precursor. Any change in isotope content of the product relative to that of the precursor represents the biochemical cleavage between labelled atoms of the precursor prior to its incorporation into the product.

Substrates doubly labelled with either radioisotopes or stable isotopes have been used extensively in biosynthetic investigations.

The most widely used radioisotopes in doubly labelled substrates are ^{3}H and ^{14}C. A mixture of the same compound labelled with the different isotopes, one containing ^{14}C tracer and the other ^{3}H label, at known specific positions and relative quantity, constitutes an intermolecularly doubly labelled substrate. The ^{3}H activity relative to ^{14}C activity in a doubly labelled substrate is normally measured as a ratio ($^{3}H/^{14}C$).

The doubly labelled substrate is administered to the test organism, and an appropriate "feeding" time is allowed to elapse. The product is isolated from the organism, rigorously purified, degraded if necessary, and the relative activities of the two radioactive tracers measured. Any change in the $^{3}H/^{14}C$ ratio usually implies cleavage of the C-H bond if the

substrate is labelled at the same relative position.

Much more versatility can be obtained if stable isotopes are used. Substrates containing ^{13}C-^{13}C, ^{15}N-^{13}C or ^{2}H-^{13}C as double labels have been employed with great success in experiments investigating biosynthetic sequences. One important criterion is that the substrate must be intramolecularly labelled to be an effective probe. Nuclear magnetic resonance analysis of the isolated product will demonstrate whether the substrate has been incorporated into the product as an intact unit (^{13}C-^{13}C, ^{15}N-^{13}C, ^{2}H-^{13}C coupling) or as a compound produced by the cleavage of the bond between the labelled atoms of the administered substrate. This technique has been restricted to biological systems, producing compounds for which optimum yield conditions have been previously established. Large quantities of the isolated products are generally required for recording satisfactory NMR spectra since sensitivity of detection of these tracers by magnetic resonance techniques is relatively poorer as compared to that of radioisotopes.

Trapping and isotope competition methods have been used primarily with radioisotopic tracers to detect possible intermediates on route to a target compound. Both techniques are similar in execution and require little or no degradation of the isolated products.

Consider a compound B, suspected of being an intermediate on route to compound C. Prior testing of the pathway

ensures that activity from compound A is non-randomly incorporated into product C. Activity from A must have passed through every intermediate on route to product. The trapping experiment entails the administration of radioactively labelled A and non-labelled B. After a sufficient incubation period, compound B and product C are isolated from the test organism and rigorously purified. If compound B were on route to C from A, then tracer from A would be found in compound B, whereas compound C would contain little if any, tracer. Compound B may then be regarded as an intermediate.

In isotopic competition experiments, the methodology is the same as in the trapping experiment, except that compound B, in this case, is not isolated. Only the target compound (compound C) is isolated and assayed for label. If the presence of non-labelled compound B reduces the efficiency of incorporation of tracer derived from compound A into C, as compared to that in the absence of compound B, then B may be regarded as an intermediate on route to compound C.

Clearly, these two methods are of limited usefulness unless they are conducted with the appropriate incorporation studies to be used as standards for comparison. Only then can these techniques provide useful information about a biosynthetic pathway.

Double labelling and isotope competition techniques

are included in the tracer studies employed to investigate the biosynthesis of pyridoxol. The results from these and other experiments will be discussed in the following chapters.

1.2.3 The Use of Enzymes

The precursors and intermediates of a product generated *in vivo*, can be identified with considerable confidence by employing mutant and tracer methodology to investigate the biosynthetic sequence of the product. This, however, is only a partial solution for the elucidation of a metabolic pathway. What remains to be established are all the parameters involved with each enzymic step (cofactors, specificity, kinetics, chemical mechanism) which effect the transformation of each intermediate on route to the product in a given organism. Information provided by enzymic studies may be regarded as conclusive proof that such a metabolic pathway exists in a particular organism.

Before the widespread use of tracer techniques, biosynthetic investigations relied solely on enzymological studies. Earlier investigations, which used crude enzyme preparations from mammalian tissues or microorganisms, were concerned with the origin and fate of primary metabolites. As a consequence, many of the enzymes which catalyze the transformation of compounds which lie on central metabolic routes, have been isolated and well-characterized. Furthermore, these studies have established the enzymological techniques which together

with tracer methodology have enhanced the elucidation of chemical mechanisms and kinetics of these transformations.

Enzymes associated with secondary metabolism, however, are not as well understood. Experimental progress is limited to those pathways whose intermediates have been recognized and whose enzymes have been isolated.

In vitamin B_6 biosynthesis, the enzymes associated with the interconvertibility of the various forms of the vitamin have been isolated from several sources[32] and have been studied. This, however, yields no information about the enzymic steps leading to the first cyclic intermediate on route to the vitamin. In the early stages of the biosynthesis, experimental progress is at the stage of discovering precursor-product relationships and hence nothing is known of the enzymic processes. Mutant studies implicate a number of enzymes thought to be involved.

Only mutant and tracer methodology has been used to acquire information regarding the early stages of vitamin B_6 biosynthesis. Recent studies employing these techniques will be briefly reviewed and the hypotheses for the biosynthesis of pyridoxol which have been advanced on the basis of these investigations, will be presented.

1.3 Knowledge Concerning the Biosynthesis of Vitamin B_6 Obtained from Enzymic, Mutant and Tracer Methods

Radioactive tracers and microbial mutants have been used to investigate the early stages of vitamin B_6 biosynthesis. Enzymic investigations have been confined to the terminal stages of the biosynthesis and have confirmed that various forms of the vitamin are interconvertible, thereby associating the vitamin with a metabolic function.

1.3.1 The terminal stages of the pathway

Pyridoxal- and pyridoxamine-5'-phosphate (5 & 6) (Fig. 1) are regarded as the biologically active forms of vitamin B_6. Pyridoxol (1) was postulated as the precursor of these compounds. Acceptance of this notion stemmed from Dempsey's earlier mutant studies.

Cell extracts of E. coli (wild type) were able to phosphorylate pyridoxol to pyridoxol-5'-phosphate (4) and to oxidize the latter to pyridoxal-5'-phosphate (5). A pyridoxineless mutant, WG2, was found to grow when the medium was supplemented with pyridoxal or pyridoxamine, but not pyridoxol. Mutant WG2, when deprived of pyridoxal (2) or pyridoxamine (3), synthesizes pyridoxol (1) and pyridoxol-5'-phosphate (4) at four to six times the rate of the wild type and excretes the newly formed compounds into the culture fluid. Thus, it appeared that mutant WG2 was unable to oxidize

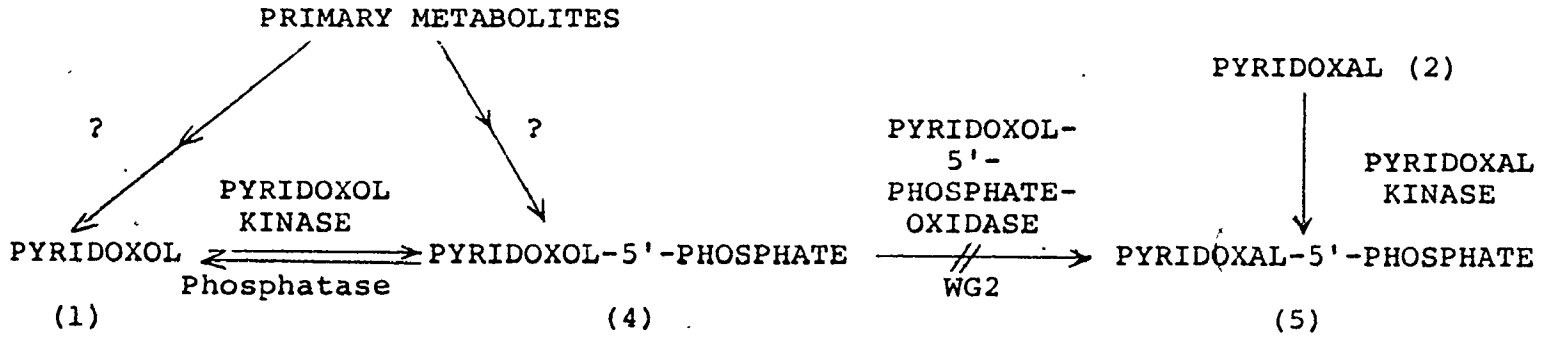
pyridoxol (1) or its 5'-phosphate (4) to pyridoxal (2) or pyridoxal-5'-phosphate (5), respectively.[41,53]

Assaying and comparing the cell extract of the wild type organism and that of mutant WG2 for the enzymes which catalyze these conversions, it was demonstrated that mutant WG2 lacked pyridoxol oxidase activity.[41,53] On this basis, Dempsey proposed a partial reaction sequence for the terminal stages of vitamin B_6 biosynthesis (Scheme 1).

This sequence places pyridoxal-5'-phosphate (4) as the end-product of the pathway. It is uncertain whether pyridoxol (1) or pyridoxol-5'-phosphate (4) is the immediate precursor for pyridoxal-5'-phosphate (5), however.

Several pyridine derivatives which are closely related to pyridoxol have been proposed as intermediates on route to pyridoxal-5'-phosphate (5) (Fig. 3).

The idea that the vitamin antagonist, 4'-deoxypyridoxine (7), could be converted to pyridoxal-5'-phosphate (5) has been advanced on the basis of the observations from two independent investigations. Pyridoxineless mutants of E. coli K12 apparently can grow in the presence of the growth inhibitor,[66] under certain conditions, i.e., when pyridoxal concentration in the cell is limited.[50] Lingens' studies demonstrated that Bacillius subtilis could phosphorylate 4'-deoxypyridoxine (7) to 4'-deoxypyridoxine-5'-phosphate. The oxidation of the 4'-methyl moiety could not be demonstrated in this organism, however.[67]



Scheme (1): Possible Sequence for the Terminal Stages of Vitamin B_6 Biosynthesis[41]

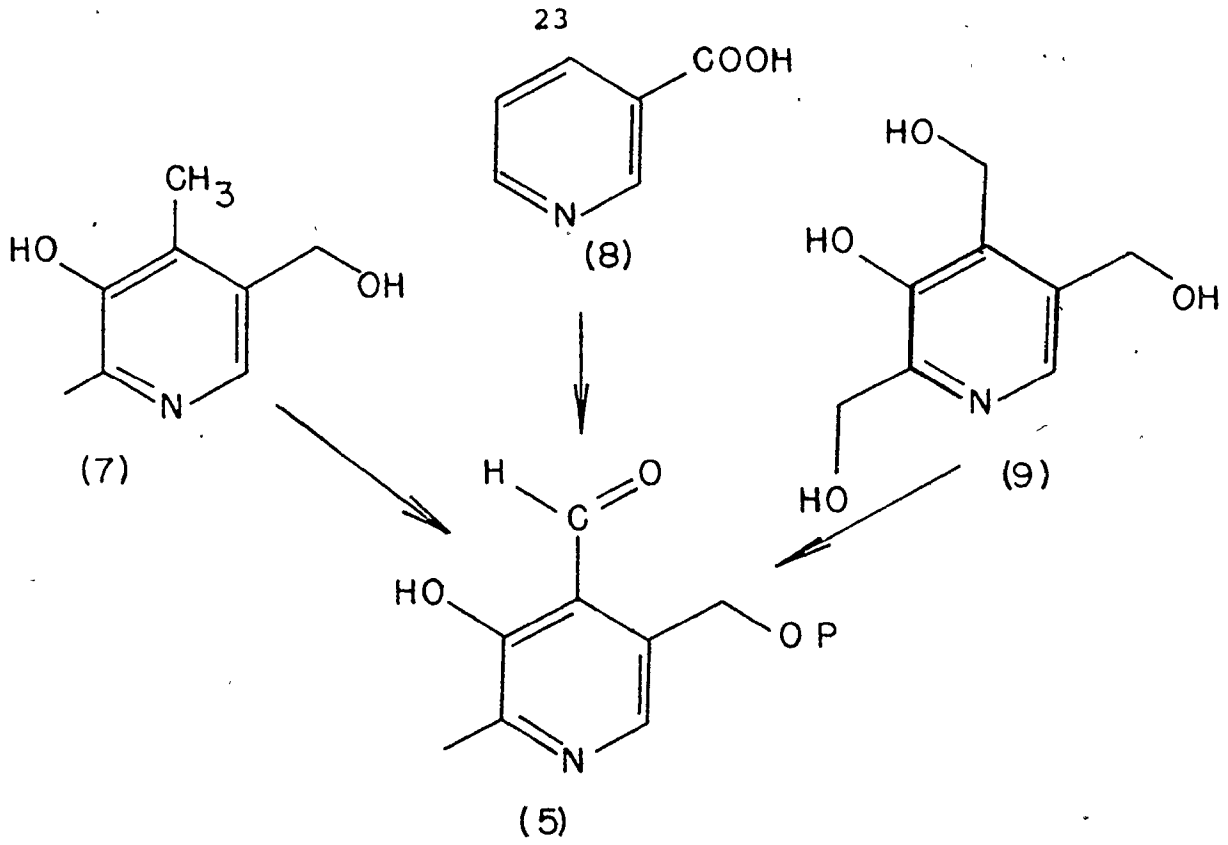
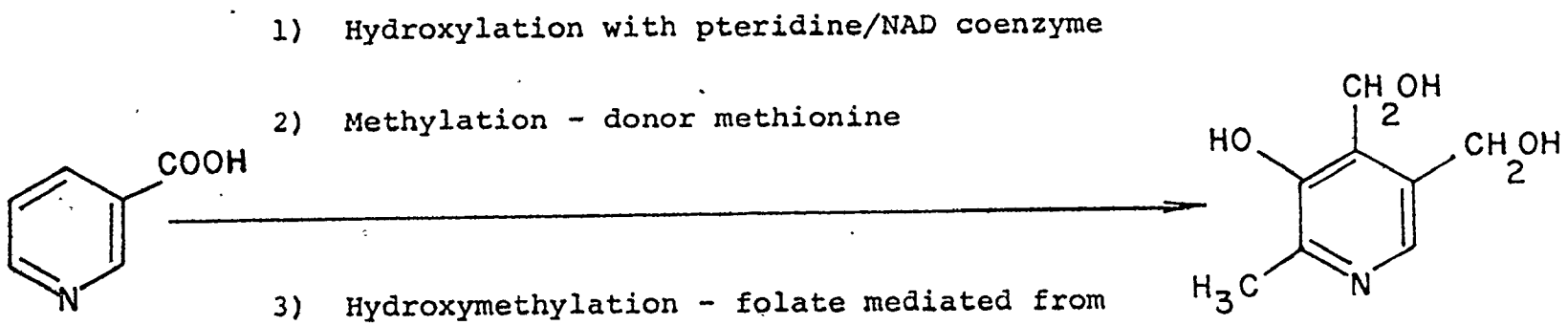


Figure 3: Postulated Cyclic Precursors for the Terminal Stages of Vitamin B_6 Biosynthesis.

The compound, 3-hydroxy-2,4,5-trihydroxymethylpyridine (9) has been shown to satisfy the vitamin B_6 requirement of Saccharomyces carlsbergensis[68] and has been postulated as an intermediate formed in Saccharomyces fragilis.[69] Scott et al. demonstrated that the vitamin dependent yeast Kloechera apiculata could grow when the pyridoxal supplement was replaced with 3-hydroxy-2,4,5-trihydroxymethylpyridine (9).[70] These observations may not necessarily reflect the course of vitamin B_6 biosynthesis since an ethyl moiety may replace the methyl group at C-2 of pyridoxol and this analogue still acts as a cofactor.[71] Interestingly, none of the five hundred pyridoxineless mutants of E. coli CR63 could grow when 3-hydroxy-2,4,5-trihydroxymethylpyridine (9) was added as a supplement to pyridoxal deprived cultures.[50]

Nicotinic acid (8) was recently noted to promote the growth of a pyridoxineless yeast mutant of Aspergillus nidulans.[72,73] In vitro studies with cell extracts of this yeast show that it produces pyridoxol when nicotinic acid is added alone or with three-carbon compounds related with glycolytic intermediates. On the basis of the nutritional and in vitro studies, a possible sequence of events is proposed by Vatsala et al. (Fig. 4). In another yeast, Saccharomyces fragilis, nicotinic acid (8) was ruled out as a possible precursor for pyridoxol.[69]



(8) (1)

Figure 4: Postulated Sequence of Vitamin B_6 Biosynthesis in Aspergillus nidulans[73]

Of the acyclic compounds examined with nicotinic acid (8) in Aspergillus nidulans, the glycolytic intermediates (dihydroxyacetone phosphate, phosphoglyceric acid, phosphoenolpyruvate and pyruvate) were shown to have a stimulatory effect on in vitro pyridoxol production. These compounds are involved in the early stages of vitamin B_6 biosynthesis and will be discussed in the following section.

1.3.2 The Early Stages of the Pathway

The objective of investigations employing tracers and mutant methodology is to identify compounds which serve as precursors or intermediates leading to pyridoxol (or pyridoxol-5'-phosphate). Studies with pyridoxineless mutants single out compounds which are able to satisfy the pyridoxal requirement of these mutants and hence are suspected as compounds on route to the vitamin. By analysis of the fate of these and related substances in an organism by tracer methodology, the non-random entry of labelled substrate into pyridoxol may be detected provided suitable degradation methods are employed. The substrate may then be considered as a precursor for the vitamin.

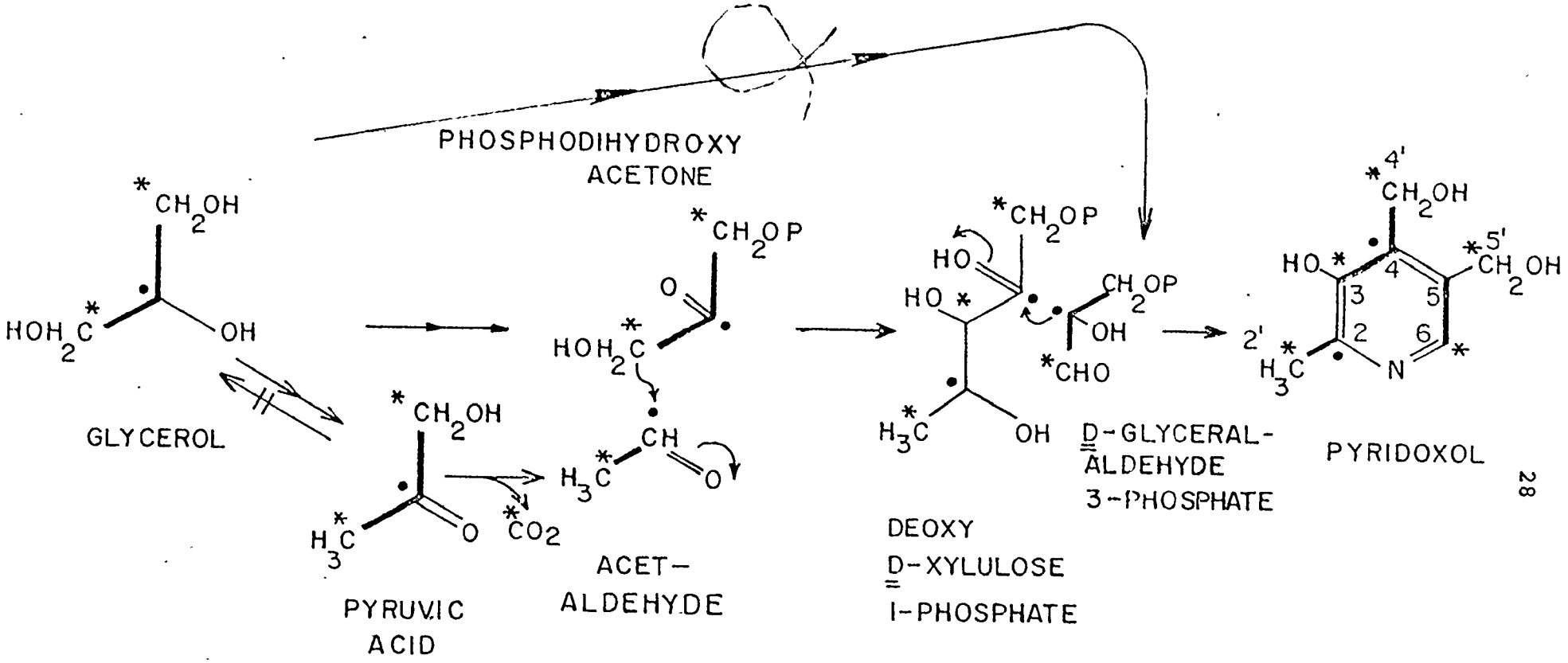
Several earlier reports in the literature yielded doubtful conclusions concerning the role of substances tested because either the isolated pyridoxol was not sufficiently purified[74] or suitable degradation methods were not employed. Such inconclusive reports will not be discussed in detail in

this section.

Precursors and Hypotheses Proposed for Vitamin B_6 Biosynthesis Based on Mutant and Tracer Studies

The most plausible sequence advanced to-date for the biosynthesis of pyridoxol-5'-phosphate stems from the thorough work of Hill, Spenser and associates.[75-79] It is considered that phosphorylated pyridoxol is derived from glycolytic intermediates: Dihydroxyacetone-1-phosphate, D-glyceraldehyde-3-phosphate and an unidentified two-carbon unit suspected to be at the oxidation level of acetaldehyde (Scheme 2). Specifically labelled glycerol, pyruvate and glucose have been shown to be non-randomly incorporated into pyridoxol, and pyridoxol-5'-phosphate in E. coli WG2. Pyridoxol-5'-phosphate was converted to pyridoxol prior to degradation.

By chemical degradation of [^{14}C]-labelled samples and by a ^{13}C-NMR study of a [^{13}C]-labelled sample of pyridoxol[79] derived from glycerol, it was shown that the entire carbon skeleton may be derived from the primary and secondary carbons of glycerol. It was shown that C-2 of glycerol yields C-2, C-4, C-5 of the vitamin and each of these three sites contained 33% of the total label of the intact pyridoxol sample. It was also shown that C-1, C-3 of glycerol yields C-2', C-3, C-4', C-5', and C-6. Each of these five sites accounted for 20% of the total pyridoxol activity. Radioactive

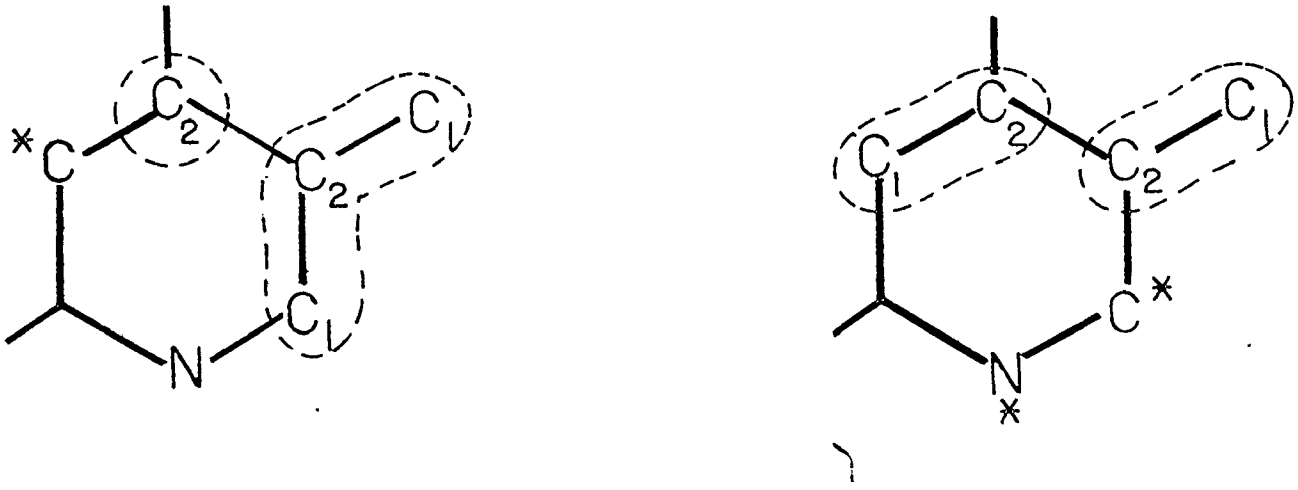


Scheme 2: Hypothetical Sequence for the Biosynthesis of Pyridoxol.[77]

pyridoxol derived from [1-^{14}C]- and [2-^{14}C]glycerol in Flavobacterium was reported to contain labelling patterns consistent with that observed by Hill and Spenser.[81] However, another investigation claims that pyridoxol is derived from both glycerol and γ-aminobutyric acid[74,82,83] by Achromobacter cycloclastes (Fig. 5).

Hill and Spenser demonstrated that samples of pyridoxol derived from [2-^{14}C]- and [3-^{14}C]pyruvate contained label only at C-2 and C-2' of the vitamin, respectively.[76,77] The carboxyl moiety was not incorporated into pyridoxol. Hence, it was concluded that pyruvate was the precursor of the two-carbon unit, thought to be at the oxidation level of acetaldehyde, which yields C-2,-2' of pyridoxol. Schroer and Frieden[69] demonstrated that in S. fragilis, unlabelled pyruvate could not spare the incorporation of [G-^{14}C]glucose, hence they concluded that pyruvate was not essential for vitamin B_6 biosynthesis in this yeast. However, they fail to comment on the observation that L-alanine, which is related to pyruvate by transamination, demonstrated an 18% reduction of incorporation of label derived from glucose (Table II in Ref. 69).

Possibly alanine and not pyruvate is transported into the yeast cells under those culturing conditions. This can be demonstrated if the respective labelled substrates are used. By chemical degradation, specific incorporation of tracer derived from labelled alanine or labelled pyruvate



Postulated to arise from the carbon atoms of Glycerol.

Postulated to arise from γ-Aminobutyric Acid.

Figure 5: Possible Mode of Incorporation of Activity into Pyridoxol Derived from ^{14}C-Labelled γ-Aminobutyric Acid and Glycerol.[74]

may be shown. However, if the isolated pyridoxol contains no radioactivity, then either labelled pyruvate or alanine, or any other cellular constituent must be isolated to demonstrate that the administered labelled substrate was transported into the cell and utilized in another manner than to synthesize pyridoxol. Then, these substrates may be excluded as a precursor for biosynthesis of the vitamin.

From mutant studies, Dempsey has shown that a pyridoxineless mutant of E. coli (pdxK) will grow to one-half normal cell density when the culture is supplemented with alanine, pyruvate or cysteine.[39] In pyridoxal-starved cultures of E. coli K12 mutant BL-1, the presence of L, D or β-alanine did not change the amount of pyridoxol excreted into the medium from that which was excreted in the absence of added compound.[84] The presence of pyruvate caused approximately a forty per cent reduction of excreted pyridoxol.

The condensation of the two triose units, postulated by Hill and Spenser, can be envisaged to occur in either of two ways (Fig. 6). Dihydroxyacetone-1-phosphate (6) could give rise to C-4',-4,-3 (Fig. 6A), or to C-5',-5,-6 of pyridoxol (Fig. 6B) while D-glyceraldehyde-3-phosphate would yield C-5',-5,-6 or C-3,-4,-4', respectively. Carbons 1, 2 and 3 of glucose yield C-1,-2,-3 of dihydroxyacetone phosphate, respectively, by aldolase cleavage (Scheme 3). Thus, C-1 labelled glucose will preferentially place label at C-1 of dihydroxyacetone-1-phosphate.

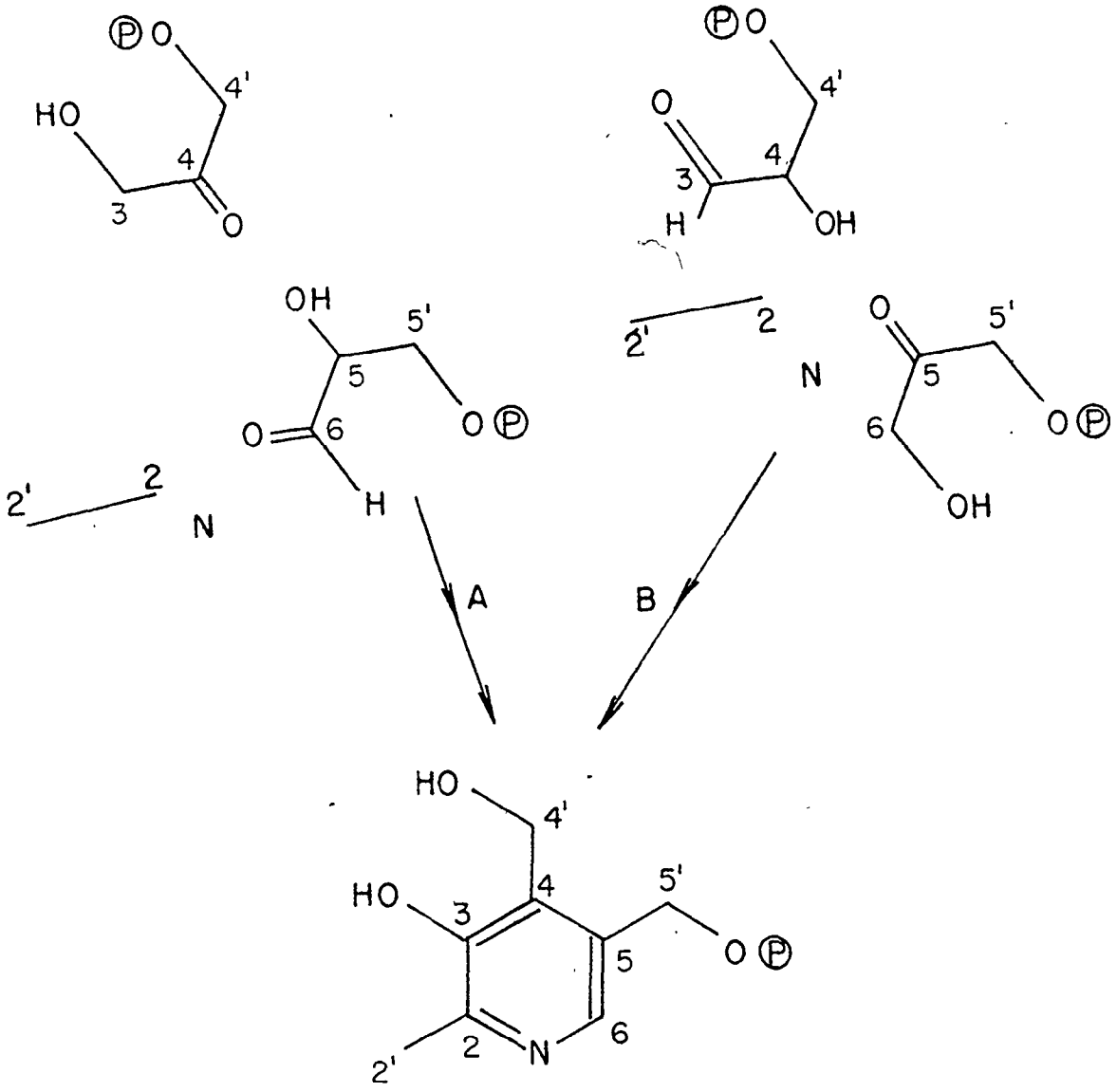
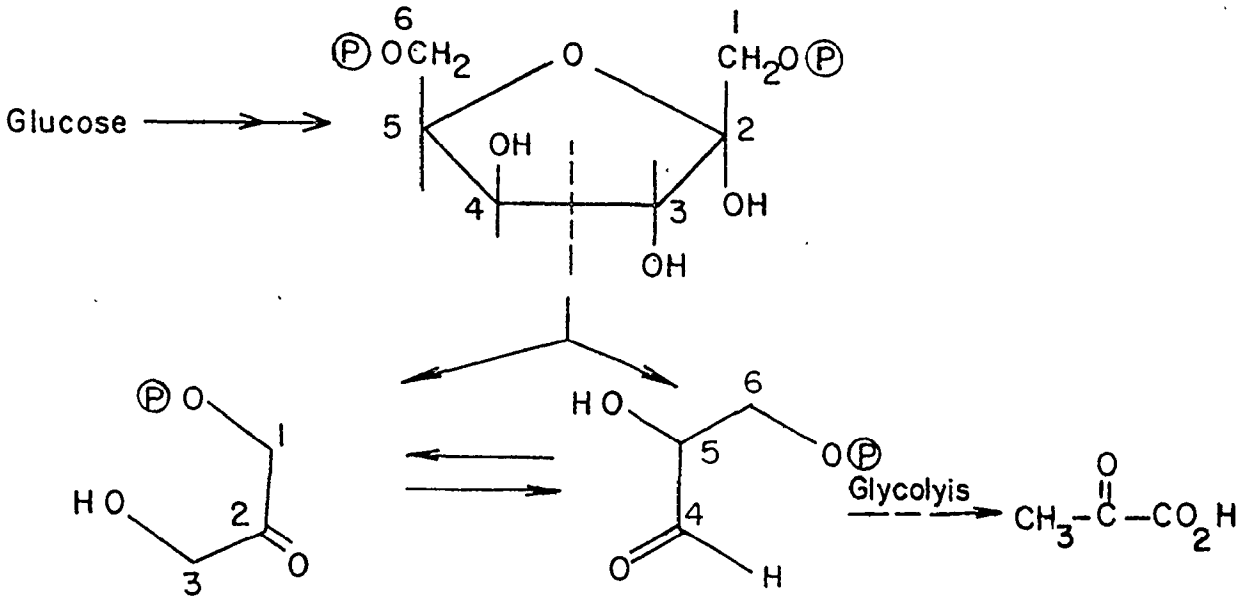
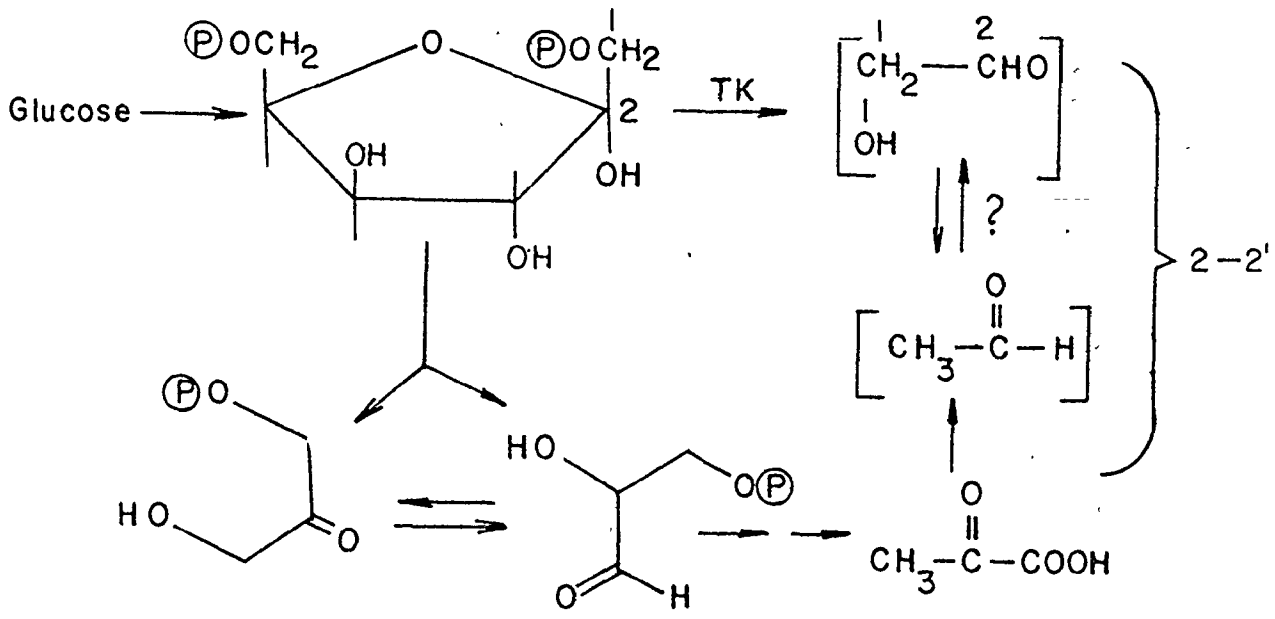


Figure 6: Possible Orientation of Triose Units in Pyridoxol Biosynthesis.



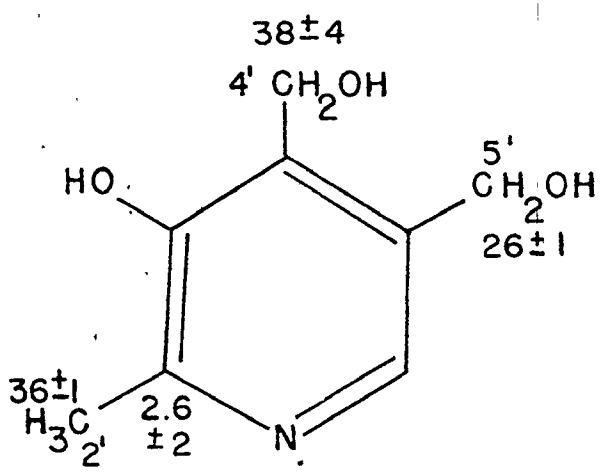
Scheme 3: Aldolase Cleavage of Fructose-1,6-Diphosphate.



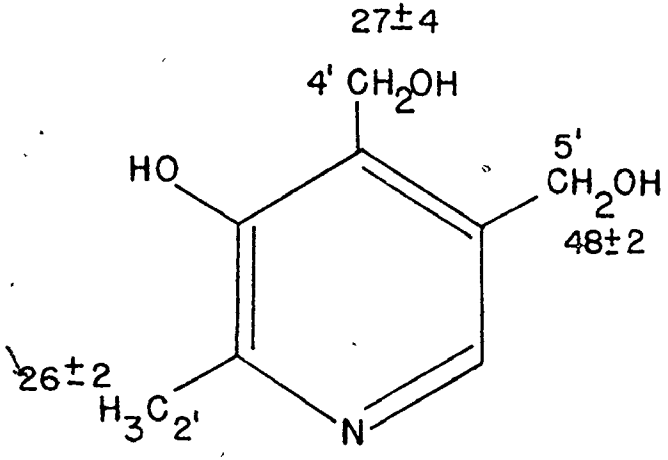
Scheme 4: Alternative Route involving Glycolaldehyde Proposed for the Origin of C-2,-2' of Pyridoxol.[78]

Tracer derived from [1-^{14}C]- and [6-^{14}C]glucose was shown by Hill and Spensér to label the same carbon atoms of pyridoxol (C-2', C-4', C-5').[78] However, the quantitative distribution of label within pyridoxol was different in the two cases (Fig. (7)). On this basis, it was considered that dihydroxyacetone-1-phosphate gives rise to C-3, C-4, C-4' and D-glyceraldehyde-3-phosphate yields C-5', C-5, C-6 of pyridoxol (Fig. 6A). The quantity of label at C-2' suggested that the C_2 unit of the vitamin is derived from dihydroxyacetone-1-phosphate rather than from pyruvate by way of D-glyceraldehyde-3-phosphate. This was not consistent with the earlier experimental observations that a pyruvate-derived unit would serve as the C_2 donor. Thus, a second route, directly from hexose, was proposed as a second source of the two-carbon unit. It was postulated that the two routes would converge in an interconvertible metabolic pool from which the two-carbon unit required for pyridoxol biosynthesis is derived (Scheme 4). Glycolaldehyde, derived from C-1,-2 of hexoses and pentoses, was considered to be involved and was predicted to give rise to C-2,-2'.

Glycolaldehyde had been implicated with the biosynthesis of vitamin B_6 as a result of earlier mutant studies.[85,86] It was not until Dempsey isolated a pyridoxineless mutant of E. coli, WG3 (pdxB), in which the pyridoxal supplement could be satisfied by glycolaldehyde, that it was considered by



[1—^{14}C] GLUCOSE



[6—^{14}C] GLUCOSE

Figure 7: Mode of Incorporation into Pyridoxol of Activity Derived from [1-^{14}C]- and [6-^{14}C]Glucose.[78]

many investigators[67,69,84,87] as an obligatory intermediate in pyridoxol biosynthesis. Nutritional studies demonstrated that the presence of glycolaldehyde increased pyridoxol production.[84] However, Lingens found that glycolaldehyde had no effect on vitamin B_6 biosynthesis in Bacillius subtilis.[67] Interestingly, of all the pyridoxineless mutants of yeast and bacteria isolated to-date, no other has been found which can grow when glycolaldehyde is added to the culture medium.

From tracer investigations, Schroer and Frieden found that the presence of unlabelled glycolaldehyde spared the incorporation of tracer derived from [G-^{14}C]glucose into pyridoxol in S. fragilis.[69] Incorporation of activity derived from [2-^{14}C]- and [1,2-^{14}C]glycolaldehyde into pyridoxol[87] has been demonstrated and activity has been found to be confined to C-5' and C-5,-5', respectively.[80] Contrary to the prediction of Hill et al., less than 3% of the incorporated activity was found at C-2,-2'.

Glycolaldehyde is normally derived from C-1,-2 of hexoses and pentoses by transketolase reaction. None of the pentoses tested could satisfy the pyridoxal requirement of several pyridoxineless mutants of E. coli B.[78] D-Xylulose was found to have no significant effect on pyridoxol production of E. coli K12 mutant BL-1.[84]

Pyridoxineless mutants of E. coli (serC)[39,88,89]

which lack 3-phosphoserine:2-ketoglutarate transaminase are unable to synthesize serine or pyridoxol. Both serine and pyridoxol are required for the growth of these mutants. This prompted Dempsey to propose that serine is required for pyridoxol biosynthesis. However, two other mutants which also are unable to synthesize serine can synthesize pyridoxol, during serine starvation, at normal rates or at greater rates than the wild type. On this basis, serine is not a precursor. Yet serine, alone or together with glycolaldehyde, stimulated pyridoxol production when added to cultures of pyridoxineless mutants of E. coli K12, BL-1.[84]

Tracer from [3-^{14}C]serine has been shown to be incorporated into pyridoxol.[69,77,90] Hill et al. found most of the activity at C-2,-2' of pyridoxol and rationalized the entry of serine via pyruvate. In contrast, Schroer et al.[69] found that label was randomly incorporated and that the presence of unlabelled serine does not spare the incorporation of label derived from [G-^{14}C]glucose into pyridoxol produced by S. fragilis. These observations further suggest that serine itself is not an obligatory intermediate, but is converted to one.

3-Phosphoserine:2-ketoglutarate transaminase has recently been shown to convert small amounts of serine to 3-hydroxypyruvate.[89] SerC mutants will synthesize normal amounts of pyridoxol when 3-hydroxypyruvate is added to a

culture deprived of serine and pyridoxol. They will grow normally when serine and 3-hydroxypyruvate are both present. Based on these and other observations from genetic studies,[89] Dempsey considers 3-hydroxypyruvate as a likely candidate to be an intermediate in the biosynthesis of pyridoxol.

Thiamin and 2-ketoglutarate have been linked to pyridoxol biosynthesis. Mutants which are unable to synthesize these compounds cannot synthesize pyridoxol. Whereas these compounds are not considered as intermediates on route to the vitamin, the enzymes with which they are associated are inferred to catalyse reactions which convert precursors to pyridoxol.[39,51] 2-Ketoglutarate(glutamate) serves as cofactor for 3-phosphoserine:2-ketoglutarate transaminase, an enzyme which appears to be essential for pyridoxol biosynthesis (vide supra). The lack of 2-ketoglutarate in citrate synthaseless mutants may explain the lack of pyridoxol synthesis. Less is known about the deficient enzyme in the thiaminless mutants but since thiamin is known to be a cofactor in the enzymic mobilization of activated two-carbon units, it is assumed that such a reaction is required for pyridoxol biosynthesis.[39,51]

Several labelled substrates tested demonstrated no incorporation[69,77,90] into pyridoxol; others yielded pyridoxol in low radiochemical yield or randomly labelled.[69,77] Activity from [2-^{14}C]acetate and [3-^{14}C]aspartate was shown to enter pyridoxol.[69,77,90]

Radioactive pyridoxol from [2-^{14}C]acetate was isolated from E. coli[77,90] and S. fragilis[69] in poor radiochemical yield. Sixty percent of the activity which entered pyridoxol was located at C-2,-2'.[77] Similarly, 60% of the activity from [3-^{14}C]aspartate was also found to reside at C-2,-2'.[77] The remaining 40% of activity is assumed to be randomly distributed over the other carbons of pyridoxol, in both cases. This mode of incorporation of activity was rationalized by the entry of labelled glycolytic intermediates formed by the reversal of glycolysis.[77] This distribution still awaits experimental confirmation. Schroer et al. found that aspartic acid partially spared the incorporation of [G-^{14}C]glucose into pyridoxol in S. fragilis. Partial degradation of pyridoxol derived from [1-^{14}C]- and [3-^{14}C]aspartate indicated that activity was randomly distributed since 25% of the incorporated label was located at C-2,-2'.[69] This observation supports Hill's rationale (entry of label by the reversal of glycolysis) since an 84 hour incubation period of the yeast culture (compared to 6 hours incubation for E. coli) is sufficient time to permit total equilibration of label via the Krebs' cycle.

The conclusion from mutant and tracer investigations is that more precursors have been proposed for pyridoxal-5'-phosphate than can be arranged in a single biosynthetic sequence. This is probably due to the interconvertibility of these precursors which lie on central pathways of metabolism.

The glycolytic intermediates derivable from glycerol and glucose have been established as precursors for the building blocks of the carbon skeleton of pyridoxol as proposed by Hill and Spenser. However, several inconsistencies remain to be explained; the isolation of any acyclic intermediate solely committed to the pathway is yet to be achieved.

The following experimental facts remain to be accommodated by a hypothetical sequence for pyridoxol biosynthesis: 1) Glycolaldehyde is incorporated specifically into C-5,-5' of pyridoxal. 2) Labelled glucose yields specifically labelled pyridoxol. 3) The pyruvate derived two-carbon unit appears to be more closely related to dihydroxyacetone-1-phosphate than to D-glyceraldehyde-3-phosphate. Furthermore, 4) the status of 3-hydroxypyruvate, 5) the function of thiamin, 6) the function of 2-ketoglutarate, and 7) the status of 4'-deoxypyridoxine in the biosynthesis of vitamin B_6 remain to be clarified.

The objective of this thesis is to provide experimental evidence which may be interpreted in accordance with the existing hypotheses and to narrow the range of possible precursors so as to facilitate their identification upon isolation.

CHAPTER II

EXPERIMENTAL

2.1 INTRODUCTION

The pyridoxineless mutants of Escherichia coli B used in this study have been described by Dempsey.[41,51] E. coli B, mutant WG2, contains a genetic lesion in the ultimate or penultimate step in pyridoxal-5'-phosphate biosynthesis and lacks pyridoxol oxidase activity. This mutant can synthesize pyridoxol, but is unable to grow unless pyridoxal or pyridoxamine is added to the culture medium. When the cells are deprived of the pyridoxal or pyridoxamine supplement, growth ceases. However, pyridoxol synthesis continues, at four to six times the rate as that of the wild type, for approximately three hours before the culture dies. During this time, pyridoxol and pyridoxol-5'-phosphate are excreted into the culture medium because the cells are unable to convert these compounds to pyridoxal or its 5'-phosphate ester.

E. coli B, mutant WG3, is a pyridoxineless mutant in which the pyridoxal requirement is satisfied by pyridoxol, pyridoxamine and glycolaldehyde.[51] This mutant, when grown in a culture medium supplemented with glycolaldehyde and deprived of pyridoxal, will biosynthesize pyridoxal-5'-phosphate, but will not excrete appreciable quantities of the vitamin into the culture medium.

Radioactive tracers were administered to cultures of mutant WG2 and WG3 at the onset of pyridoxal deprivation. Incubation of these cells was carried out for about six hours.

Radioactive pyridoxol-5'-phosphate released into the medium by mutant WG2 was hydrolysed to pyridoxol.[77] Unlabelled pyridoxol hydrochloride was then added to the hydrolysate, as carrier, to facilitate the isolation. The mixture of pyridoxol synthesized by mutant WG2 and pyridoxol "carrier" was reisolated by several chromatographic techniques.

To isolate pyridoxal-5'-phosphate biosynthesized by mutant WG3,[90] it was necessary to lyse the cells. Pyridoxal-5'-phosphate was reduced to pyridoxol-5'-phosphate with sodium borohydride. Hydrolysis of the 5'-phosphate ester and isolation of pyridoxol hydrochloride is accomplished in the same manner as that for mutant WG2.

Purification of pyridoxol hydrochloride was accomplished by several crystallizations until constant specific activity of each recrystallized batch was achieved. Purification by high vacuum sublimation and further dilution with recrystallized pyridoxol hydrochloride prepared each sample for degradation and radioactive assay.

The chemical degradation of pyridoxol hydrochloride by the sequence leading to the isolation of sodium acetate (11) as N-acetyl-α-naphthylamine(12) permitted the assay of radioactivity at carbons 2 plus 2' of pyridoxol. A portion of the sodium acetate was further degraded by the Schmidt reaction

to methylamine hydrochloride (13) representing carbon 2' of pyridoxol, and was isolated as 1-methyl-3-phenylthiourea (15) or N-methylphthalimide (14). The C_2 fragment C-5',-5 was isolated as N-phthaloylglycine (21). Carbon 5' of pyridoxol was recovered as benzoic acid (18).

2.2 MATERIALS AND METHODS EMPLOYED IN THIS STUDY

2.2.1 Microorganisms

Two pyridoxine auxotrophs of Escherichia coli B, mutants WG2 and WG3, isolated by W.B. Dempsey,[41,51] were used. The pyridoxal requirement of the latter mutant is satisfied by glycolaldehyde.

The culturing conditions for the two mutants were as follows: A minimal medium composed of a carbon source (either glycerol or glucose) and inorganic salts (KH_2PO_4; K_2HPO_4; $(NH_4)_2SO_2$; $MgSO_4$; $CaCl_2$) prepared according to the published procedure of Rickenberg et al.[91] The growth of E. coli B mutant WG3 can be maintained only if the minimal medium, containing 0.2% glycerol[44,51] as a general carbon source, is supplemented with pyridoxal, pyridoxol or glycolaldehyde. For growth, mutant WG2 requires that the minimal medium, containing the same general carbon sources, is supplemented with pyridoxal.

In some of the tracer experiments, the carbon source concentration was varied as described in Tables (2) and (6). In experiments using mutant WG3 (Experiments 1 and 3), the same salts medium was used, the carbon source concentration

was maintained at 0.2% (2 g/l) of glycerol, and the glycolaldehyde concentration (required for the growth of mutant WG3) varied from 0.45 mmol/l (Experiment 1^{87}) to 1 mmol/l (Experiment 3). The pyridoxal supplement was omitted. The minimal media used for culturing mutant WG2 for all the tracer experiments contained the same salts. The glycerol concentration was reduced to 0.1% (1 g/l) in all but two experiments in which the glycerol concentration was maintained at 0.2% (2 g/l) (Experiment 8) in one case and reduced to 0.05% (0.46 g/l) (Experiment 5) in the other. The pyridoxal supplement was omitted. In the isotope competition experiments (Experiments 4, 11, 12 and 13) the unlabelled substrates were added to the culture medium in concentrations equal to that of glycerol (10 mmol/l). In one competition experiment (Experiment 6), the glycolaldehyde concentration was 0.075 g/l, whereas the glycerol concentration was maintained at 1 g/l.

Mutant stocks, maintained on slants, were reisolated by replica plating and by growth of single colonies before each tracer experiment. The minimal medium supplemented with pyridoxol or pyridoxal was inoculated with freshly isolated cells and the culture was incubated on a rotary shaker (37°C at 200 RPM) for 6 hours. The optical density of the culture was monitored at 620 nm (Pye-Unicam UV/Visible spectrophotometer Model SP 1800) to determine the exponential phase of growth. The cells were harvested by centrifugation for 10 minutes at

8,000 g and washed with sterile distilled water (3 x 100 ml).

The cells were resuspended in four 500 ml quantities of minimal salts medium containing glycerol (1 g/l for mutant WG2) or glycerol (1 g/l) plus glycolaldehyde (0.060 g/l for mutant WG3) in 2 litre Erlenmeyer flasks. The solution containing the radioactive tracer was administered equally to each of the culture flasks, which were then incubated for six hours on a rotary shaker (37°C at 200 RPM). The medium was centrifuged for 10 min at 8,000 g and the supernatant was decanted and stored at 4°C.

2.2.2 Labelled Compounds

The radioactively labelled compounds used in the tracer experiments are listed in Table (1). In eight of these experiments, [2-^{14}C]glycerol was used either as the only labelled substrate in the presence of unlabelled compounds such as glycolaldehyde (Experiments 3 and 4), sodium pyruvate (Experiment 11), sodium acetate (Experiment 12) and lithium 3-hydroxypyruvate (Experiment 13), or together with [2-^{14}C]glycolaldehyde (Experiments 5 and 6) or together with [1-^{3}H]glycerol (Experiment 14). In the remaining experiments, samples of [2-^{14}C]-glycolaldehyde (Experiments 1,[87] 2 and 7), [2-^{3}H]glycolaldehyde (Experiment 7), [2-^{14}C]pyruvaldehyde (Experiment 8), [1-^{14}C]-acetate (Experiment 9) and [1-^{14}C]ribose (Experiment 10) were used as tracers.

Labelled samples obtained from commercial suppliers (New England Nuclear Corp. and Radiochemical Center) in sterile

Table 1 RADIOACTIVE SUBSTRATES

Expt. #	Mutant	Substrate	Nominal Specific Activity (mCi/mmol)	Nominal Total Activity (μCi)	Source
1	WG3	[2-^{14}C]Glycolaldehyde	0.40	360	a
2	WG2	[2-^{14}C]Glycolaldehyde	0.21	137	d
3	WG3	[2-^{14}C]Glycerol	(40)0.023	500	c
4	WG2	[2-^{14}C]Glycerol	(15.3)0.023	250	b
5	WG2	[2-^{14}C]Glycolaldehyde	0.035	345	e
		[2-^{14}C]Glycerol	(16.6)0.036	360	b
6	WG2	[2-^{14}C]Glycolaldehyde	1.28	198	d
		[2-^{14}C]Glycerol	(40)0.012	250	c
7	WG2	[2-^{3}H]Glycolaldehyde	0.53	816	d
		[2-^{14}C]Glycolaldehyde	0.045	70	d
8	WG2	[2-^{14}C]Pyruvaldehyde	0.225	515	e
9	WG2	[1-^{14}C]Acetate	56.9	1000	b
10	WG2	[1-^{14}C]Ribose	53	250	b
11	WG2	[2-^{14}C]Glycerol + Sodium Pyruvate	(16.5)0.036	390	b

Table 1 continued

12	WG2	[2-^{14}C]Glycerol + Sodium Acetate	(15.3)0.023	250	b
13	WG2	[2-^{14}C]Glycerol + Lithium, 3-Hydroxypyruvate	(16.4)0.023	250	b
14	WG2	[1,3-^{3}H]Glycerol	(2500)0.230	5000	c
		[2-^{14}C]Glycerol	(40)0.012	250	c

a - quoted from ref. 87, experiment III-8
b - New England Nuclear
c - Amersham/Searle
d - see Table 3
e - see Table 8

solution were added to the culture medium, without further sterilization, by means of a sterile disposable syringe. Other labelled substrates were dissolved in distilled water (10 ml) and were sterilized by filtration through a millipore (0.2 μ) filtering apparatus. The filtrate was then added to the culture medium.

Synthesis of Labelled Compounds

[2-^{14}C]Glycolaldehyde and [2-^{3}H]glycolaldehyde were prepared from commercial samples of DL-[3-^{14}C]serine (New England Nuclear) and DL-[3-^{3}H]serine (Radiochemical Center), respectively, by the adaptation of a published procedure.[87,92] The preparation of [2-^{14}C]pyruvaldehyde from a commercial sample of [2-^{14}C]acetone (New England Nuclear) was carried out by a modification of published procedures.[93,94]

[2-^{14}C]Glycolaldehyde: DL-[3-^{14}C]Serine (100 μCi) was added to a solution of (±)-serine (70 mg) in a little water. The mixture was evaporated and the residue was recrystallized from an ethanol/water mixture (4:1 v/v). The product (70 mg) was dried and redissolved in water (10 ml). Phosphoric acid (0.05 ml, 85% w/w) was added to the stirred solution, followed by ninhydrin (238 mg) and the mixture was heated at 60° until colourless (∿ 2 hrs). After cooling, the suspension was filtered through a bed of celite. A mixture (1:1) of Dowex 1 (OH^- form, 100-200 mesh, 5 ml) and Dowex 50 (H^+ form, 100-200

mesh, 5 ml) was added to the filtrate and the mixture was stirred for 30 min and then filtered. A sample of the filtrate was chromatographed on two TLC systems (methanol:pyridine:water (20:1:5 v/v) on silica gel, glycolaldehyde: R_f 0.66, serine R_f 0.16; and ether:ethanol (95:5 v/v), on silica gel, glycolaldehyde: R_f 0.76, serine R_f 0.28) and was found to contain a single radioactive zone corresponding in R_f to glycolaldehyde (Packard Radiochromatogram scanner, model 7201).

The filtrate, containing [2-^{14}C]glycolaldehyde, was filter-sterilized before addition to the culture medium.

All the samples of [2-^{14}C]glycolaldehyde used in the tracer experiments were prepared in an analogous manner.

[2-^{3}H]Glycolaldehyde: [2-^{3}H]Glycolaldehyde was prepared similarly, from DL-[3-^{3}H]serine (1 mCi) and (±)-serine (140 mg).

[2-^{3}H,2-^{14}C]Glycolaldehyde: [2-^{3}H,2-^{14}C]Glycolaldehyde was prepared by mixing samples of [2-^{3}H]glycolaldehyde and [2-^{14}C]glycolaldehyde (^{3}H/^{14}C ratio 11.8 ± 0.1). A small sample of the mixture (approximately 5 μCi of ^{14}C) was added to a solution containing glycolaldehyde (20 mg) in aqueous ethanol (50% v/v, 5 ml). Dimedone (95 mg) was added and the mixture was heated 30 min on a steam bath. The dimedone derivative of glycolaldehyde was filtered off, washed with cold water (20 ml), dried and recrystallized from 95% ethanol.

m.p. 229-231°C (literature[92,95] m.p. 224-226°C, $^{3}H/^{14}C$ ratio 9.8 ± 0.1).

[2-^{14}C]Pyruvaldehyde: The preparation of [2-^{14}C]-pyruvaldehyde was carried out on a vacuum line apparatus equipped with a three-port manifold, a McCloud gauge, and two liquid nitrogen traps containing a solution (10 ml) of 2,4-dinitrophenylhydrazine dissolved in ethanol (Fig. 8).

The reaction vessel, equipped with a magnetic stirrer and containing 0.5 g (0.005 moles) of selenium dioxide dissolved in p-dioxane (2.5 ml) and water (0.2 ml), was connected to the first port of the manifold. A break-seal tube containing [2-^{14}C]-acetone (1 mCi) was attached to the second port and a 10 ml round bottom flask containing carrier acetone (250 mg) was connected to the third port. All the vessels attached to the manifold were frozen with liquid nitrogen and the apparatus was evacuated to 0.025 mm of mercury. The stopcock to the vacuum pump was closed in preparation for the transfer of the labelled acetone to the reaction vessel.

The break-seal was broken and the tube was warmed to room temperature to ensure the transfer. In a similar manner, the carrier acetone was transferred from the third port to the reaction vessel.

The stopcock to the reaction vessel was closed and the reaction mixture was warmed to room temperature. A fine red suspension of reduced selenium metal was evident after approxi-

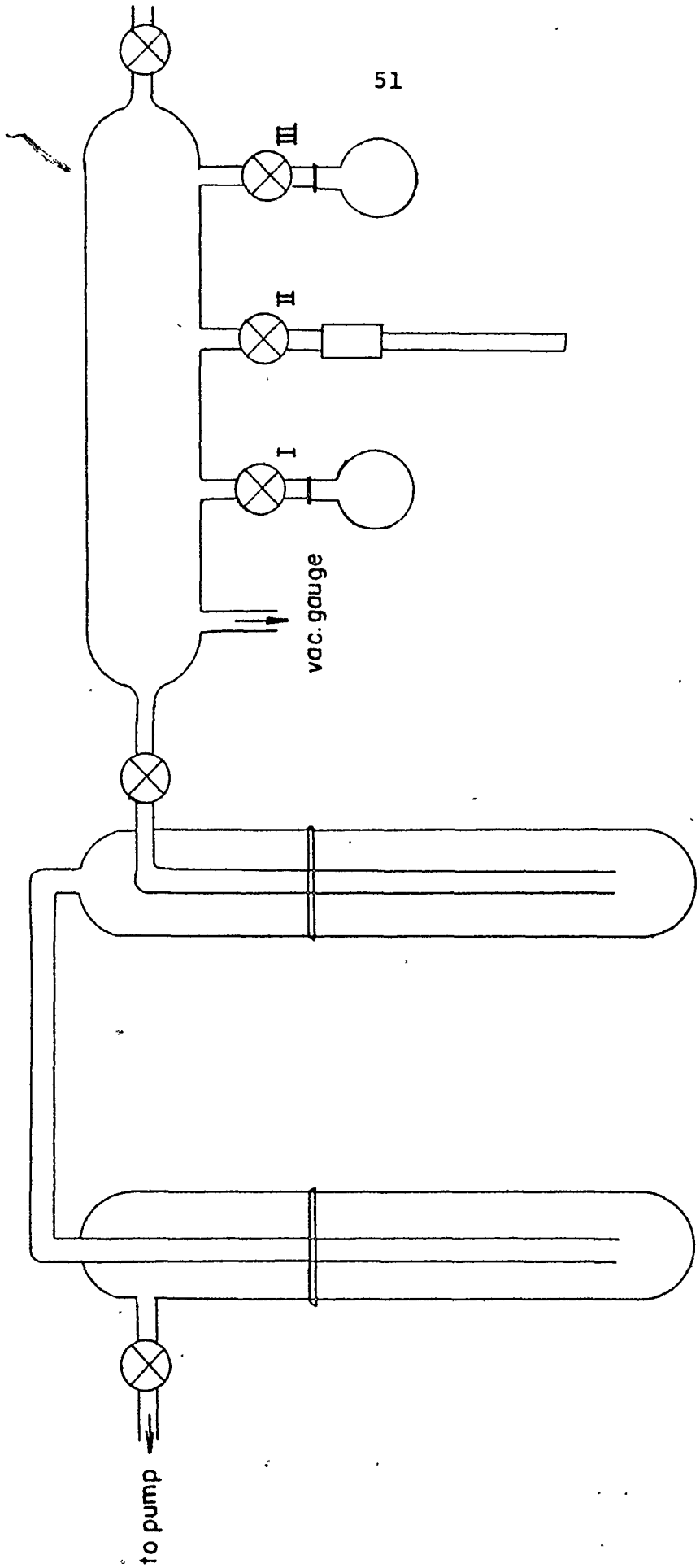


Figure 8: Apparatus Used for the Synthesis of [2-^{14}C]Pyruvaldehyde.

mately 30 min. The reaction mixture was left stirring at room temperature overnight.

With the pump operating and the stopcock to the reaction vessel closed, the stopcock to the liquid nitrogen traps was opened. The stopcocks to ports two and three were also opened at this time. The system, excluding the reaction vessel, was allowed to reach atmospheric pressure which permitted the replacement of the vessels attached to ports two and three with 10 ml round bottom flasks. The system was then evacuated to 0.025 mm of mercury in preparation for distillation.

The product was obtained by distillation under vacuum in the following manner: The stopcock to the liquid nitrogen traps and to the second port were closed. The reaction vessel and the receiver on the third port were frozen with liquid nitrogen and after about 30 min the stopcock to the reaction vessel was opened very slowly. The system was allowed to equilibrate for about 10 min. The liquid nitrogen trap surrounding the reaction vessel was replaced with a dry ice/acetone bath which commensed the distillation and was continued for 3 hrs. During the distillation, the pressure in the system rose to 0.05 mm of mercury. The stopcocks to the reaction vessel and to the receiver flask, containing fraction I, were closed.

Similarly, fraction II (collected between -78°C and 0°C) and fraction III (0°-65°) were collected.

Analysis of the three fractions collected was based on a previous synthesis of [2-^{13}C]pyruvaldehyde followed by ^{13}C

analysis. Fraction I was shown to contain unreacted acetone. Fraction III contained water, dioxane and an unidentified selenium complex, in which red selenium precipitated from solution within a few hours. Fraction II was found to contain the bulk of the product, pyruvaldehyde, along with some unreacted acetone, Fig. (9).

Fraction II was dissolved in distilled water (10 ml) and a sample (3 x 10 µl) of this solution was counted to determine total activity and radiochemical yield. Another sample of this solution was chromatographed on a thin layer system (ethyl acetate/benzene/acetic acid; 4:1:1: v/v on silica gel, pyruvaldehyde, R_f 0.13) and was found to contain a single radioactive zone corresponding in R_f to freshly distilled pyruvaldehyde (Packard Radiochromatogram Scanner Model 7201).

The solution containing [2-^{14}C]pyruvaldehyde was immediately filter sterilized and administered to the culture medium.

<u>[1-^{3}H,2-^{14}C]Glycerol</u>: [1-^{3}H,2-^{14}C]Glycerol was prepared by mixing commercial samples of [1-^{3}H,2-^{14}C]glycerol (Radiochemical Center) and [2-^{14}C]glycerol (New England Nuclear) and made up to volume (10 ml) with distilled water. Samples (3 x 10 µl) were withdrawn from the solution for liquid scintillation counting (^{3}H/^{14}C ratio 19.8 $\pm$ 0.02). A small sample of the mixture (approximately 5 µCi of ^{14}C) was added

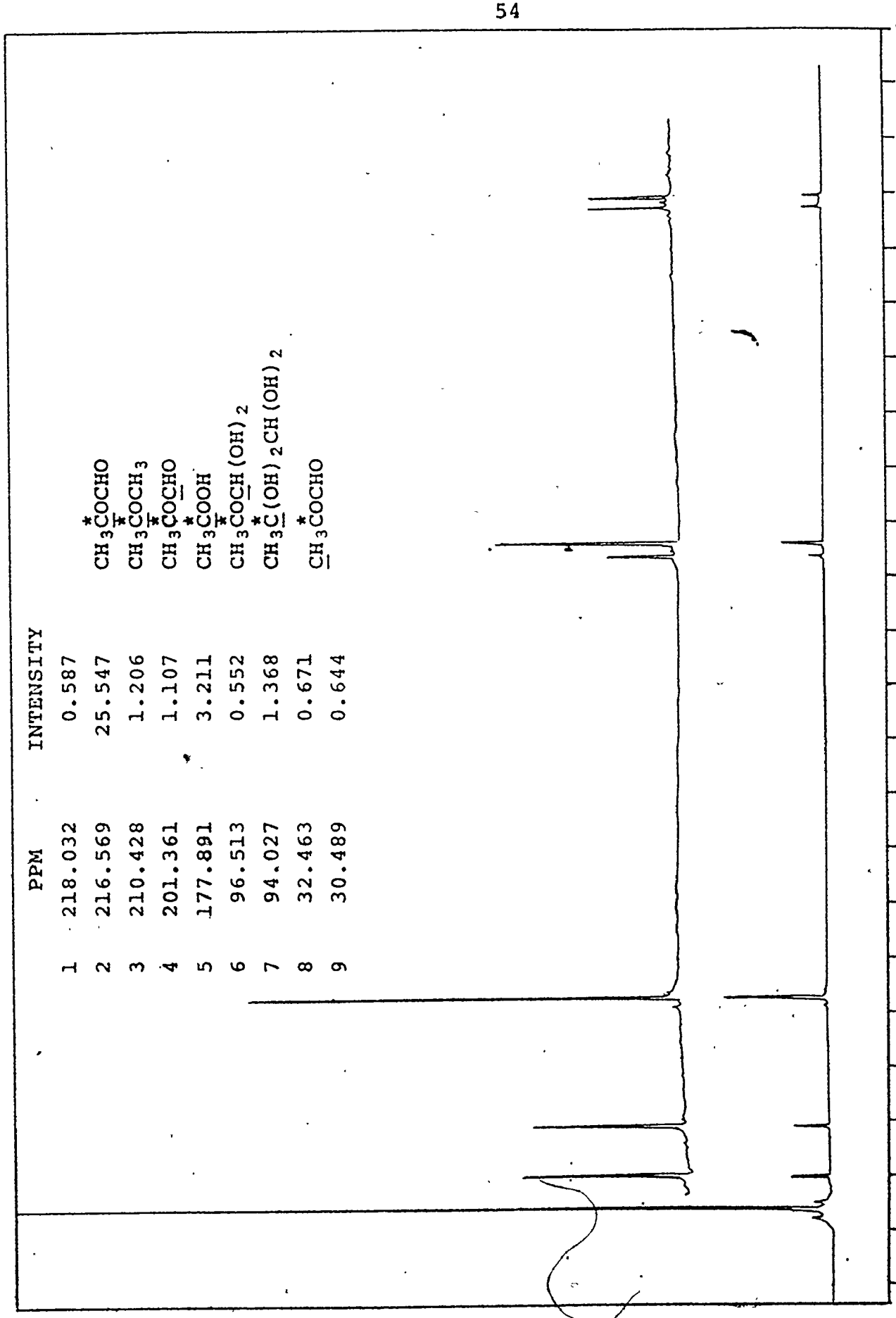


Figure 9: ^{13}C-NMR Spectrum of Fraction II Containing [2-^{13}C]Pyruvaldehyde.

to a solution containing glycerol (500 mg) in distilled water (1 ml). Sodium hydroxide solution (10% w/v, 10 ml) was added followed by benzoylchloride (2.5 ml) which was added over 15 min. The reaction mixture was shaken vigorously after each addition of the acid chloride. A white flocculant precipitate was isolated by centrifugation, washed repeatedly with water and recrystallized three times from 95% ethanol. Glycerol tribenzoate had a melting point of 75-76°C. (Literature[96] m.p. 76°, $^{3}H/^{14}C$ ratio 18.7 $\pm$ 0.5.)

The solution containing the intermolecularly labelled glycerol was filter-sterilized and administered to the culture fluid in Experiment 14.

2.2.3 Isolation of Pyridoxol

Samples of radioactive pyridoxol were isolated from the cultures of *E. coli* mutant WG2 by carrier dilution (30-40 mg) as previously described.[77] Radioactive pyridoxal from mutant WG3 was obtained by carrier dilution and chemically reduced with sodium borohydride to pyridoxol by a published method.[90]

The pyridoxol hydrochloride samples were repeatedly crystallized, from methanol by the addition of anhydrous diethyl ether, until constant specific activity was observed. The product was finally purified by high vacuum sublimation (2×10^{-2} mm at 120-130°C). The yield of the purified pyridoxol hydrochloride (m.p. 205-206°C with decomposition) corresponded to approximately 75 to 85% of the total weight of inactive carrier

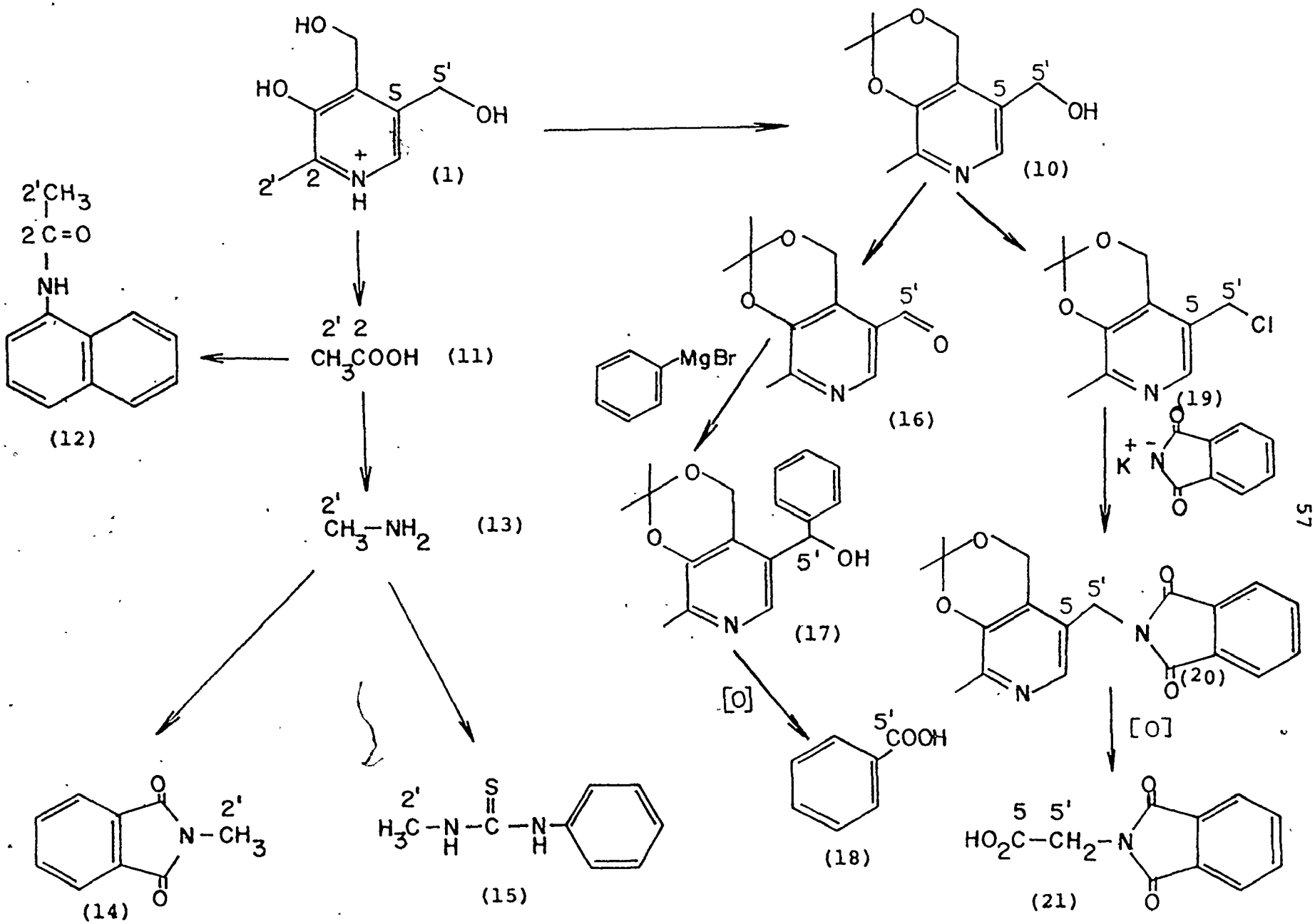
which had been added. The sublimed product was diluted further by the addition of recrystallized inactive carrier and used for chemical degradation.

2.2.4 Degradation of Radioactive Pyridoxol to Locate the Sites of Labelling

In each of the tracer experiments, the radioactive sample of pyridoxol hydrochloride was degraded to determine which sites contained radioactivity. The degradation sequences leading to the isolation of individual carbons or carbon fragments employed methods which were previously published[77,80] (Scheme 5).

Kuhn-Roth Oxidation - Carbons 2 and 2' of Pyridoxol as Sodium Acetate:[97,77] Pyridoxol hydrochloride (1) (40 mg) was dissolved in a solution of dilute sulfuric acid (10 ml, 10% v/v) containing chromium trioxide (2 g). The reaction mixture was heated and slowly distilled while a stream of nitrogen was bubbled through the solution. The volume in the reaction vessel was maintained by the repeated addition of water. The distillate (80 ml), containing the acetic acid, was collected over 4 hours. Neutralization with sodium hydroxide (0.1 N) to pH 7.5 facilitated the isolation of sodium acetate after evaporation (90°C) of the solution. Yield was 16 mg.

Carbon Atoms 2 plus 2' as N-acetyl-α-naphthylamine (12):[77] A portion of the sodium acetate, obtained by Kuhn-Roth oxidation



Scheme 5: Degradation Sequences for Pyridoxol.

of pyridoxol, was purified by conversion to N-acetyl-α-naphthylamine. Crude sodium acetate (5 mg) was dissolved in a little water. To this, a solution containing a slight molar excess of 1-naphthylamine hydrochloride (15 mg) dissolved in water (1 ml) followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (40 mg) was added. The product, N-acetyl-α-naphthylamine, which precipitated from a stirred solution at room temperature was filtered off, washed with water and recrystallized from a benzene/cyclohexane (1:4) mixture. Final purification was effected by sublimation at 90° and 2×10^{-2} mm.

Carbon Atom 2' as 1-Methyl-3-phenylthiourea (15) or N-methylphthalimide(14):[98] A portion of the sodium acetate obtained in the Kuhn-Roth oxidation was further degraded to methylamine hydrochloride by the Schmidt reaction.[77] For purification and radioactive assay, the methylamine hydrochloride was converted to 1-methyl-3-phenylthiourea (15) or to N-methylphthalimide (14).

1-Methyl-3-phenylthiourea (15): Methylamine hydrochloride (12 mg) was dissolved in a drop of water. Ethanol (5 ml) was added, followed by phenylisothiocyanate (0.25 ml). The mixture was stirred 10 min at 0°C, saturated aqueous potassium bicarbonate (0.5 ml) was added, and the mixture heated for 15 min. After cooling, water (15 ml) was added, and the product was filtered off, washed with water and ether, and dried *in vacuo*. 1-Methyl-3-phenylthiourea was sublimed

at 85-93°C and 10^{-2} mm of mercury. Yield was 6 mg. Melting point 113-114°C (literature[99] 112-113°C), ms. m/e 166 (M^+).

N-Methylphthalimide (14): Methylamine, trapped in a dilute solution of hydrochloric acid (3 ml; 0.1 N) from the Schmidt reaction,[77] was converted directly to the phthalimide derivative. Carboethoxyphthalimide (18 mg) dissolved in ethanol (1 ml) was added to the acidic solution containing methylamine. Solid sodium bicarbonate was carefully added to the mixture until pH 8 was obtained. The solution appeared slightly yellow. On cooling at 0°C, a white crystalline solid was obtained. The crude product was filtered off, washed with a little water and dried in vacuo. The melting point of the crude product was 126-131°C. Sublimation of the crude solid (63-69°C at 3 x 10^{-2} mm) yielded N-methylphthalimide (4 mg) m.p. 132-133°C (literature[100] 134°).

Isolation of Carbon Atom 5' as benzoic acid (18)[77] and Carbon Atoms 5 plus 5' as N-phthaloylglycine (21):[80] Pyridoxol hydrochloride was converted to the 3,4'-O-isopropylidene derivative by the adaptation of a published procedure.[77] A sample of this compound was counted, after rigorous purification by recrystallization (95% ethanol) and sublimation (110-120°C at 2 x 10^{-2} mm Hg). It was essential that the measured molar specific activity of this derivative be the same, within experimental error, as that of the parent compound, pyridoxol hydrochloride. Otherwise, the parent compound had to be re-

crystallized and resublimed until the molar specific activities of these two compounds were in agreement; only then was this sample subjected to further degradation.

The degradation sequences for the isolation of carbon 5' as benzoic acid (18) and carbon atoms 5 plus 5' as N-phthaloylglycine (21) from 3,4'-O-isopropylidenepyridoxol hydrochloride (10) have been described elsewhere and are summarized in Scheme (5).

2.2.5 Determination of Radioactivity

Radioactivity was assayed by liquid scintillation counting (Mark I liquid scintillation computer, model 6860, Nuclear Chicago). Samples (0.5 mg to 2 mg) were dissolved in an appropriate solvent (water, dioxane, dimethylformamide 1 to 3 drops) and dispersed in "Aquasol" (New England Nuclear) (10 ml). Triplicate samples of each compound were counted under comparable conditions of quenching. The efficiency of counting was determined by comparison with an external standard of ^{133}Ba (approximately 80% for samples containing either ^{3}H or ^{14}C; approximately 50% for ^{14}C and approximately 30% for ^{3}H in samples containing both ^{3}H and ^{14}C). Correction for background radioactivity was applied for all samples. Confidence limits shown in the tables are standard deviations of the mean.

CHAPTER III

BIOSYNTHESIS OF PYRIDOXAL

3.1 GLYCOLALDEHYDE AS A PRECURSOR OF PYRIDOXAL

3.1.1 Introduction

3.1.1.1 Origin and Metabolism of Glycolaldehyde

Glycolaldehyde, $HOCH_2$-CHO (22), Fig. 10, has been known as a product of metabolism since the turn of the century. It was first isolated as a product of yeast fermentation[101] and was shown to accumulate in the culture medium of other microorganisms growing on various pentoses.[102,103] An early chemical investigation demonstrated that glycolaldehyde could be formed by the aldolase cleavage of pentoses.[104]

In 1938, Dickens suggested that glycolaldehyde was derived from D-ribose-5-phosphate.[105] However, subsequent experiments by Horecker et al. and Racker et al., provided evidence that glycolaldehyde is derivable from the ketoses, D-xylulose-5-phosphate, D-fructose-6-phosphate and D-sedoheptulose-7-phosphate, by the action of a transketolase enzyme.[106-109] Glycolaldehyde, derived by the transketolase reaction, has been demonstrated to be complexed to thiamin pyrophosphate ("active glycolaldehyde") (27) Scheme (6). The function of the transketolase enzyme is to transfer a C_2 unit from a ketose donor to an acceptor by way of this "active glycolaldehyde" intermediate. "Active

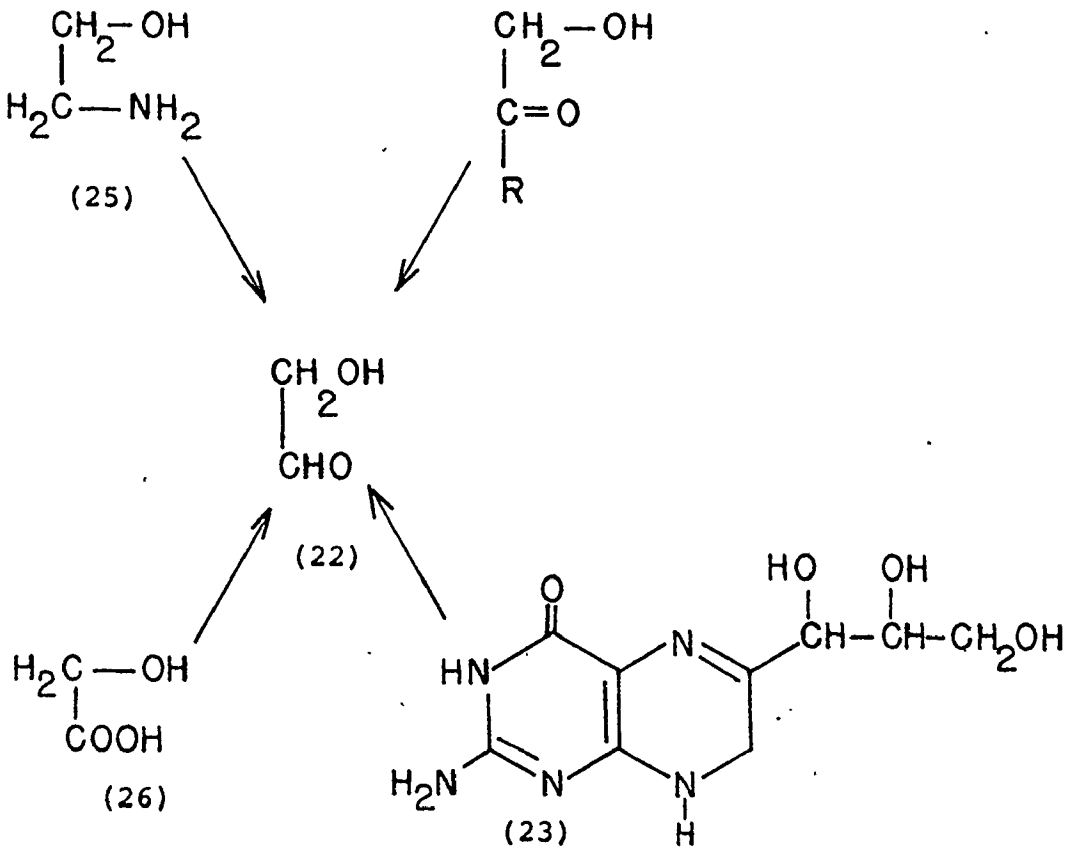
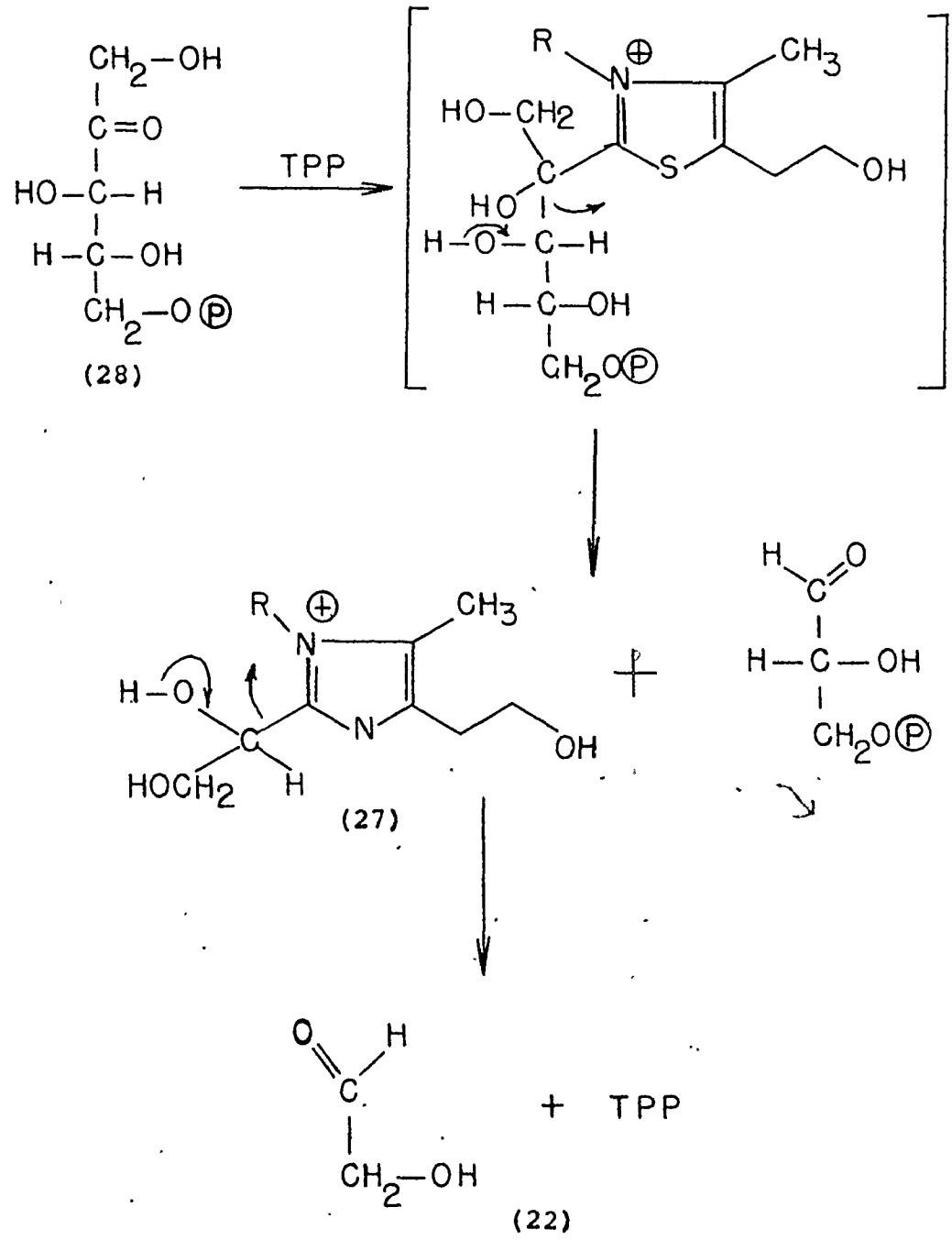


Figure 10: Origin of Glycolaldehyde.

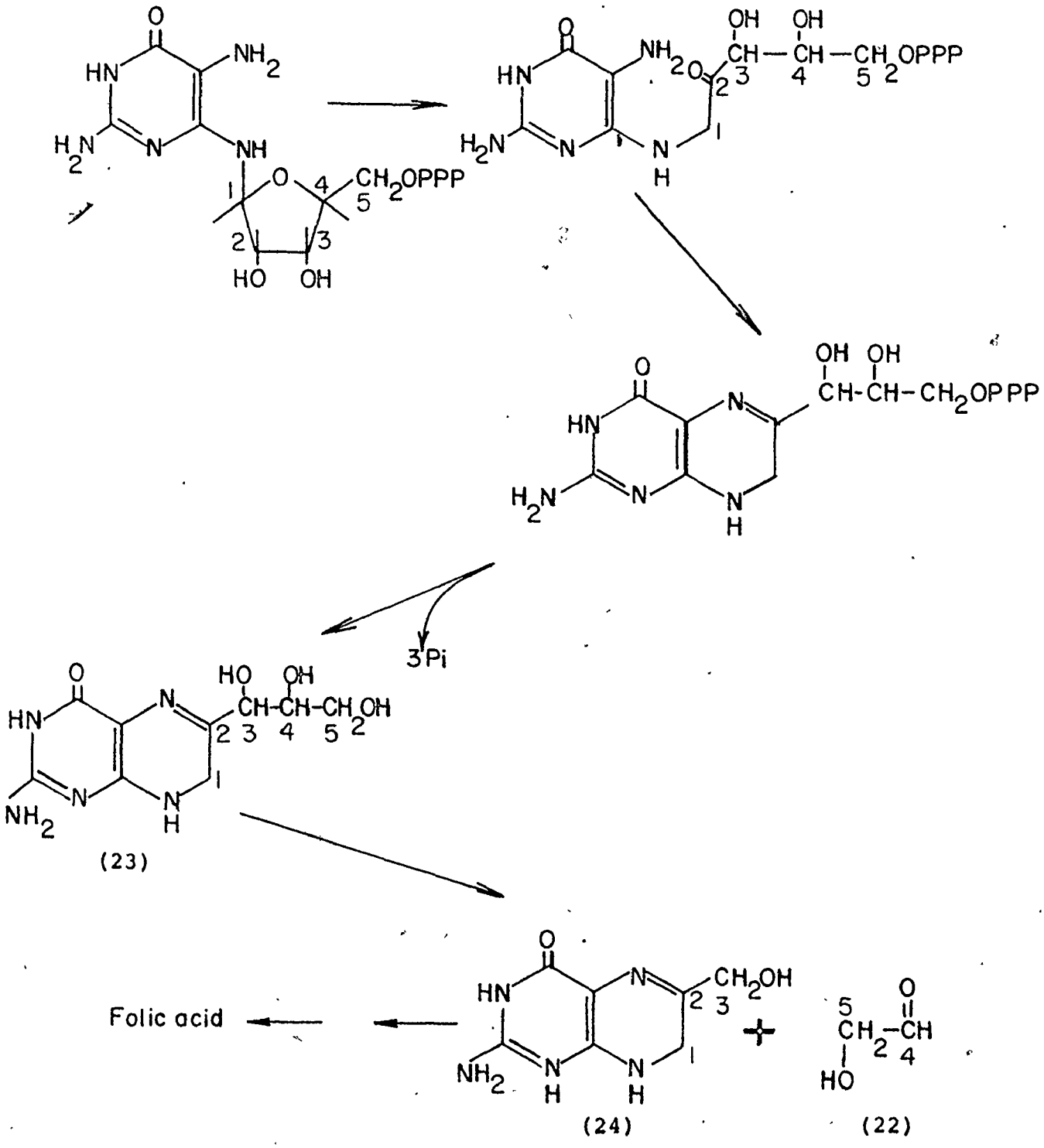


Scheme 6: Glycolaldehyde derived from D-Xylulose-5-Phosphate by Transketolase Catalysis.

glycolaldehyde" or 2-(α,β-dihydroxyethyl)thiamin pyrophosphate (27) has been proposed as an intermediate in another enzymic reaction, similar to that catalysed by transketolase, which has been observed in pentose fermenting bacteria. In these organisms, the phosphoketolase catalyses the formation of acetylphosphate from ketoses[113-115] by a rearrangement of "active glycolaldehyde".[116,117] "Active glycolaldehyde" from both of these enzymic reactions is derived from C_1 and C_2 of hexulose or pentulose (Fig. 10).

In agreement with Dickens' prediction, glycolaldehyde can be derived from C-4 and C-5 of ribose-5-phosphate in an indirect manner. During the elucidation of the biosynthesis of the pterine compound of folic acid, it was demonstrated that only C_1, C_2 and C_3 of ribose were incorporated into folic acid,[118] and that the two-carbon compound produced during the biosynthesis of the pterine moiety of folic acid was glycolaldehyde.[119] The enzyme which catalyses the transformation of 2-amino-4-hydroxy-6-(D-6-erythro-1',2',3'-trihydroxypropyl)-7,8-dihydropterine (23) (commonly known as neopterine) to 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropterine (24) (Scheme (7)) has been purified.[120]

Glycolaldehyde can originate from compounds other than hexoses or pentoses (Fig. 10). It is formed by the enzymic oxidation of ethanolamine (25),[121] Fig. 10, in microorganisms, plants[122-123] and insects.[124] In mammals, phosphorylated inter-



Scheme 7: Origin of Glycolaldehyde during Folic Acid Biosynthesis.

mediates may be involved in the catabolism of phosphoethanolamine. Several enzymes which utilize these phosphorylated intermediates have been isolated.[125,126] Oxidation of ethanolamine to glycolate and glyoxylate via glycolaldehyde was thought to occur in rats and pigeons.[127] This could not be confirmed, however.[128]

The metabolic fate of glycolaldehyde has been investigated in mammals.[127] Glycolaldehyde is readily converted to carbon dioxide, presumably via glycolate. Oxidation of glycolate to glyoxylate and oxalate is well established.[129] In the rat, glycolaldehyde is thought to be converted to glycine, and to contribute to glycogen formation via the sequence glycolaldehyde → glycine → serine → hydroxypyruvate → 3-phosphoglyceric acid → glucose. It is not appreciably converted to acetate.[92] The metabolism of glycolaldehyde in microorganisms has not been fully investigated.

3.1.1.2 Glycolaldehyde and Pyridoxal Biosynthesis

The first indication that glycolaldehyde was implicated in the biosynthesis of vitamin B_6 was obtained by Morris and Woods.[85,86] They found that two *E. coli* B mutants, which were auxotrophic for vitamin B_6, could grow if the pyridoxal supplement was replaced by either serine or by glycine, together with glycolaldehyde.[85,86]

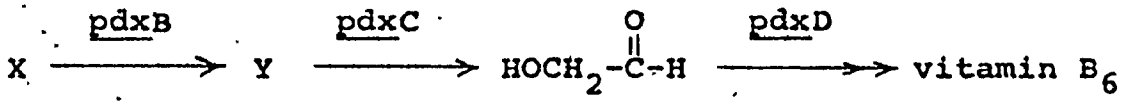
It was Morris' work which prompted Dempsey[51] to include

glycolaldehyde among the compounds which he tested as nutritional substitutes for pyridoxal, in pyridoxineless mutants. This led to the characterization of the Group I (pdxB, pdxC, pdxD, Fig. 2) mutants in which the pyridoxal requirement can be satisfied by pyridoxol, pyridoxamine or glycolaldehyde (E. coli B, WG3). Although not all mutants in this group responded to glycolaldehyde in the same manner, this observation strongly implicated glycolaldehyde or a metabolite readily derived from it, in vitamin B_6 biosynthesis.

Only two of the three closely linked phenotypes (pdxB, pdxC, Fig. 2) in the Group I mutants responded to glycolaldehyde. This suggested that the metabolic block in pdxB and pdxC mutants occurs earlier in the pathway than that of the pdxD mutants.[43,44] Accordingly, Dempsey proposed a sequence which placed glycolaldehyde on route to vitamin B_6 (Scheme 8).

The proposed association of glycolaldehyde with the biosynthesis of vitamin B_6 was subsequently tested by Tani and Dempsey by administering [^{14}C]glycolaldehyde to a Group I mutant of E. coli WG3.[87] The tracer experiments with labelled glycolaldehyde demonstrated that radioactivity from this substrate was incorporated into pyridoxal. Chemical degradation of these radioactive samples revealed that glycolaldehyde serves as a specific precursor for the C_2 unit, C-5' and C-5.[80]

On the basis of these observations, Tani suggested



(22)

(or Derivative)

X and Y are unknown substances

Scheme (8): Glycolaldehyde Implicated as a Precursor of Vitamin B_6.[51]

that glycolaldehyde was a compound necessary for the biosynthesis of pyridoxal. Furthermore, the observation that the pyridoxal requirement of mutant WG3 is satisfied by glycolaldehyde led to the inference that glycolaldehyde responsive mutants are lacking an enzyme required for glycolaldehyde biosynthesis. When mutant WG3 was tested with glycolate as a nutritional substitute for glycolaldehyde, no growth was observed. Tani proposed that the mutant was deficient in the enzyme, glycolaldehyde dehydrogenase.[130] Hence, it was suggested that glycolaldehyde was formed by reduction of glycolate (26) by glycolaldehyde dehydrogenase (Fig. 10).

Tani and coworkers conducted an extensive study to test this suggestion. They examined the wild-type organism for other enzymes known to catalyse the formation of glycolaldehyde, and compared the results with those obtained with mutant WG3. Both organisms exhibited what was thought to be ethanolamine oxidase activity. However, since ethanolamine formation by the decarboxylation of serine could not be demonstrated, it was assumed that no ethanolamine was present in the organisms and that the observed oxidase activity was attributed to a non-specific monoamine oxidase. The only significant difference between the two strains which was observed was in glycolaldehyde dehydrogenase activity.[131]

Glycolaldehyde dehydrogenase was isolated from the wild-type organism and partially purified. It was composed

of three isozymes. Glycolaldehyde dehydrogenase isolated from mutant WG3, however, was found to possess only one of these isozymes.[131-134] The relationship of this enzyme to vitamin B_6 biosynthesis is not yet certain.

In an independent investigation, Shimizu and Dempsey concluded from genetic studies, that glycolaldehyde dehydrogenase had no relationship with the pdxB gene (pdx genes in E. coli are coded for the enzymes associated with pyridoxal biosynthesis). They observed that the pyridoxal requirement of the other pdxB mutants (of which WG3 is one) could not be satisfied even though glycolaldehyde dehydrogenase activity was detected.[135] From these observations, Dempsey concluded that a secondary mutation must have occurred in mutant WG3 and that pyridoxal biosynthesis was not dependent on glycolaldehyde dehydrogenase activity. The nature of the secondary mutation is not yet known.[39]

The utilization of glycolaldehyde, in mutant WG3, for the biosynthesis of pyridoxal remains to be explained. If glycolaldehyde were a true intermediate on route to pyridoxal, it would be expected to be incorporated into the carbon skeleton of pyridoxol produced by mutant WG2, a mutant whose metabolic block occurs in the final stages of pyridoxal biosynthesis.

In mutant WG2, the entire carbon skeleton of pyridoxol is derived from the carbon atoms of glycerol.[75-77,79,80] A tracer experiment with [^{13}C]glycerol followed by ^{13}C-NMR analysis of the isolated pyridoxol[79] confirmed the results

obtained from experiments with [^{14}C]glycerol followed by chemical degradation of the labelled product.[77,80] These experiments provide unequivocal evidence that five carbon atoms of pyridoxol ($C_{2'}$, C_3, $C_{4'}$, $C_{5'}$, and C_6) are derived from the primary carbons of glycerol whereas the remaining three carbon atoms of pyridoxol (C_2, C_4, and C_5) are derived from the secondary carbon of glycerol. Tracer experiments with [1-^{14}C]- and [6-^{14}C]-glucose[77,78] provide further information regarding the mode of incorporation of triose units into pyridoxol. On the basis of these observations, a hypothesis for the biosynthesis of pyridoxol in E. coli B, which was consistent with the tracer evidence, was advanced (Scheme (2)).

The non-random incorporation of [^{14}C]glycolaldehyde into pyridoxal by mutant WG3, yielding labelled carbon atoms 5 and 5' could not be explained by the original hypothesis. In mutant WG2, these sites are supplied by the carbon atoms of glycerol.

It was the objective of this investigation to clarify this apparent contradiction and to determine whether glycolaldehyde plays an obligatory role in the biosynthesis of vitamin B_6.

3.1.2 Results

The mutants of Escherichia coli, WG2 and WG3, used in this study require pyridoxal for growth. Mutant WG2 excretes pyridoxol into the culture medium.[41] In mutant WG3, glycol-

aldehyde can satisfy the pyridoxal requirement for sustained growth.[51] Radioactive samples of pyridoxol were isolated by carrier dilution from the culture medium in the experiments with mutant WG2 (Experiments 2, 4, 5, 6 and 7). In the experiments with WG3 (Experiments 1 and 3), radioactive pyridoxal was isolated similarly from the cells and converted into pyridoxol.[90]

Samples of [2-^{14}C]glycolaldehyde (prepared by the oxidative decarboxylation of DL-[3-^{14}C]serine (Table 3)) in the presence of inactive glycerol (Experiments 1 and 2) were administered to cultures of mutants WG3 and WG2. Similarly, the reciprocal experiments, in which samples of [2-^{14}C]-glycerol were administered in the presence of non-radioactive glycolaldehyde (Experiments 3 and 4), were carried out with both mutants. Samples of [2-^{14}C]glycolaldehyde and [2-^{14}C]-glycerol in each other's presence (Experiments 5 and 6), and a sample of [2-^{3}H,2-^{14}C]glycolaldehyde (Experiment 7) were administered to mutant WG2. The details of these experiments are summarized in Table 2.

The samples of radioactive pyridoxol isolated from experiments 1-6 were converted to appropriate derivatives and then partially degraded by established reaction sequences[77,80] to determine the distribution of radioactivity. The results of the radioactivity assays for each of the degradation products isolated for each of the experiments (1-6) are shown in

Table 2: INCORPORATION OF LABELLED SUBSTRATES INTO PYRIDOXOL

Expt	E. coli B strain	Labelled substrate: Labelled substrate	Nominal specific activity (mCi/mmol)	Nominal total activity (μCi)	Initial glycerol content: Total volume (L)	Weight (g) (including weight of radioactive sample if any)	Concentration (g/L)	Concentration (mmol/L)	Initial glycolaldehyde content: Weight (μg) (including weight of radioactive sample if any)	Concentration (μg/L)	Concentration (nmole/L)	Pyridoxol: Inactive pyridoxol added before workup (mg)	Specific activity of isolated pyridoxol·HCl (dpm per nmole x 10^{-4})
1[a]	WG3[c]	[2-^{14}C]glycolaldehyde	0.40	360	2	4	2	21.7	54	27	0.45	500[b]	1.10 ± 0.04[d]
		[2-^{14}C]glycolaldehyde[d]	0.21	137	2	2	1	10.9	39	20	0.33	117	2.10 ± 0.01
3	WG3	[2-^{14}C]glycerol[e]	(40)0.023	500	1	2	2	21.7	60	60	1	137	1.17 ± 0.04
4	WG2	[2-^{14}C]glycerol[f]	(15.3)0.023	259	1	1	1	10.9	600	600	10	210	3.41 ± 0.13
5	WG2	[2-^{14}C]glycolaldehyde[d]	0.035	345	2	0.92	0.46	5	600	300	5	1.50	1.77 ± 0.04
		[2-^{14}C]glycerol[f]	(16.6)0.036	369									
6	WG2	[2-^{14}C]glycolaldehyde[d]	1.28	198	2	2	1	10.9	9	4.5	0.075	287	4.[illegible] ± 0.02
		[2-^{14}C]glycerol[e]	(10)0.012	250									
7	WG2	[2-^{3}H]glycolaldehyde[d]	0.53	816	1	1	1	10.9	93	93	1.6	36	0.[illegible] ± 0.2 (^{3}H)
		[2-^{14}C]glycolaldehyde[d]	0.045	70									

(a) [illegible] from Experiment III-B
(b) See References 87 and 90
(c) See Reference 69 Table 1
(d) See Table 3
(e) Aerobic Scale
(f) Anaerobic Scale

Table 3 : SYNTHESIS OF LABELLED SAMPLES OF GLYCOLALDEHYDE

DL-Serine						Glycolaldehyde						
						Labelled glycolaldehyde					Sample used in tracer experiment	
Expt. No	Label (source)	Nominal specific activity (mCi per mmol)	Nominal total activity (μCi)	Inactive DL-serine added (mg)	Nominal specific activity of diluted serine (mCi per mmol)	Position of Label	Total activity (μCi)	Yield %	Weight (mg)	Inactive glycol-aldehyde added (mg)	Total weight (mg)	Nominal specific activity (mCi per mmol)
2	3-^{14}C[a]	46.9	250	125	0.21	2-^{14}C	137	55	39	0	39	0.21
5	3-^{14}C[a]	48.6	500	140	0.37	2-^{14}C	345	69	56	544	600	0.035
6	3-^{14}C[a]	47.7	1000	80	1.28	2-^{14}C	198	20	9	0	9	1.28
7	3-^{3}H[b]	8900	1000	140	0.75	2-^{3}H	816	82	65	0	} 93	0.53
	3-^{14}C[a]	46.9	100	70	0.15	2-^{14}C	70	70	28			0.045

[a] New England Nuclear

[b] Amersham/Searle

Tables (4) to (6).

In the samples of pyridoxol from the experiments with [2-^{14}C]glycolaldehyde in the presence of unlabelled glycerol (Experiments 1 and 2) (Table (4)), within experimental error, all activity resided in the C_2 unit C-5',-5 (isolated as N-phthaloylglycine), and more particularly, at C-5' (isolated as benzoic acid) (Experiment 1). The C_2 unit, C-2,-2' (isolated as N-acetyl-α-naphthylamine) was essentially devoid of activity. Furthermore, pyridoxol from a culture of mutant WG2, to which intermolecularly doubly labelled [2-^{3}H,2-^{14}C]-glycolaldehyde (^{3}H/^{14}C ratio of the dimedone derivative was 9.8 ± 0.1) had been administered (Experiment 7), showed a ^{3}H/^{14}C ratio of 9.8 ± 0.6, indicating complete retention of ^{3}H, relative to ^{14}C.

Each of the radioactive pyridoxol samples, isolated from the experiments with [2-^{14}C]glycerol in the presence of unlabelled glycolaldehyde (Experiments 3 and 4) (Table (5)), was found to contain approximately one-third of the total activity in the C_2 fragment C-2,-2' (Kuhn-Roth acetate) isolated as the N-acetyl-α-naphthylamine. Furthermore, all of this activity was present at C_2 (Kuhn-Roth acetate minus Schmidt methylamine). Whereas the C_2 unit C-5',-5 (N-phthaloylglycine), of the sample from mutant WG2 (Experiment 4) contained one-third of the total activity, this C_2 unit in the pyridoxol sample isolated from mutant WG3 (Experiment 3) was essentially devoid of activity.

In the experiments with mutant WG2, in which [2-^{14}C]-

Table 4 : INCORPORATION INTO PYRIDOXOL OF [2-^{14}C]GLYCOLALDEHYDE IN THE PRESENCE OF NONRADIOACTIVE GLYCEROL

Expt. No.		1						2	
E. Coli B Strain		WG3						WG2	
Products	C-Atoms of Pyridoxol	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]
Pyridoxol HCl(1)	All	1.18±0.04[c]	100±3	0.69±0.02[c]	100±3			2.10±0.01	100±1
3,4'-O-Isopropylidenepyridoxol(10)	All	1.20±0.04	102±4	0.70±0.02	101±4	0.56±0.02[d]	100±4	2.12±0.02	101±1
3,4'-O-Isopropylidene-5'-N-phthaloyliso pyridoxamine(20)	All							2.09±0.02	100±1
N-Phthaloylglycine(21)	5',5					0.55±0.03	99±6	2.01±0.01	96±1
Benzoic acid(18)	5'	1.11±0.02	94±3						
Kuhn-Roth acetate (as N-acetyl-α-naphthylamine(12))	2',2			0.01±0.003	2±0.4			0.06±0.01	3±1

[a] Specific activity (dpm per mmole) x 10^{-4}.

[b] Relative specific activity (per cent)(pyridoxol = 100)

[c] Quoted from Ref. 80;Table 1

[d] Obtained from isopropylidenepyridoxol, SA 1.20(±0.04)x 10^4 dpm per mmol, by dilution with inactive carrier

Table 5: INCORPORATION INTO PYRIDOXOL OF [2-^{14}C]GLYCEROL IN THE PRESENCE OF NONRADIOACTIVE GLYCOLALDEHYDE

Expt. No		3		4	
E. coli B Strain		WG 3		WG 2	
Products	C-Atoms of pyridoxol	SA[a]	RSA[b]	SA[a]	RSA[b]
Pyridoxol HCl(1)	All	1.16±0.02	100±1	3.38±0.06	100±2
3,4'-O-Isopropylidenepyridoxol(10)	All			3.37±0.04	100±2
N-Phthaloylglycine(21)	5',5	0.01±0.01	1±0.2	1.08±0.02	32±1
Kuhn-Roth acetate (as N-acetyl-α-naphthylamine(12))	2',2	0.39±0.01	34±1	1.07±0.02	32±1
Schmidt Methylamine (as 1-methyl-3-phenyl-thiourea(15))	2'	0.004±0.006	0.4±0.5		

[a] Specific activity (dpm per mmol)x10^{-4}

[b] Relative specific activity (per cent) (pyridoxol HCl = 100)

Table 6: INCORPORATION INTO PYRIDOXOL OF [2-^{14}C]GLYCOLALDEHYDE AND [2-^{14}C]GLYCEROL IN EACH OTHER'S PRESENCE IN MUTANT WG2

Expt. No		5		6	
Precursors:		Concentration (mmol per l)	Specific Activity (mCi per mmol)	Concentration (mmol per l)	Specific Activity (mCi per mmol)
[2-^{14}C]Glycolaldehyde		5	0.035	0.08	1.28
[2-^{14}C]Glycerol		5	0.036	10.9	0.012
Products	C-Atoms of Pyridoxol	SA[a]	RSA[b]	SA[a]	RSA[b]
Pyridoxol HCl(1)	All	2.70±0.04	100±2	4.00±0.02	100±1
3,4'-O-Isopropylidenepyridoxol(10)	All	2.68±0.02	99±2	3.99±0.03	100±1
3,4'-O-Isopropylidene-5'-N-phthaloylisopyridoxamine(20)	All	2.56±0.04	95±2	3.98±0.03	99±1
3,4'-O-Isopropylidene-5'-phenylpyridoxol(17)	All	2.65±0.02	98±2	3.97±0.03	99±1
N-Phthaloylglycine(21)	5',5	0.93±0.02	34±1	1.83±0.02	46±1
Benzoic acid (18)	5'	0.09±0.01	3±1	0.88±0.01	22±0.3
Kuhn-Roth acetate (as N-acetyl-α-naphthylamine)(12)	2',2	0.85±0.01	32±1	0.95±0.02	24±1

[a]SA = Specific activity (dpm per mmole) x 10^{-4}

[b]RSA = Relative specific activity (per cent)(pyridoxol HCl = 100)

glycolaldehyde and [2-^{14}C]glycerol were administered in each other's presence (Experiments 5 and 6) (Table (6)), the pyridoxol samples were found to contain activity in both the C_2 units, C-5,-5' (N-phthaloylglycine) and C-2,-2' (Kuhn-Roth acetate) in the two cases. However, the distribution of label within the two samples differed. In the experiment (Experiment 5) in which the two tracers were administered in equimolar concentrations and possessing the same specific activity, the resulting pyridoxol sample was found to contain approximately one-third of its total radioactivity in each of the C_2 units, C-2,-2' and C-5,-5'. Within the C_2 unit, C-5,-5', very little of the activity was present at C-5' (benzoic acid). In the experiment (Experiment 6) in which the specific activity of [2-^{14}C]glycolaldehyde far exceeded that of [2-^{14}C]glycerol, and the glycerol concentration was much higher than that of glycolaldehyde, an entirely different distribution of label was observed within pyridoxol. The C_2 unit, C-2,-2', accounted for only one-quarter of the total activity, whereas the C_2 unit, C-5,-5', contained approximately one-half of the total radioactivity of the intact pyridoxol. Within the C_2 unit, C-5,-5', the activity was approximately equally distributed between C-5' (benzoic acid) and C-5 (N-phthaloylglycine minus benzoic acid).

3.1.3 Discussion

The mode of incorporation of radioactivity from a number of labelled substrates into the carbon skeleton of pyridoxol has

been examined in two pyridoxal-requiring mutant strains of E. coli B, WG2 and WG3. The mutants were characterized by W.B. Dempsey.[41,51,53] Evidence, acquired from genetic studies or tracer experiments with the two mutants, concerning vitamin B_6 biosynthesis will be briefly reviewed.

3.1.3.1 Mutant WG2

E. coli WG2 is among several pyridoxineless mutants belonging to the Group IV (pdxH, Fig. (2)) category.[136] These mutants are blocked in the ultimate or penultimate step in pyridoxal-5'-phosphate biosynthesis and lack pyridoxol phosphate oxidase activity, hence these mutants require pyridoxal or pyridoxamine for growth.[41,51] When deprived of pyridoxal, the Group IV mutants will synthesise pyridoxol at an enhanced rate and hence large amounts of both pyridoxol and its -5' phosphate accumulate in the culture fluid, under certain conditions. These mutants, and particularly WG2, would best represent the wild type organism.

Tracer experiments with this mutant have provided the evidence for the currently accepted[137] hypothesis for the biogenesis of pyridoxol. In this mutant, the carbon skeleton of pyridoxol is derived from the carbon atoms of glycerol.[77,79] It was shown by chemical degradation of ^{14}C-labelled samples, that C-2 of glycerol yields C-2, C-4 and C-5 of pyridoxol. Each of these three sites contains one-third of the label of

the intact pyridoxol[75-77] (Scheme (9), Fig. (A1)). In a ^{13}C NMR study of a ^{13}C labelled sample of pyridoxol, it was shown that C-1,-3 of glycerol yields C-2', C-3, C-4', C-5' and C-6 of pyridoxol and each of these five sites contains one-fifth of the label[75-77,79] (Scheme (9), Fig. (A2)). The mode of incorporation of radioactivity from [1-^{14}C]- and [6-^{14}C]glucose[77,78] and from [2-^{14}C]- and [3-^{14}C]pyruvate[75-77] extends the evidence that pyridoxol in WG2 is biosynthetically derived from trioses related to glycerol and generated by glycolysis. The evidence shows conclusively that in E. coli B mutant WG2, the pyridoxol skeleton can be derived entirely and specifically from glycerol.

3.1.3.2 Mutant WG3

E. coli mutant WG3, is another pyridoxineless mutant and is characterized as a Group I (pdx B, Fig. (2)) mutant.[51] In this mutant, the pyridoxal requirement can be satisfied by pyridoxol, pyridoxamine, or glycolaldehyde. This suggests that glycolaldehyde is implicated in the biosynthesis of pyridoxal. This idea was first advanced in 1959 by J.G. Morris[85,86] and coworkers. He had studied a number of pyridoxol-requiring mutants of E. coli. Two of these mutants grew in the absence of pyridoxol if glycolaldehyde was supplied together with either serine or glycine. Although recent genetic discoveries suggested that the original mutants were likely to contain more than one mutation[138] Morris' work prompted Dempsey to include

A

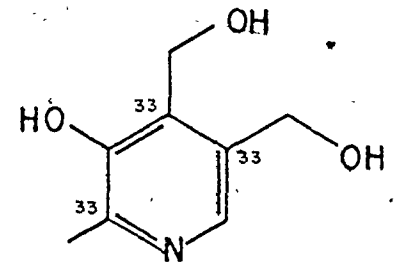


Fig. 1: [2-^{14}C]Glycerol[75-77]

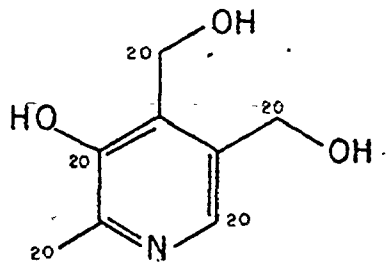


Fig. 2: [1-^{14}C]Glycerol[75-77]
[1,3-^{13}C]Glycerol[79]

B

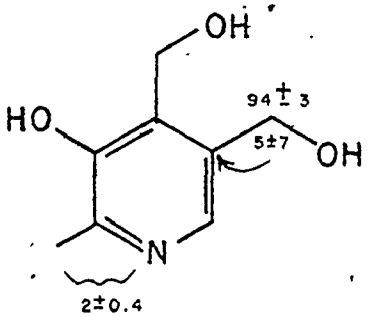


Fig. 3: Experiment 1, WG3

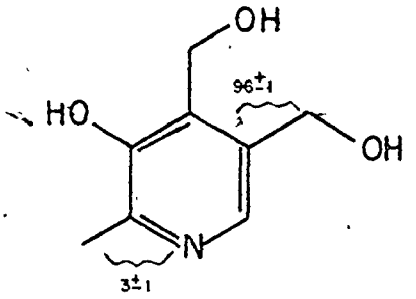


Fig. 4: Experiment 2, WG2

C

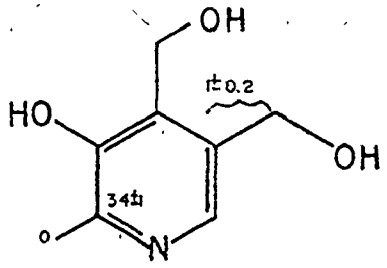


Fig. 5: Experiment 3, WG3

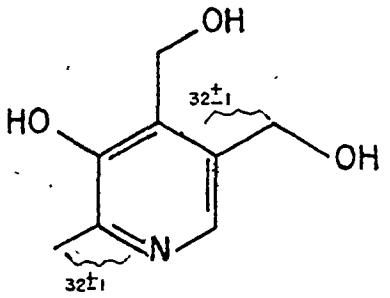


Fig. 6: Experiment 4, WG2

D

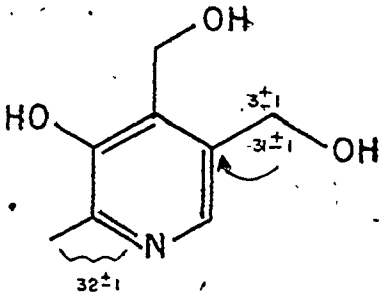


Fig. 7: Experiment 5, equimolar

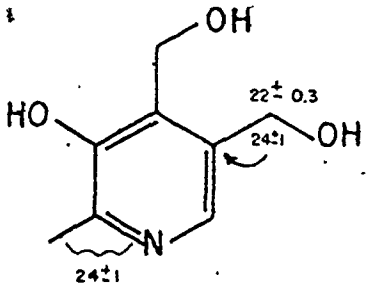


Fig. 8: Experiment 6, nonequimolar

Scheme 9: Distribution of Activity within Pyridoxol Derived from ^{14}C-labelled Substrates (Activity of Intact Pyridoxol = 100; Per cent of Total Activity at Indicated Carbon Atoms).

Continued.....

Scheme 9 (Continued)

A. Distribution of label from [1-^{14}C]- and [2-^{14}C]glycerol in pyridoxol from E. coli B, mutant WG2.

B. Incorporation of [2-^{14}C]glycolaldehyde in the presence of unlabelled glycerol.

C. Incorporation of [2-^{14}C]glycerol in the presence of unlabelled glycolaldehyde.

D. Incorporation of [2-^{14}C]glycolaldehyde plus [2-^{14}C]-glycerol in mutant WG2.

glycolaldehyde among the compounds which were tested in the course of the investigations which eventually led to the characterization of the Group I mutant, WG3, whose pyridoxal requirement is satisfied by pyridoxol and by glycolaldehyde. The metabolic block in mutant WG3 has been shown by genetic experiments, to be located at an early stage on the pathway to pyridoxal (Fig. 2).

A tracer experiment which suggests that glycolaldehyde was also implicated in pyridoxol biosynthesis in yeast was reported by Schroer and Frieden.[69] It was found that in the presence of glycolaldehyde in the culture medium, less radioactivity from [G-^{14}C]glucose was incorporated into pyridoxol than in the absence of glycolaldehyde, even though the quantity of pyridoxol present in the culture was unchanged.

More recently, the tracer experiments with labelled glycolaldehyde led to the observation that radioactivity from this substrate was incorporated into pyridoxal, from mutant WG3.[87] Controlled chemical degradation demonstrated that all the radioactivity was located at C-5' when [2-^{14}C]glycolaldehyde was the substrate and at C-5,-5' when [1,2-^{14}C]-glycolaldehyde was used in the incubation.[80] Based on these findings, it was considered that glycolaldehyde served as a specific precursor of the C_2 unit, C-5,-5'. The distribution of label within pyridoxol, derived from a sample of pyridoxal phosphate which was isolated from a culture of E. coli B

mutant WG3, which had been incubated with [2-^{14}C]glycolaldehyde (Experiment 1)[80] is shown in Scheme (9), Fig. (B3).

Not only is the distribution in pyridoxol of radioactivity derived from glycolaldehyde quite characteristic, but in addition, the incorporation of label from radioactive glycolaldehyde into C-5' and C-5 of pyridoxal, in mutant WG3, takes place without change in concentration of radioactivity, i.e., the pyridoxal which is formed has the same molar specific activity as the glycolaldehyde that was added to the culture medium.[87] It follows that the C_2 unit, C-5',-5 of pyridoxal is derived solely from the glycolaldehyde which is supplied, and neither the glycolaldehyde nor any of its metabolites on route to pyridoxal are diluted by endogenously formed material.

On this basis, the C_2 unit C-5',-5 of pyridoxal is generated specifically by glycolaldehyde in mutant WG3. The derivation of the other six carbon atoms of the pyridoxal formed in this mutant is unknown. In mutant WG2, on the other hand, the corresponding carbon atoms, C-5',-5 of pyridoxol originate from C-1 and C-2 of glycerol, respectively, as part of the C_3 unit C-5',-5,-6 which is derived from the intact C_3 chain of glycerol. The remainder of the pyridoxol molecule, formed in this mutant, is also derived from glycerol in a specific manner (Scheme (9), Figs. (A1) and (A2)).

3.1.3.3 Interpretation of Tracer Results in Mutants WG2 and WG3

That glycolaldehyde is a required nutrient for mutant WG3

and that its specific incorporation, without dilution, into carbons 5 and 5' of pyridoxol from this mutant, is incompatible with the tracer evidence obtained from the investigations with mutant WG2. Several models can be constructed to rationalize this apparent contradiction.

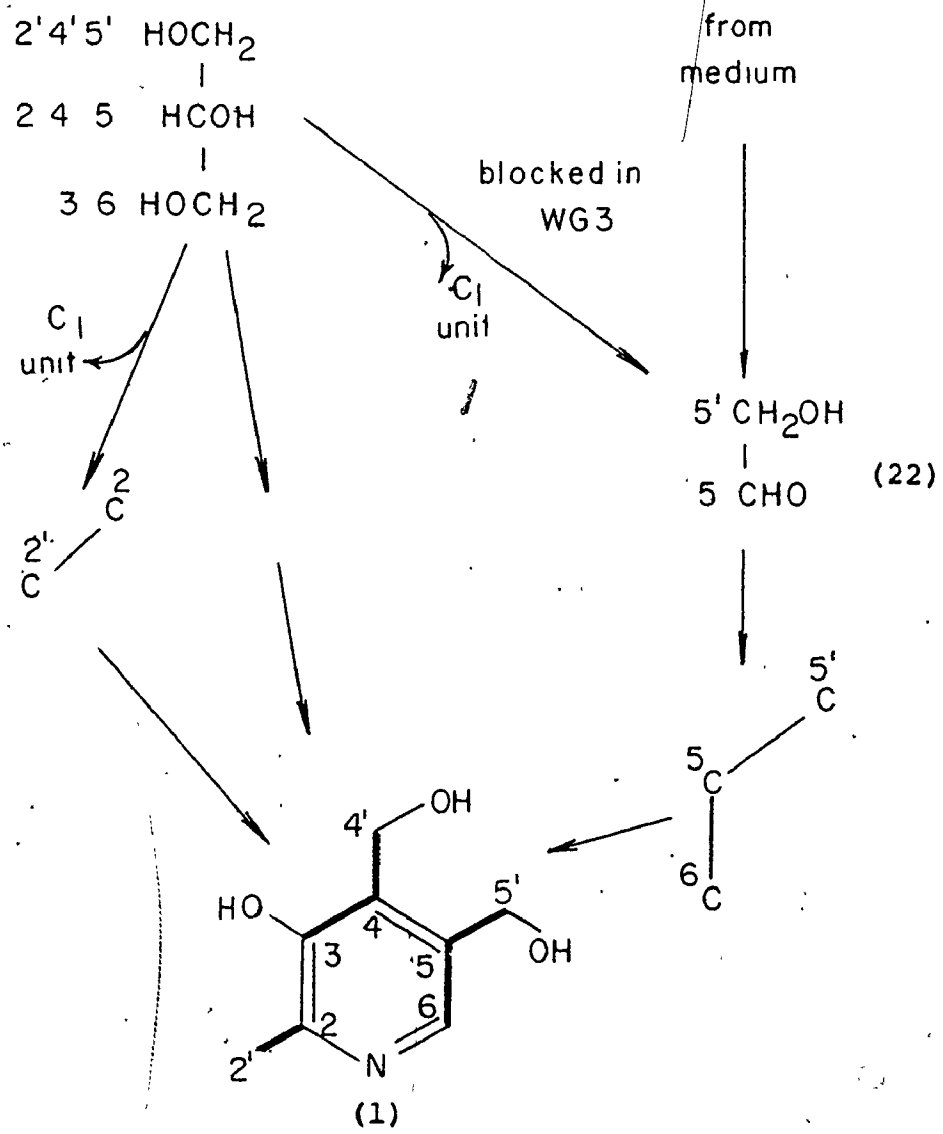
The first of these that must be considered places glycolaldehyde in the role as a compound which lies on the committed pathway to pyridoxal. The observation that in mutant WG3 label from glycolaldehyde is incorporated specifically and without dilution into carbons 5' and 5 clearly raises the possibility that glycolaldehyde is indeed an obligatory intermediate of pyridoxol biosynthesis not only in mutant WG3 but also in mutant WG2 and hence also in the wild type of E. coli B. Incorporation of label from glycolaldehyde into the C_2 unit C-5',-5 of pyridoxol would be a necessary prerequisite for further consideration of this model. It is now shown that label from [2-^{14}C]glycolaldehyde enters this fragment and no other site of pyridoxol in mutant WG2 (Experiment 2, Scheme (9), Fig. (B4)). Furthermore, an intermolecularly labelled sample of [2-^{3}H,2-^{14}C]glycolaldehyde is incorporated into pyridoxol without loss of tritium relative to ^{14}C (Experiment 7).

In the experiment with mutant WG3, radioactivity was incorporated into pyridoxal without dilution. In other words, the molar specific activity of the isolated pyridoxal was

identical with that of the administered glycolaldehyde,[87] whereas in the experiments with mutant WG2 (Experiments 2 and 7), the molar specific activity of the biosynthesized pyridoxol (approx. 0.006, 0.0005 mCi/mmole)[139] was lower by two orders of magnitude than that of the glycolaldehyde which had served as substrate (0.21, 0.045 mCi/mmole).

These findings are consistent with the model which assigns to glycolaldehyde the role of an obligatory intermediate: Since, in mutant WG2, not only C-1 and C-2 of glycolaldehyde but also C-2 and C-1 of glycerol[76,77] are incorporated into C-5,-5' (Scheme 9, Figs. A1 and A2), the model requires that glycolaldehyde is generated from glycerol by loss of one of its primary carbon atoms. In mutant WG3, which, in the absence of exogenous pyridoxal, requires glycolaldehyde for growth but cannot grow on glycerol alone, this conversion is blocked by the mutation and glycolaldehyde is incorporated into pyridoxal without dilution. In mutant WG2, on the other hand, this conversion takes place, by a hitherto unknown process, and since administered labelled glycolaldehyde is diluted by endogenously formed glycolaldehyde from glycerol, dilution of label is expected (Scheme (10)).

According to this model, it is predicted that label from [2-^{14}C]glycerol, when incubated with mutant WG3, should enter C-2, and C-4 of pyridoxal but not C-5, to yield a pyridoxal sample containing 50% at each of C-2 and C-4.



Scheme 10: Biogenesis of Pyridoxol, with Glycolaldehyde as an Obligatory Intermediate (now disproven).

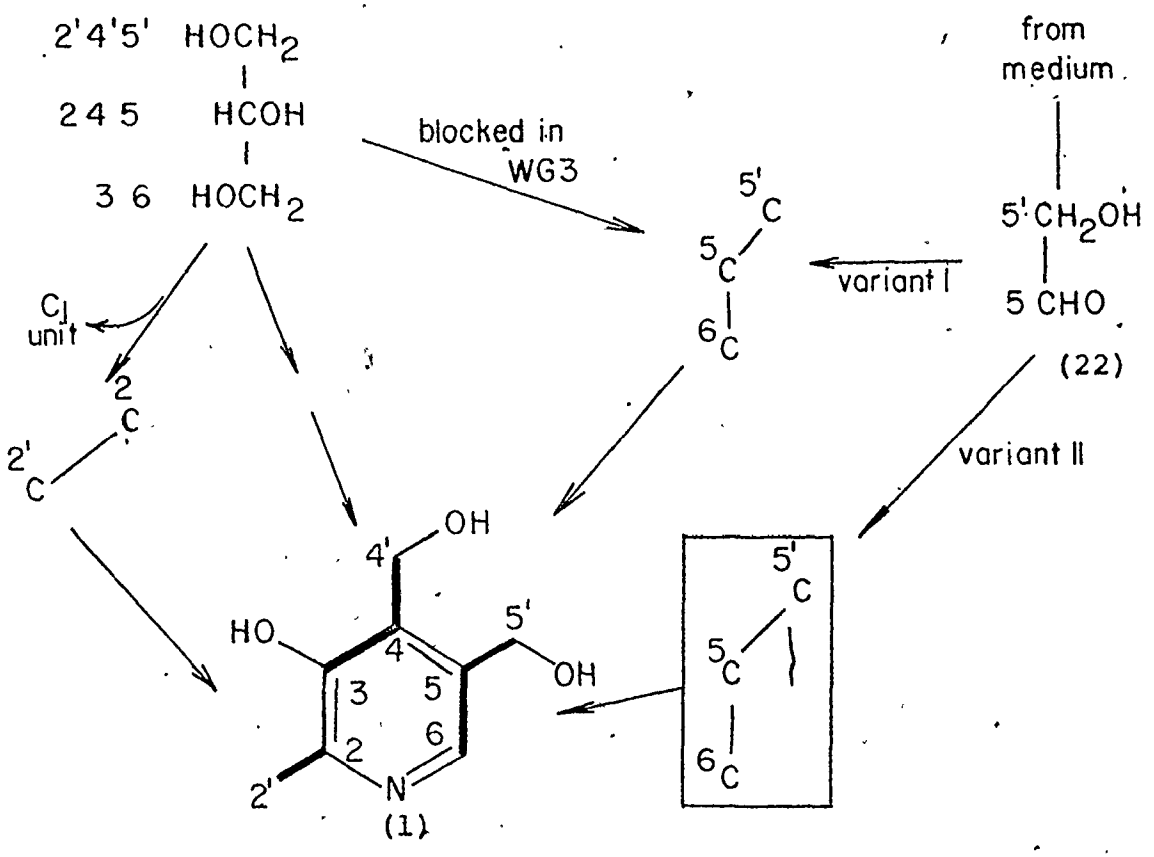
In mutant WG2, the model demands that the presence of non-radioactive glycolaldehyde in the culture medium should spare the incorporation of label from [2-^{14}C]glycerol into carbon-5 of pyridoxol, and should not affect its entry into C-2 and C-4. Hence, the isolated pyridoxol should contain more than 33% of its total activity at each of C-2 and C-4 (e.g., 40 to 45% at each) and less than 33% at C-5 (e.g., 20 to 10%). In the limiting case (i.e., 50% at each of C-2 and C-4), the distribution would be identical with that expected in WG3.

Experiments 3 and 4 were carried out to test the predictions based on this model. In WG3, activity from [2-^{14}C]-glycerol does not enter the C_2 unit, C-5',-5, a result which is consistent with the predictions based on the model. However, contrary to prediction, only one-third, rather than one-half of the activity was present at C-2 of pyridoxal (Scheme (9), Fig. (C5)). In mutant WG2, the predictions based on this model are also not fulfilled, since the presence of non-radioactive glycolaldehyde in the culture medium did not change the distribution within pyridoxol of activity derived from [2-^{14}C]glycerol (Scheme (9), Fig. (C6)) from that observed in the absence of glycolaldehyde (Scheme (9), Fig. (A1)).

The model which placed glycolaldehyde on route from glycerol to pyridoxol as an obligatory intermediate must, therefore, be rejected.

A second model, which is consistent with the available evidence is presented in Scheme (11). This model is based on the notion that there are two routes, a major one from glycerol, and a minor one from glycolaldehyde, leading to C-5',-5,-6 of pyridoxol in mutant WG2. In WG3, the major route from glycerol to the intermediate which gives rise to carbons 5',5,6 is blocked due to the mutation, so that the minor route from glycolaldehyde becomes the sole pathway when glycolaldehyde is supplied to a culture of WG3. Whether or not glycolaldehyde utilization for pyridoxal biosynthesis is normally operative in mutant WG2, and therefore also in the wild type, or whether glycolaldehyde induces an enzyme when it is supplied to the medium is a question that cannot be answered on the basis of available knowledge.

It is not yet possible to distinguish with certainty between two variants of the model, i.e., whether the major and minor routes converge at a common intermediate (Variant I), or whether two separate C_3 intermediates are formed from glycerol and from glycolaldehyde (Variant II) on route to the C_3 unit, C-5',-5,-6. Preliminary evidence favours the latter variant. Whereas in mutant WG2, carbon 6 of pyridoxol is derived from C-1 of glycerol[79] when the major pathway operates, the result of experiment 3 suggests, solely by inference, on the basis of incomplete recovery of label, that C-6 may be derived from C-2 of glycerol when the glycolaldehyde pathway operates. This inference is based on the hitherto unproven assumption that the remaining carbons, C-2,-2',-3,-4,-4' of



Scheme 11: Biogenesis of Pyridoxol: Two Independent Routes leading to the C_3 Unit, C-5',-5,-6.

pyridoxal in mutant WG3 are of similar origin as those of the pyridoxol in mutant WG2, i.e., that C-3, C-4' of pyridoxal are derived from C-1 of glycerol and that C-4 of pyridoxal is derived from C-2 of glycerol. Since it is now shown (Scheme (9), Fig. (C5)) that C-2', C-5 and C-5' of pyridoxal, derived from [2-^{14}C]glycerol, contain no radioactivity and that one-third of the label is located at C-2, it is assumed that the remaining activity is divided between C-4 and C-6. Since there was insufficient sample to carry out further degradations on the pyridoxol obtained from Experiment 3, to locate the remaining activity, the inference from this incomplete experiment requires experimental confirmation.

The relative contribution of the two sources of the C_3 unit in mutant WG2 to the biosynthesis of pyridoxol was examined by means of two experiments (Experiments 5 and 6) in which a mixture of [2-^{14}C]glycerol and [2-^{14}C]glycolaldehyde was administered to the culture medium. Since label from [2-^{14}C]-glycerol enters C-5 of pyridoxol but not C-5' (Scheme (9), Fig. (A1)), whereas label from [2-^{14}C]glycolaldehyde enters C-5' but not C-5 (Experiment 1, Scheme (9), Fig. (B3)), assay of activity at these two carbon atoms of pyridoxol derived from a mixture of the two labelled substrates serves as a diagnostic test of their relative importance as precursors, and thus of the relative importance of the two sources of the C_3 unit in mutant WG2.

When the two substrates were added in equimolar concentration and with equal specific activities (Experiment 5, Scheme (9), Fig. (D7)), glycolaldehyde contributed less than 10% of the C_3 unit, C-5',-5,-6, while glycerol contributed over 90%. The results of this experiment and of the earlier one (Experiment 4) in which the presence of equimolar non-radioactive glycolaldehyde did not detectably alter the distribution of label from [2-^{14}C]glycerol are in full agreement with each other. Predictably an entirely different distribution of label was observed in Experiment 6, in which the glycerol concentration in the medium greatly exceeded that of glycolaldehyde while the specific activity of [2-^{14}C]glycolaldehyde was much higher than that of [2-^{14}C]glycerol. Under these conditions, it was found that each substrate contributed approximately equal amounts of radioactivity to carbons 5 and 5' of pyridoxol (Scheme (9), Fig. (D8)). It is not surprising that approximately equal amounts of radioactivity should enter the product even though less than 1% of the pyridoxol is generated by the glycolaldehyde pathway. The molar specific activity of [2-^{14}C]glycolaldehyde was 106 times that of [2-^{14}C]-glycerol, which means that one in fifty molecules of glycolaldehyde present in the medium is labelled, whereas only one in 5300 molecules of glycerol carries radioactivity. Hence, 106 molecules of glycerol containing radioactivity must be converted into pyridoxol for every labelled glycolaldehyde molecule which enters the product.

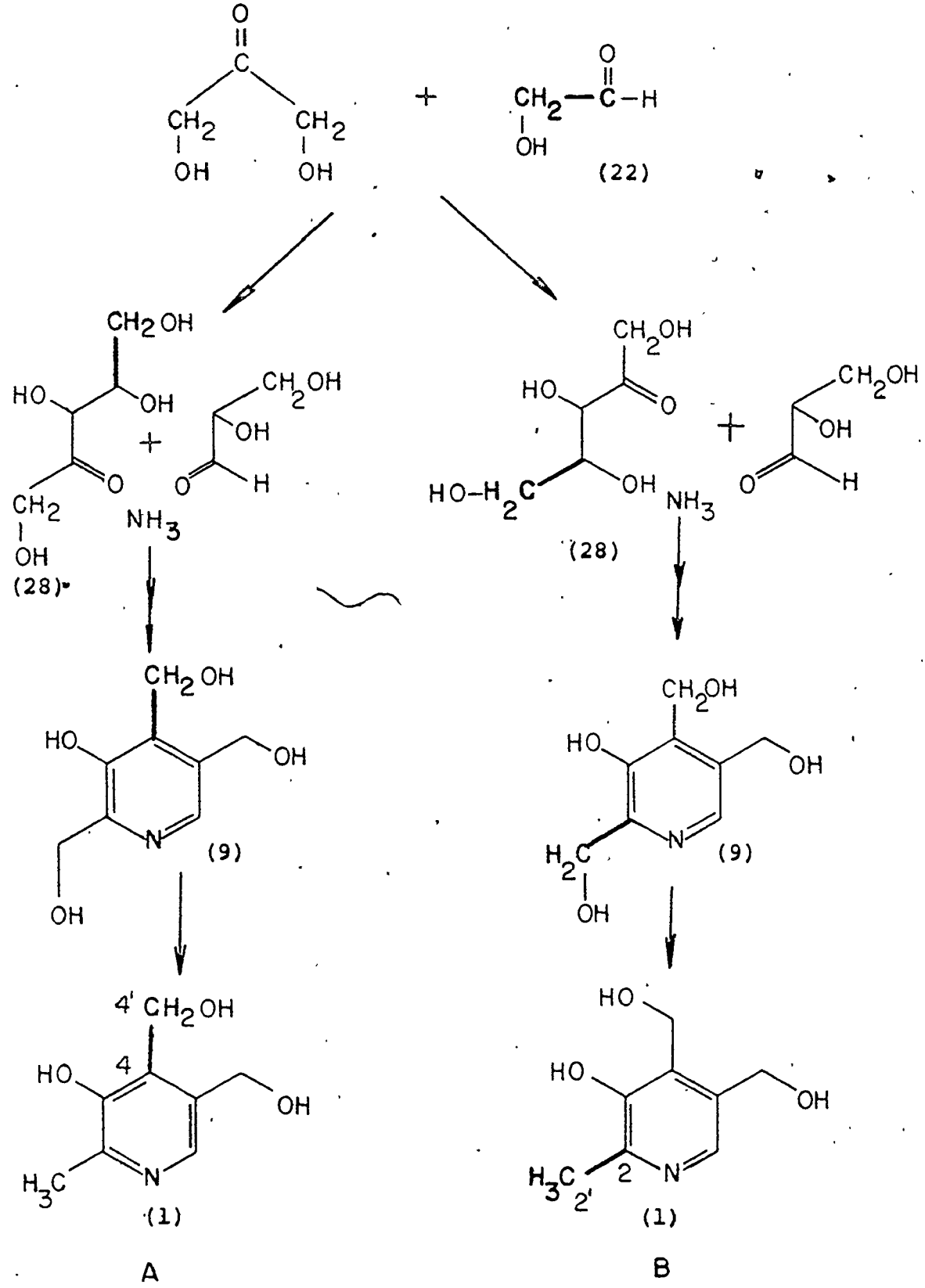
The mode of incorporation of label from glycolaldehyde in the presence of glycerol into pyridoxol in E. coli B mutant WG2 demonstrates that whereas glycolaldehyde does not lie on the major route from glycerol to pyridoxol, it can serve as donor of C-5',-5 of pyridoxol by way of a secondary minor route.

Recently Dempsey reported that a secondary mutation is required for glycolaldehyde utilization in WG3.[39] Based on genetic studies, this secondary mutation has been shown to be tightly linked to the isoleucine-valine biosynthetic gene in one group of mutants, to threonine genes in another group and to a glycolaldehyde dehydrogenase gene in a third group. A correlation between glycolaldehyde dehydrogenase activity and pyridoxal biosynthesis was assumed to exist.[130-134,140,141] No relationship between pyridoxal requirement and absence of glycolaldehyde dehydrogenase activity was observed in numerous pyridoxineless mutants tested.[135] Dempsey and Shimizu concluded that glycolaldehyde is not a normal precursor and must be converted to one by the enzyme whose specificity has been altered by the secondary mutation. This, however, does not explain the utilization of glycolaldehyde in pyridoxol biosynthesis by mutant WG2, whose only metabolic block occurs after the pyridine ring is formed, nor does it explain the stimulatory effect on pyridoxol production by starved pyridoxineless mutants of E. coli K12 when glycolaldehyde is present in the culture medium.[84]

3.1.4 Schemes Proposed for the Utilization of Glycolaldehyde in Pyridoxal Biosynthesis

Since the first report published by Morris in 1959, investigators studying the biosynthesis of pyridoxol have tried to explain how glycolaldehyde is utilized in pyridoxal-requiring strains of bacteria and yeast. It was not until Dempsey demonstrated that glycolaldehyde could satisfy the pyridoxal requirement of a pyridoxal auxotroph of Escherichia coli (mutant WG3),[51] that glycolaldehyde was seriously considered as a precursor leading to the vitamin.

Schroer and Frieden[69] proposed a scheme for the biosynthesis of vitamin B_6 involving glycolaldehyde, xylulose (28) and 2'-hydroxypyridoxine (9) (Scheme (12)). This scheme was based on the hypothesis proposed by Hill and Spenser[77] suggesting that 5-deoxyxylulose, formed by the condensation of dihydroxyacetone and acetaldehyde, provides carbons 4',4,3,2 and 2' and that glyceraldehyde-3-phosphate provides carbons 5', 5 and 6 of the vitamin. It was suggested that glycolaldehyde, in place of acetaldehyde,[142] underwent an aldolase reaction with a triose phosphate to form xylulose; reaction with glyceraldehyde phosphate and a nitrogen source would lead to 3-hydroxy-2,4,5-trihydroxymethylpyridine (9) as an intermediate which would then be converted to pyridoxol by the yeast Saccharomyces carlsbergensis.[68] It was not specified, however, which carbons of pyridoxol would originate from glycolaldehyde (Scheme (12), Fig. A or Fig. B).

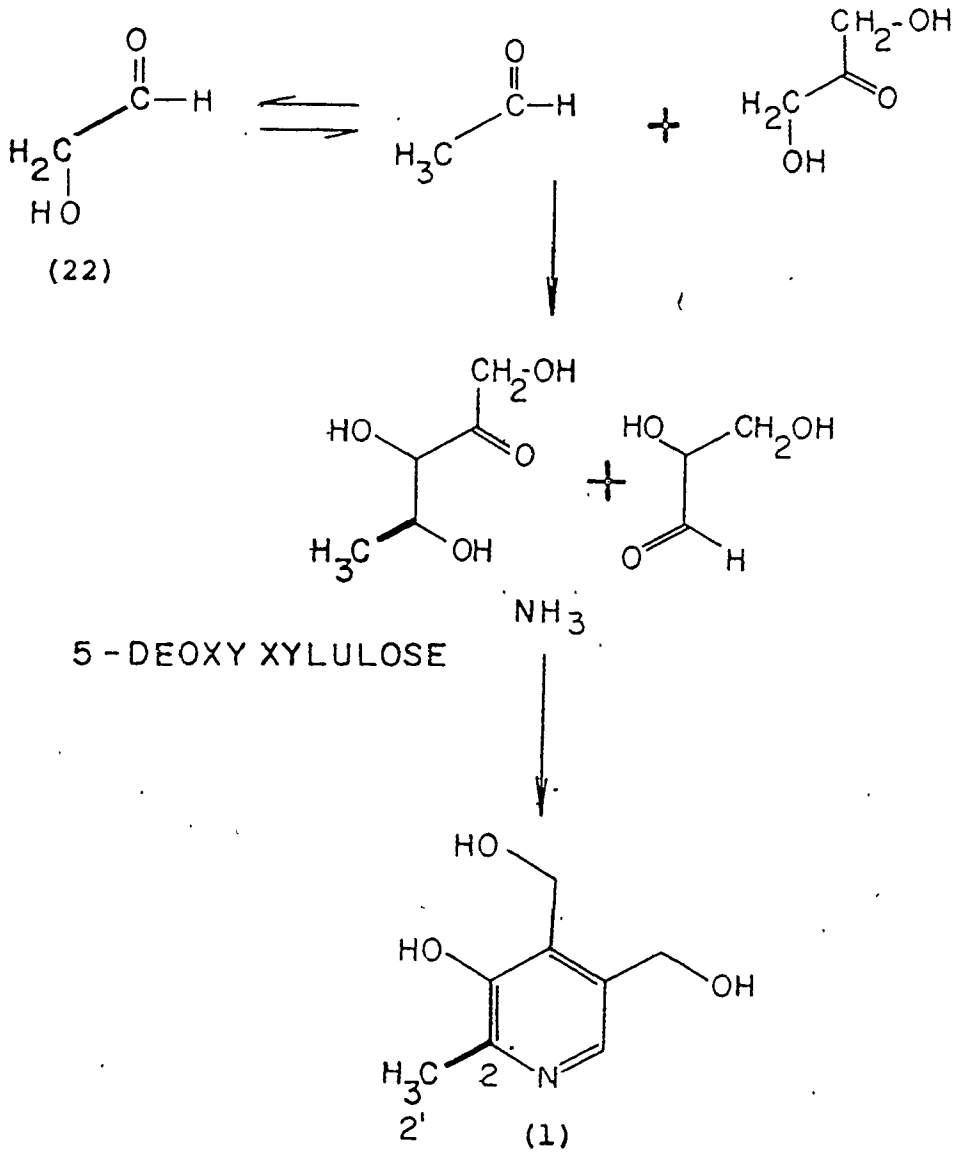


Scheme 12: Sequence Proposed for Glycolaldehyde Utilization in Saccharomyces fragilis for Pyridoxol Biosynthesis.[69]

Hill and Spenser had originally reported a scheme for the biosynthesis of pyridoxol which did not include glycolaldehyde.[77] It was proposed that the pyridoxol carbon skeleton was made up of two 3-carbon units related to glycerol and a C_2 unit also derivable from glycerol. Dempsey's tracer studies with [1,2-^{14}C]glycolaldehyde and [2-^{14}C]glycolaldehyde demonstrated that the two-carbon compound, glycolaldehyde, served as an efficient precursor of pyridoxal in mutant WG3. Hill and Spenser speculated that if glycolaldehyde were a precursor leading to pyridoxol, it might serve as the progenitor of the two-carbon unit C-2,-2' of the vitamin. This notion, however, was not consistent with the tracer evidence which supported a two-carbon unit at the oxidation level of acetaldehyde as a precursor of carbons 2 and 2' of pyridoxol; an intramolecular redox reaction had to be invoked[78] (Scheme (13)).

The possible intermediacy of "active glycolaldehyde" (27), derivable from pentuloses via transketolase reaction, in pyridoxol biosynthesis stimulated numerous mutant and tracer studies with pentoses. Dempsey and coworkers tested several pentoses (L-arabinose, D-arabinose, D-xylose, D-ribose) and pentuloses (D-xylulose, L-xylulose, D-ribulose-5-phosphate, D-xylulose-5-phosphate and 5-deoxy-D-xylulose) and demonstrated that none of these compounds were able to satisfy the pyridoxal requirement of *E. coli* B_6 auxotrophs.[78]

Hill and Spenser obtained the radioactive samples of pyridoxol derived from [^{14}C]glycolaldehyde which Dempsey had



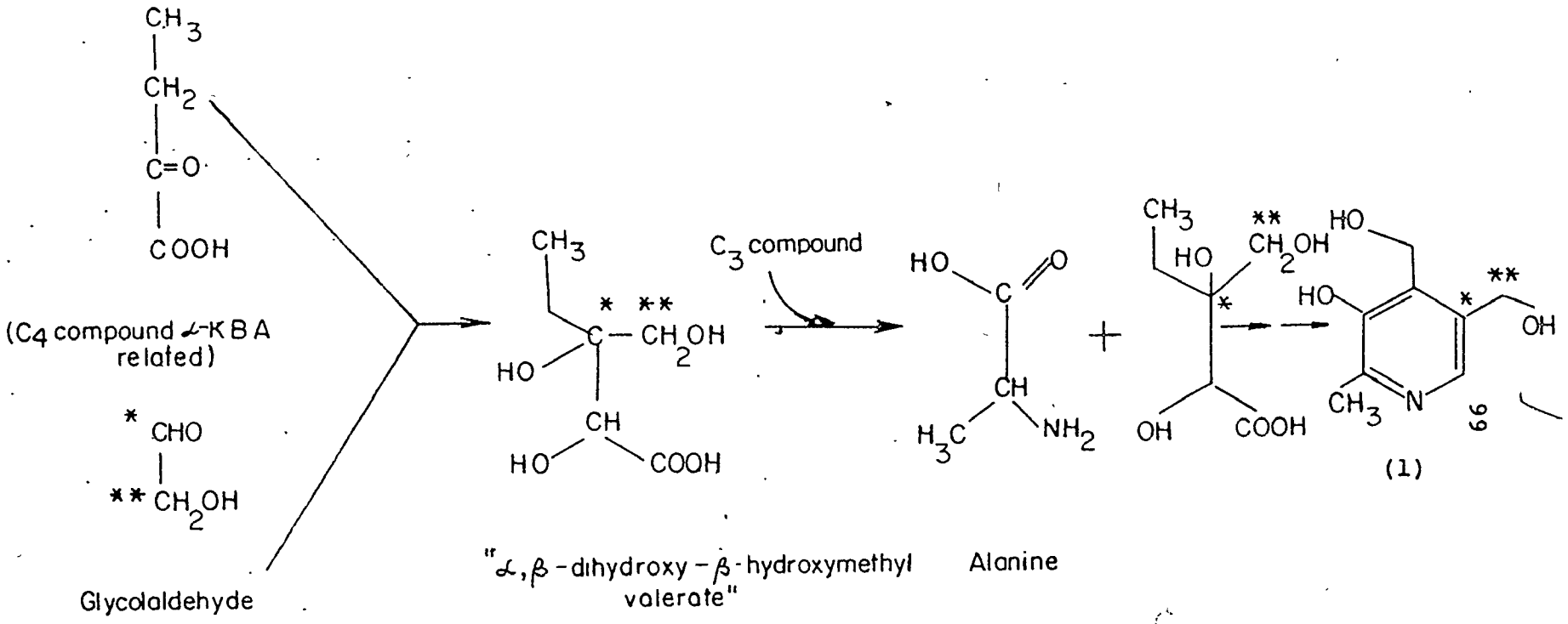
Scheme I3: The Possible Intermediacy of Glycolaldehyde as the Progenitor of C-2,-2' of Pyridoxol.[78]

isolated. It was shown by unambiguous chemical degradation of these pyridoxol samples that the carbon fragment C-2,-2' of pyridoxol was devoid of radioactivity derived from labelled glycolaldehyde.[78,80] All the radioactivity from these samples was shown to be confined to carbons 5 and 5' of the vitamin.

The non-random incorporation of glycolaldehyde into C-5,-5' of pyridoxal disproved the hypotheses advanced by Schroer and Frieden (Scheme (12)) and the prediction proposed by Hill and Spenser. The original hypothesis of Hill and Spenser did not include glycolaldehyde. This hypothesis has now been extended to include a minor pathway from glycolaldehyde leading to pyridoxol.

Two other schemes have recently been published. Tani and Dempsey[37,38,87] and Lingens[67] have advanced similar hypothetical sequences in an attempt to account for the utilization and non-random incorporation of glycolaldehyde into pyridoxal in bacteria. The proposed schemes involve biochemical transformations analogous to those involved in the biosynthesis of the branched chained amino acids, isoleucine and valine.

Tani's proposed sequence (Scheme (14)) for the biosynthesis of pyridoxal was based on the origin of glycolaldehyde in bacteria and its non-random incorporation into the pyridoxal carbon skeleton. Lingens' extensive studies with vitamin B_6 deficient mutants of Bacillus subtilis, provided

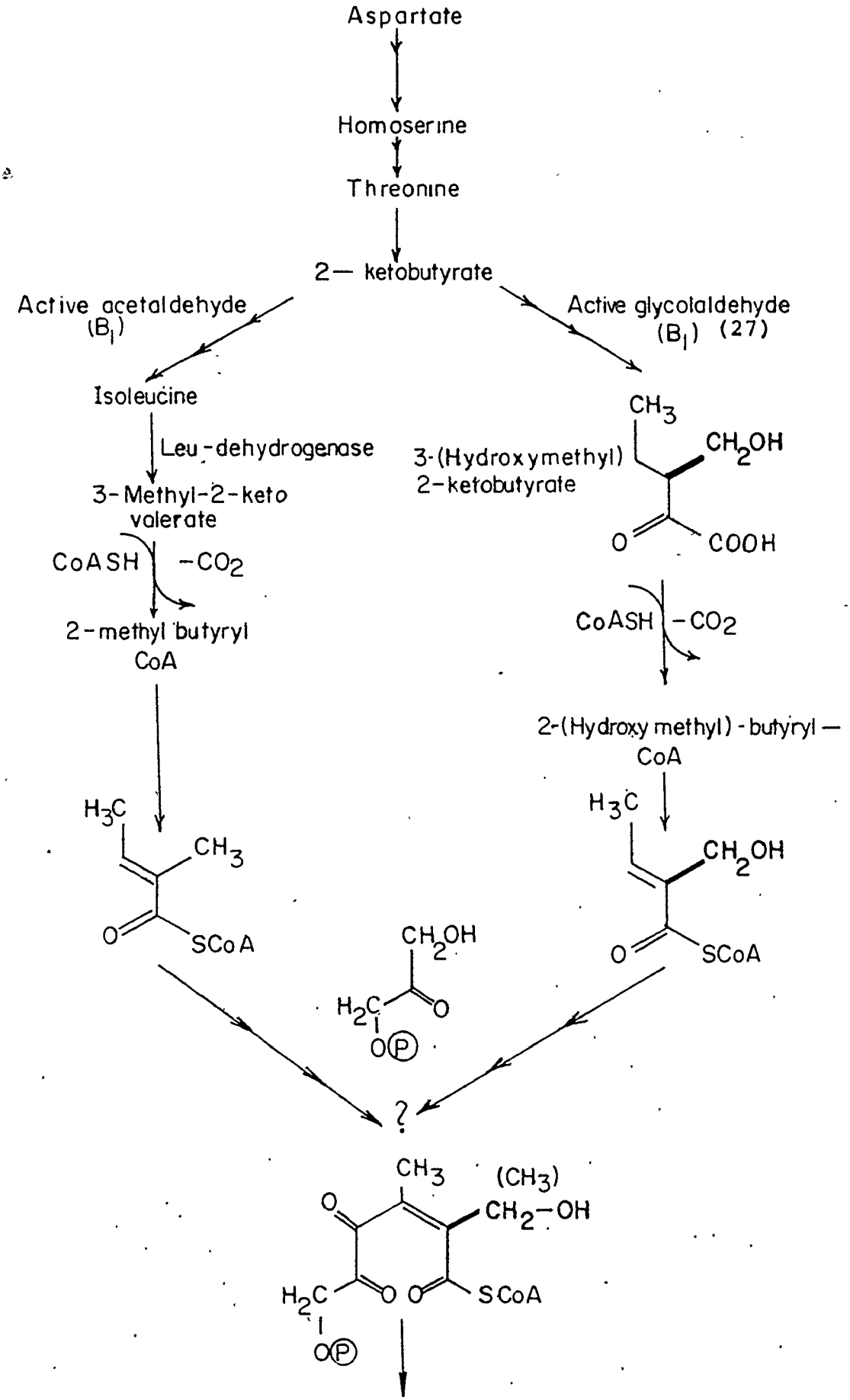


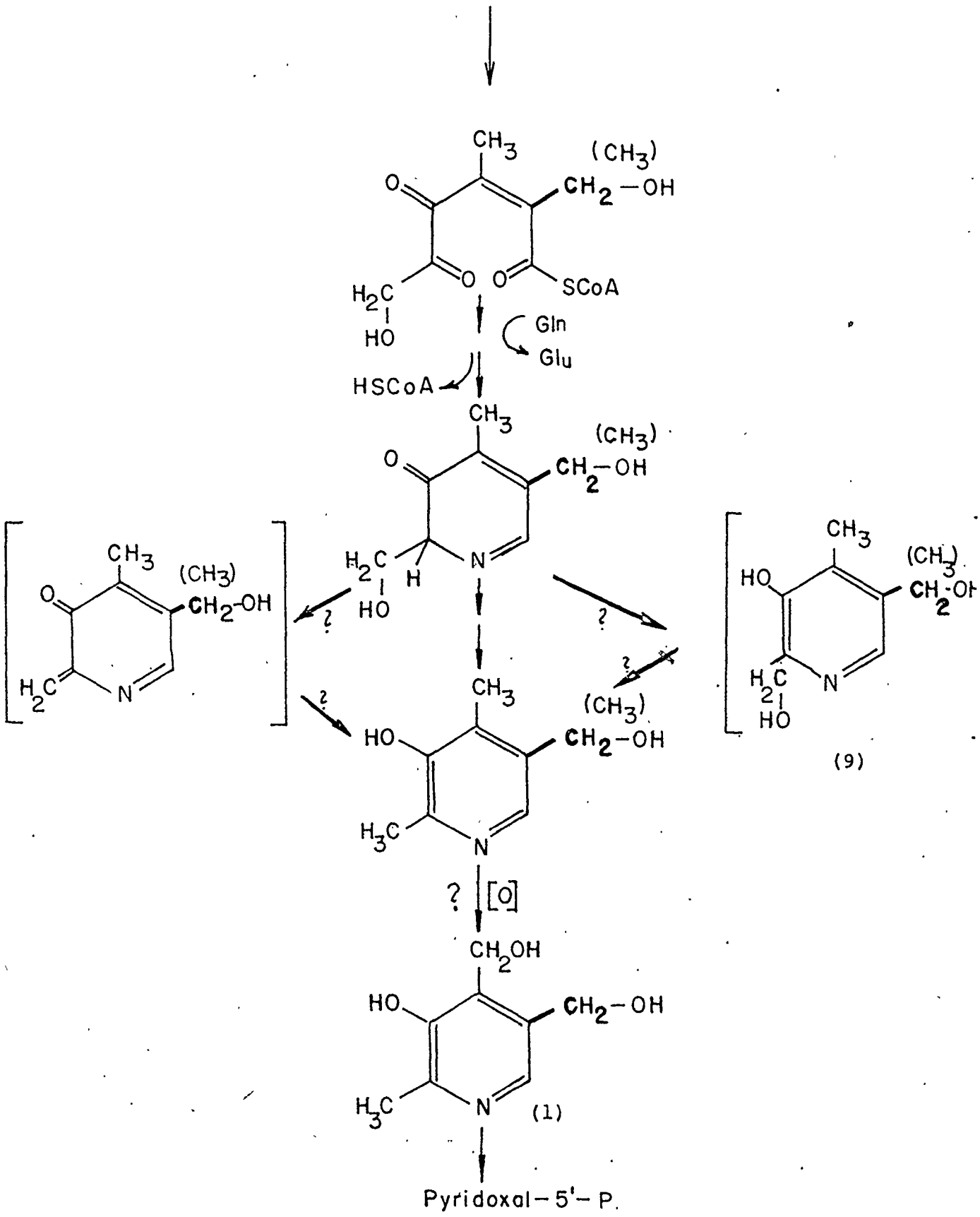
Scheme 14: Hypothetical Pathway for the Specific Incorporation of Glycolaldehyde into C-5,-5' of Pyridoxol.[128]

evidence which suggested a similar sequence (Scheme (15)) for the biosynthesis of the vitamin. The evidence for these hypothetical schemes is still meager, yet it is tempting to consider this sequence as a likely secondary minor route to pyridoxal. The evidence presented by Lingens and the mutant work of Dempsey will be briefly considered.

All mutants of B. subtilis, isolated by Lingens, were vitamin B_6 deficient and were able to grow when the culture medium was supplemented with either pyridoxal, pyridoxol, pyridoxamine and 4'-deoxypyridoxine. It was observed that neither nicotinic acid nor glycolaldehyde could support the growth of any of these mutants. In some mutants, the pyridoxine requirement was satisfied by isoleucine or by 3-methyl-2-ketovaleric acid. In other mutants, the addition of isoleucine in the culture medium stimulated growth of these cells.

It is interesting to note that the amino acids threonine and isoleucine are able to postpone the effects of pyridoxine starvation when they were added to starving pyridoxineless mutants of E. coli B, i.e., cell density, nucleic acid synthesis and protein synthesis were found to increase.[143] On analysis of the activities of the enzymes relevant to isoleucine-valine biosynthesis, Dempsey found transaminase B to be completely inactive in cell extracts of pyridoxine starved mutants.[39] He concluded that the threonine-isoleucine biosynthetic pathway was the most sensitive to pyridoxol starvation.


Aspartate
Homoserine
Threonine
2— ketobutyrate
Active acetaldehyde (B1)
Active glycolaldehyde (B1) (27)
Isoleucine
Leu-dehydrogenase
3-Methyl-2-keto valerate
CoASH
-CO2
2-methyl butyryl CoA
3-(Hydroxymethyl) 2-ketobutyrate
CoASH
-CO2
2-(Hydroxy methyl)-butyryl — CoA
SCoA
SCoA
?
(CH3)
SCoA



Scheme 15: Hypothetical Pathway for the Biosynthesis of Vitamin B_6 in Bacillus subtilis.[67]

That these intermediates or the enzymes associated in the isoleucine-valine biosynthetic pathway might be involved in the biosynthesis of pyridoxal was demonstrated in crossfeeding experiments carried out by Dempsey.[39] The culture fluid of a pyridoxineless mutant, WG25 (pdxG, Fig. (2)), was found to "feed" a threonine deaminaseless "Km" mutant, WG1285. The latter mutant was shown to require either pyridoxine or isoleucine for growth.[45] Hence, mutant WG25 must excrete either isoleucine or a metabolite which can be used by mutant WG1285 to synthesize pyridoxal. The "Km" mutant, WG1285, was found to crossfeed WG3, a pyridoxine auxotroph which can utilize glycolaldehyde for growth. Hence, mutant WG1285 must excrete either glycolaldehyde or a precursor required for pyridoxal biosynthesis which can be used by mutant WG3. Inconsistent with this trend of crossfeeding, however, was the observation that WG25 and WG3 do not crossfeed each other. These results do not lead to any firm conclusion regarding the involvement of the intermediates or enzymes along the branched-chain amino acid biosynthetic pathway, yet a relationship, which is not understood, does exist.[39]

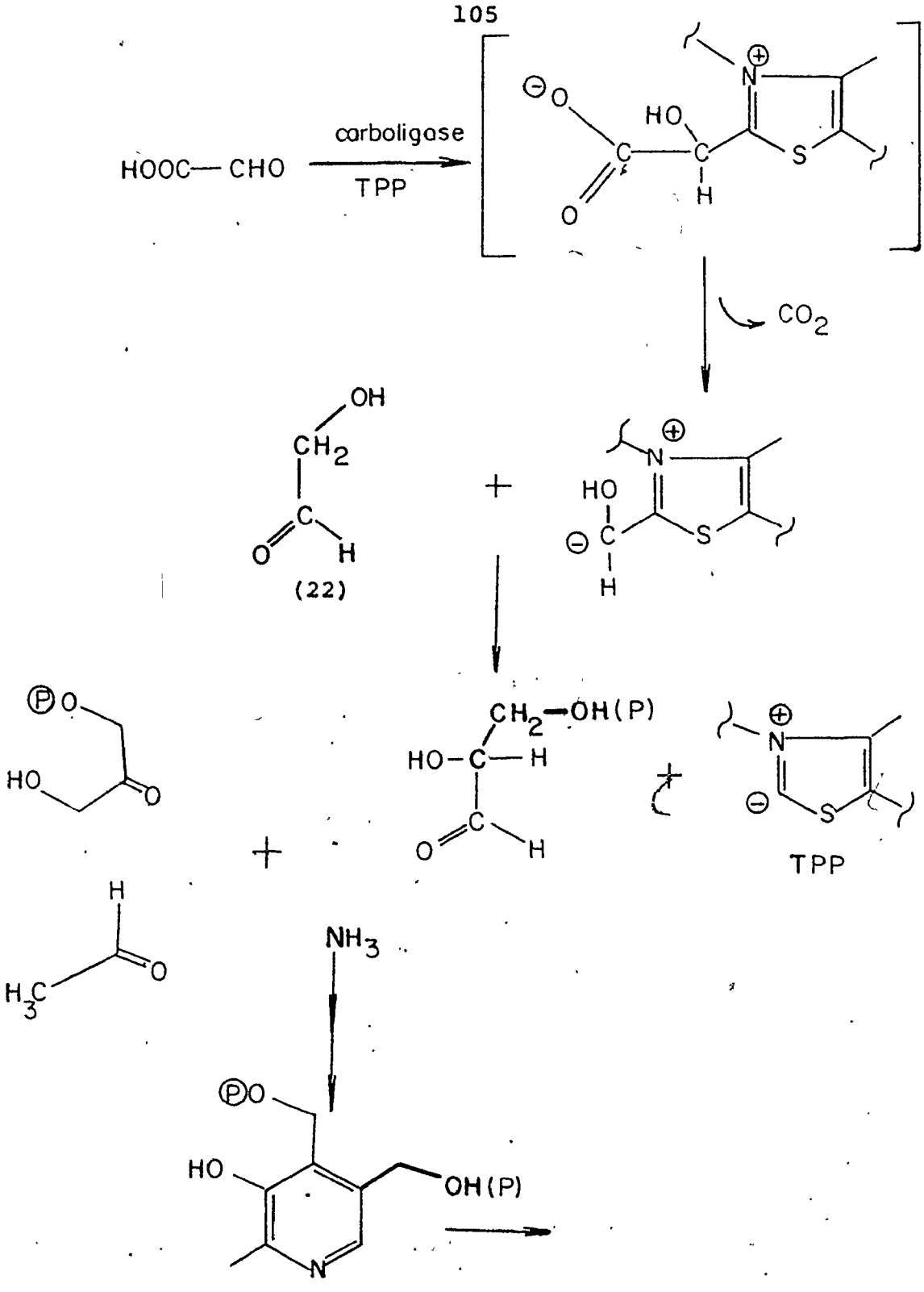
Considering the intermediates which are advanced in the hypothetical sequence for the biosynthesis of pyridoxol proposed by Lingens, it is suggested that 4'-deoxypyridoxol (7) is converted to pyridoxol by the oxidation of the 4'-methyl to the 4'-hydroxymethyl moiety. In testing this hypothesis, Lingens found that 4'-deoxypyridoxol (7), added to the culture

medium, promoted the growth of vitamin B_6 deficient mutants of B. subtilis. Furthermore, 4'-deoxypyridoxine-5'-phosphate was found to accumulate in the culture fluid. Lingens concluded that a kinase was present in B. subtilis, but was unable to demonstrate the enzymic oxidation of 4'-deoxypyridoxine to pyridoxol in these mutants, a step predicted by him and also by Scott and coworkers.[84]

Scott et al. demonstrated that the action of 4'-deoxypyridoxine in "oxidase" mutants of E. coli K12 causes inhibition of growth. However, under certain conditions, 4'-deoxypyridoxine can promote the growth of the pyridoxal-requiring mutants. The stimulation of the growth of these mutants suggested that at low pyridoxal concentrations, the organism could convert 4'-deoxypyridoxine to pyridoxal.[84]

The vitamin B_6 analogues, 4'-deoxypyridoxine and its 5'-phosphate ester are known to be powerful inhibitors of pyridoxal oxidase.[19,66] Their mode of action is to inhibit the binding of pyridoxal-5'-phosphate to the apoenzyme in a competitive manner. The regulatory function of 4'-deoxypyridoxine suggested by Scott and the role of this analogue as an intermediate in pyridoxine biosynthesis suggested by Lingens and Scott await experimental confirmation since 4'-deoxypyridoxine has yet to be isolated from a natural source.

Another scheme may be envisaged for the utilization of glycolaldehyde in the biosynthesis of vitamin B_6 (Scheme (16)).



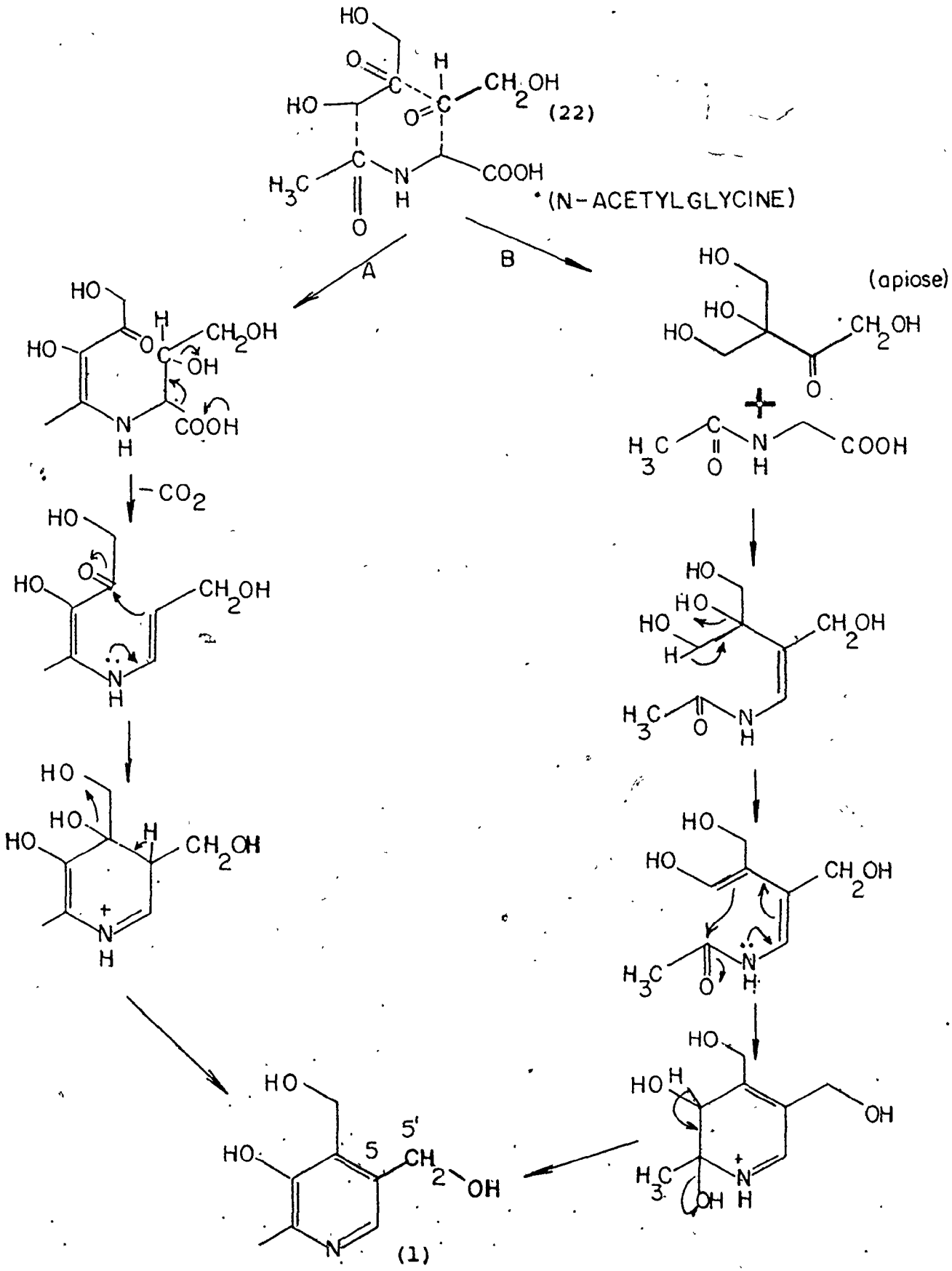
Scheme 16: Possible Conversion of Glycolaldehyde to Glyceraldehyde.

Decarboxylation of glyoxylic acid by the carboligase reaction in E. coli has been shown to be thiamin pyrophosphate dependent.[144,145] Hydroxymethyl thiamin pyrophosphate ("active formaldehyde") is supposedly formed in the reaction.[146] Carbanion formation of "active formaldehyde" with subsequent aldolase reaction of glycolaldehyde would lead to the formation of glyceraldehyde.

Glyceraldehyde formation from glycolaldehyde via the carboligase reaction would permit entry of C_1 and C_2 of glycolaldehyde specifically into C-5 and -5', respectively, of pyridoxal according to the sequence proposed by Hill and Spenser. This sequence (Scheme (16)) is in agreement with the tracer evidence obtained from Experiment 3 and is consistent with the prediction that C_6 of pyridoxol, synthesized by way of the glycolaldehyde minor route, should be derived from C_2 of glycerol.

Other biosynthetic sequences which also are consistent with the data presented above are presented in Scheme (17A and B).

Considering that in the biosynthesis of pyridoxol, not one compound has yet been isolated whose sole function is to serve as a precursor of the pyridoxal carbon skeleton, these hypothetical sequences (Schemes (16) and (17)) attempt to limit the possible intermediates on route to pyridoxal as well as putative precursors which may then be tested as intermediates.



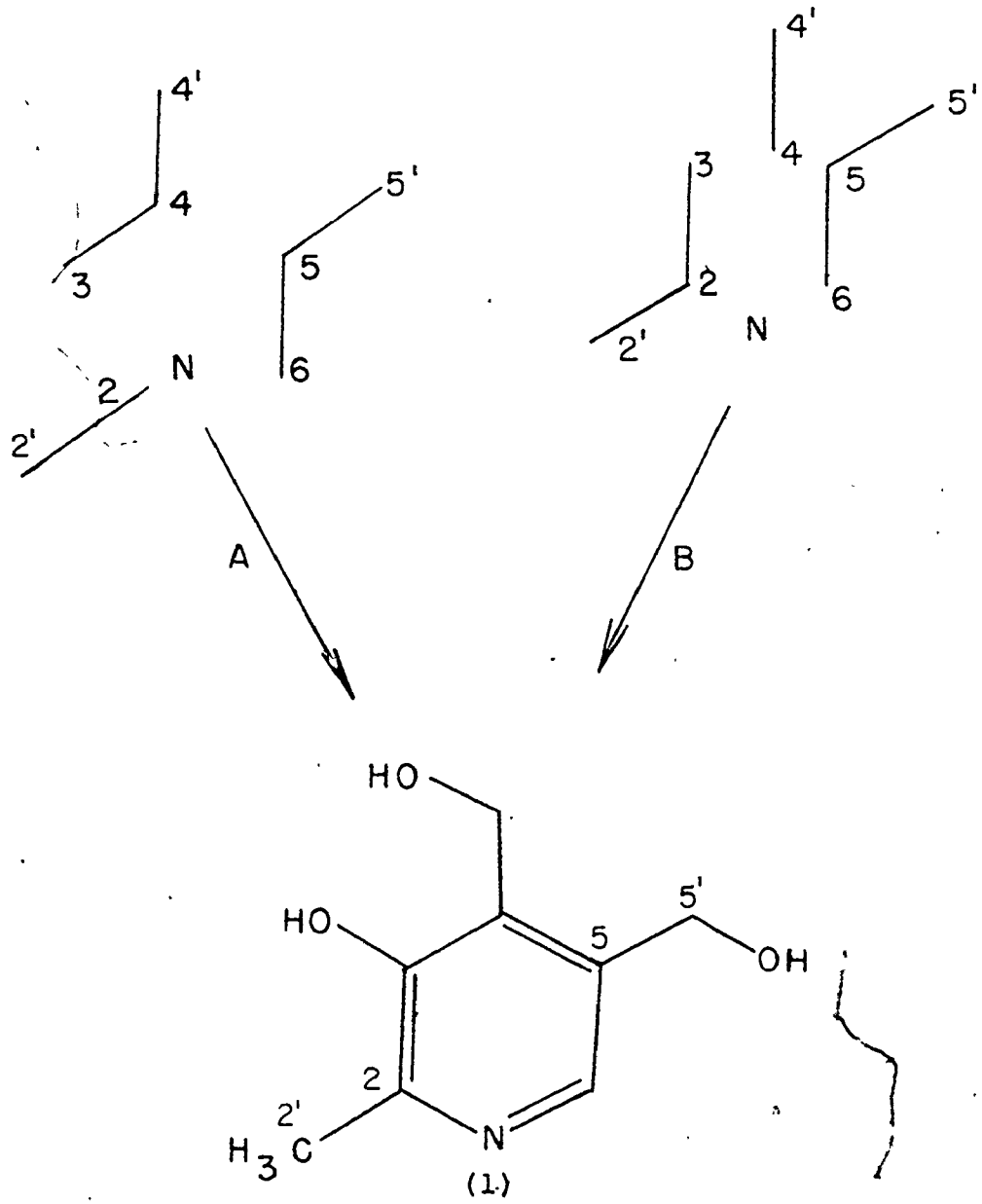
Scheme 17: Intermediacy of Dihydroxyacetone, Glycolaldehyde and N-Acetylglycine in a Hypothetical Sequence for the to Pyridoxol.

3.2 Origin of C_2 Unit C-2,-2' of Pyridoxol

3.2.1 Introduction

The mode of incorporation of label into pyridoxol derived from [^{14}C]- and [^{13}C]glycerol confirms that the entire carbon skeleton of pyridoxol can be derived from the carbon atoms of glycerol.[75-77,79,80] The eight-carbon structure of pyridoxol can be envisaged to be formed from two triose units related to glycerol and a two-carbon unit derivable from C-1 and C-2 of glycerol. The two-carbon unit may be incorporated into the carbon skeleton of pyridoxol in either of two ways: It may give rise to the carbon fragment C-2,-2' (Scheme 18A) or C-4,-4' (Scheme 18B) of pyridoxol.

In an attempt to identify which two carbons of pyridoxol originated from a C_2 unit, it was found that radioactive samples of pyridoxol, derived from [2-^{14}C]- and [3-^{14}C]-pyruvate, contained most of the activity at C-2 and C-2', respectively.[76,77] Hence, it was concluded that the methyl and carbonyl moieties of pyruvate yielded carbons 2' and 2, respectively, of pyridoxol. This observed mode of entry does not reveal whether or not C-1 of pyruvate is incorporated into C-3 of pyridoxol. In an experiment with [1,3-^{14}C]-pyruvate, in which activity at C-1 (52% $\pm$ 1) and at C-3 (48% $\pm$ 1) was approximately the same, it was shown that over 90% of the total pyridoxol radioactivity, derived from [1,3-^{14}C]-pyruvate, was located at C-2' of the vitamin. It follows that the carboxyl moiety of pyruvate is not incorporated into



Scheme 18: A Two-carbon Unit as the Progenitor of

A) C-2,-2', or

B) C-4,-4' of Pyridoxol.

pyridoxol. Thus, it is the carbonyl and methyl carbons (C-2 and C-3, respectively) of pyruvate which give rise to the two-carbon fragment, C-2,-2' of pyridoxol (Scheme 18A).[77]

From the non-random incorporation of activity into pyridoxol derived from labelled pyruvate and from labelled glycerol, it was inferred that the triose units, related to glycerol, and the C_2 compound, derivable from both C-1 and C-2 of glycerol and from C-2 and C-3 of pyruvate, which make up the carbon skeleton of the vitamin, are generated by glycolysis.[76,77]

The mode of incorporation of radioactivity into pyridoxol derived from [1-^{14}C]- and [6-^{14}C]glucose extended this supposition. It was shown that activity from both labelled substrates which entered pyridoxol was located at C-2', C-4', and C-5'.[77,78] Further, it was found that more activity from [1-^{14}C]glucose than from [6-^{14}C]glucose,[78] was incorporated into C-2' of pyridoxol. This mode of incorporation was not consistent with the idea that pyruvate generated by glycolysis provided the C_2 unit C-2,-2' of pyridoxol. Hence, to rationalize the observed distribution of radioactivity within pyridoxol derived from [1-^{14}C]- and [6-^{14}C]glucose, it was necessary to propose that a second route, directly from hexoses, also supplies the two-carbon unit (Scheme 4).[78]

A two-carbon unit directly derivable from hexoses or pentoses is "active glycolaldehyde" (27). Yet, it has been shown that glycolaldehyde is not associated with the origin of C-2,-2'

of pyridoxol.[80,147]

Little is known about the pyruvate-derived two-carbon unit. From the earlier work of Hill et al.,[75,76] it was suggested that the two-carbon unit required for pyridoxol biosynthesis must be of an oxidation level of acetaldehyde. This conclusion was based on the relatively poor incorporation of radioactivity into pyridoxol derived from [2-^{14}C]acetate. Also, the amount of activity found at carbons 2' and 2 of pyridoxol derived from labelled acetate was less than the total amount of activity which entered pyridoxol. Hence, the incorporation of label into pyridoxol derived from [2-^{14}C]-acetate was not confined to one carbon and it was inferred that acetate was not the pyruvate-derived C_2 unit required for pyridoxol biosynthesis.

The identity of the C_2 unit originating from pyruvate is unknown and the route which leads from pyruvate to the C_2 unit, required for pyridoxol biosynthesis, remains to be established. It is informative, in this context, to consider the origin of C-methyl groups present in other natural products. Information regarding the source of the C-2,-2' unit of pyridoxol has also been derived from nutritional studies of pyridoxineless mutants. These topics will be briefly discussed in the following sections.

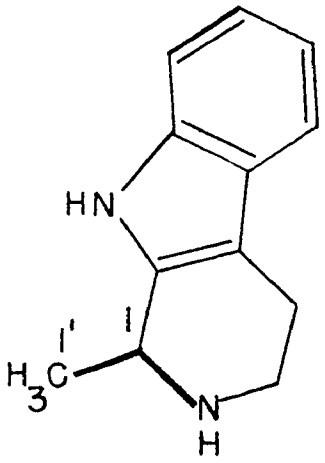
Alkaloids requiring a C_2 unit

A structural analogy can be drawn between pyridoxol

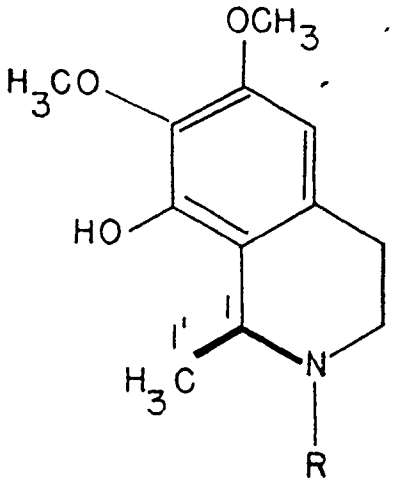
and a number of alkaloids: The indole alkaloids, eleagnine (29) and harmine (30) and the tetrahydroisoquinoline alkaloids, pellotine (32) and anhalonidine (31), Fig. (11). These alkaloids, like pyridoxol, contain a CH_3-C-N unit. In the biosynthesis of the isoquinoline and indole alkaloids, the major portion of the carbon skeleton is derived from a phenethylamine and a tryptamine moiety, respectively. In addition, a two-carbon fragment is required to complete the carbon skeleton of these compounds. The identity of the two-carbon unit involved in the biosynthesis of these alkaloids has been a subject of contraversy which has only recently been resolved and may have some bearing on the origin of C-2,-2' of pyridoxol.

The biosynthesis of the isoquinoline alkaloids was envisaged by Hahn, in 1935, to occur by the condensation of an appropriate α-keto acid and the amino acid tyrosine.[148] Battersby et al. and Leete et al., while investigating the biosynthesis of pellotine (32) confirmed Hahn's prediction.

It was shown that label derived from [1-^{14}C]acetate yielded radioactive pellotine (32). Half the total radioactivity of pellotine (32) was located at the C-1 + C-1' fragment of the alkaloid. Further, this activity was shown to be equally divided between these two carbons. The mode of incorporation of activity derived from [^{14}C]acetate into pellotine (32) showed clearly that acetate was not the direct precursor of this two-carbon unit C-1, C-1' of pellotine (32).[149,150]

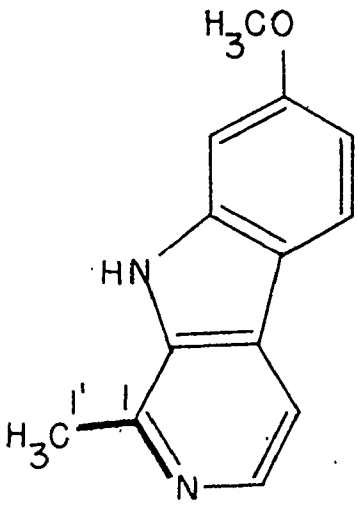


ELEAGNINE (29)

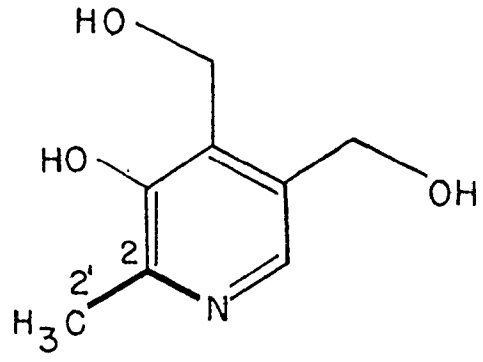


R = H, ANHALONIDINE (31)

R = CH_3, PELLOTINE (32)



HARMINE (30)



PYRIDOXOL (1)

Figure 11: Structural Similarity between Pyridoxol and Various Alkaloids.

In a similar alkaloid (31), Leete et al. demonstrated that high activity from [3-^{14}C]pyruvate was incorporated into C-1' of anhalonidine (31)[151] and further the methyl (C-3) and carbonyl (C-2) moieties of pyruvate, but not of acetate, served as the donor of the C_2 fragment C-1,-1' of the isoquinoline alkaloids, pellotine (32) and anhalonidine (31).[152]

Similarly, in the biosynthesis of the β-carboline nucleus of the indole alkaloid harmine (30),[153] label derived from [^{14}C]acetate was randomly incorporated into the carbon skeleton of the alkaloid, whereas activity from [2-^{14}C]- and [3-^{14}C]pyruvate was located at C-1 and C-1', respectively.

The C_2 unit, C-1,-1', of eleagnine (29) is of similar origin. Most of the activity of this alkaloid, derived from [3-^{14}C]pyruvate, was located at the C-1 methyl moiety.[154]

These observations are in agreement with the findings of Stolle et al. and support the work of Battersby and of Leete which defines pyruvate as the immediate precursor of the C-1,-1' carbon unit of the isoquinoline alkaloids. Furthermore, Hahn's hypothesis regarding the origin of C-1 and its substituent in tetrahydroisoquinoline alkaloids may be extended to include the benzylisoquinoline alkaloids[155] and the indole alkaloids described above.

The specific incorporation of the methyl and carbonyl moieties of the α-ketoacid, pyruvate, into pyridoxol without the involvement of acetate may be a further example of Hahn's hypothesis. However, until the actual C_2 unit required for

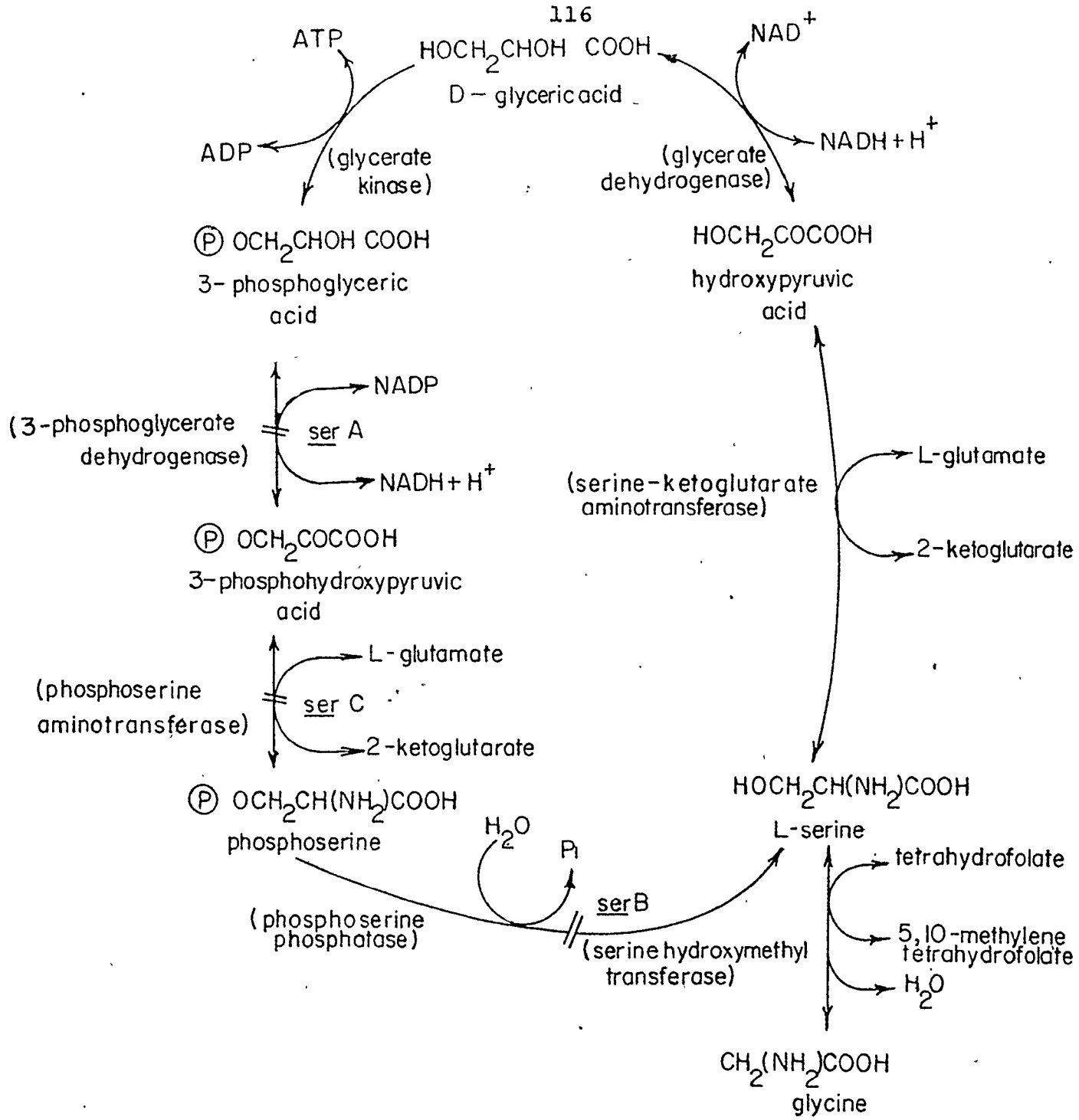
the biosynthesis of these alkaloids is identified and shown to be the same as that of pyridoxol, this relationship must be regarded as being coincidental.

Nutritional studies with pyridoxineless mutants

An empirical approach which has yielded much information regarding the biosynthesis of vitamin B_6 has been the testing of various compounds which could nutritionally satisfy the pyridoxal requirement of pyridoxineless mutants. Dempsey has conducted the most comprehensive mutant study and has characterized several hundred pyridoxineless mutants of E. coli. From nutritional, starvation and genetic experiments with these mutants, data have been accumulated indicating several compounds which might serve as potential precursors for the pyridoxal carbon skeleton.[39] A brief description of some of these mutants and the compounds which they require for growth and/or pyridoxal biosynthesis will follow. Preliminary tracer experiments have been carried out on this basis and will be dealt with in section 3.2.3 of this chapter.

3-Phosphoserine, Serine and 3-Hydroxypyruvate

The relationship of 3-phosphoserine, serine and 3-hydroxypyruvate in E. coli is of considerable interest with respect to pyridoxal biosynthesis. There are two pathways known for the biosynthesis of serine (Scheme (19)). The route involving the phosphorylated intermediates appears to be the major source of this amino acid in E. coli, where



Scheme 19: Two Pathways for the Biosynthesis of Serine. The pathway via non-phosphorylated intermediates does not occur in E. coli.

phosphoserine has been shown to be the precursor of serine.[156-159]

Several mutants of E. coli have been isolated which clearly show a dependence for serine.[160-162] These mutants have been characterized according to their gene product, i.e., enzyme deficiency: serA mutants lack 3-phosphoglycerate dehydrogenase, serB mutants lack 3-phosphoserine phosphatase, and serC mutants lack (3-phosphoserine:2-ketoglutarate transaminase (Scheme (19)).

In two of these mutant classes (serA and serB) pyridoxal production was observed. However, in serC mutants, no pyridoxal synthesis occurs. In fact, Dempsey showed that serC mutants were also pyridoxineless mutants (Group III - originally designated as pdxF , Fig. (2)), which required both serine and pyridoxal for growth.[138]

Initially, serine was thought to be a precursor of pyridoxal. In fact, serine can be excluded as a precursor of pyridoxal because serA and serB mutants, which also have a serine requirement can synthesize normal amounts of pyridoxal. Further, neither growth nor pyridoxal synthesis occurs when serC mutants are cultured on a medium with serine as the lone supplement.

Originally, it was thought that 3-phosphoserine, rather than serine[138] was the precursor of pyridoxal because the enzyme which catalyses the formation of 3-phosphoserine was lacking in serC mutants. However, in a recent investigation,[89]

Dempsey found that the pyridoxal requirement of serC mutants was satisfied by 3-hydroxypyruvate, i.e., when serC mutants were cultured on a medium supplemented with serine plus 3-hydroxypyruvate, normal growth and pyridoxal production was observed. When these mutants were deprived of serine and pyridoxol but their medium was supplemented with 3-hydroxypyruvate, normal amounts of pyridoxal were detected, but no growth of the culture occurred. This finding demonstrates that 3-hydroxypyruvate cannot satisfy the serine requirement (for growth), i.e., 3-hydroxypyruvate is not converted to serine. Furthermore, it demonstrates that serine is not converted to 3-hydroxypyruvate (vide supra).

From these results, Dempsey concluded that the function of 3-phosphoserine transaminase in the wild type organism was not only to transaminate the phosphorylated compounds, but also to convert small amounts of serine to hydroxypyruvate sufficient for pyridoxal biosynthesis. He further speculated that pyridoxal biosynthesis is a function of the 3-hydroxypyruvate pool.[89] The proposed role of 3-hydroxypyruvate as a precursor of the carbon skeleton of pyridoxal remains to be investigated by tracer methods.

The earlier observation that radioactivity from [3-^{14}C]-serine was incorporated into pyridoxol from Saccharomyces fragilis[69] and from E. coli[77,90,163] can be rationalized to take place via pyruvate, a known product of serine catabolism.[164-166]

Pyruvate and Alanine

It has been shown by tracer studies that the methyl and carbonyl moieties of pyruvate provide carbons 2' and 2, respectively, of pyridoxol. From mutant studies, the Group V (coded for pdxJ and pdxK Fig. (2)) mutants have been shown to contain two different phenotypes: One (pdxJ) of these can grow only if supplemented with pyridoxol, the other (pdxK) can use D- or L-alanine, pyruvate or cysteine. However, sub-optimal growth rates are achieved with the three substrates.

Alanine was one of several amino acids (isoleucine, aspartate, and glutamine) which stimulated pyridoxol production in a pyridoxineless mutant of Bacillus subtilis.[67]

Thiamin and 2-Ketoglutarate

Starvation experiments with two non-pyridoxineless mutants WG1225 and WG1390, have demonstrated that thiamin and 2-ketoglutarate (glutamate), respectively, are required for pyridoxal biosynthesis. When deprived of thiamin or 2-ketoglutarate (glutamate), respectively, these mutants cease production of vitamin B_6. In other words, mutants which are not able to synthesize 2-ketoglutarate (glutamate) or thiamin do not produce pyridoxol.[51]

Mutants (WG1390) which have an absolute requirement for 2-ketoglutarate (glutamate) have been shown to be deficient in citrate synthase[167] which is required for the synthesis of 2-ketoglutarate (glutamate). Although 2-ketoglutarate itself

is not required for pyridoxal biosynthesis[51] the 2-ketoglutarate:glutamate pair may be linked to the 3-phosphohydroxypyruvate:glutamate transaminase reaction which has been shown to be essential for pyridoxal biosynthesis in the serC mutants,[89,138] Scheme (19). In the citrate synthaseless mutants, the 3-phosphoserine:3-hydroxypyruvate transaminase may be inactivated due to the lack of 2-ketoglutarate. As a consequence, production of pyridoxal ceases. It has been suggested that the 2-ketoglutarate:glutamate transaminase reaction may supply the pyridine nitrogen[39] of pyridoxol. This, however, remains to be tested.

The relationship of thiamin with the biosynthesis of vitamin B_6 was first recognized in yeast.[168-170] Although this relationship is not understood, it is thought, that due to the structural similarity of pyridoxal and the pyrimidine component of thiamin, Fig. 12, a competition between the two vitamins can exist.[171] It has been shown that the pyrimidine moiety of thiamin functions as a pyridoxal-5'-phosphate antagonist in Saccharomyces carlsbergensis, Spreptococcus faecalis, Neurospora and E. coli. Harris found that in Neurospora, an excess of vitamin B_6 caused inhibition of both growth and thiamin synthesis. This inhibition could be relieved by the addition of thiamin or of the pyrimidine component of thiamin.[172] Lewin et al. discovered that pyridoxal phosphate prevented thiamin biosynthesis by inhibiting the enzymic condensation

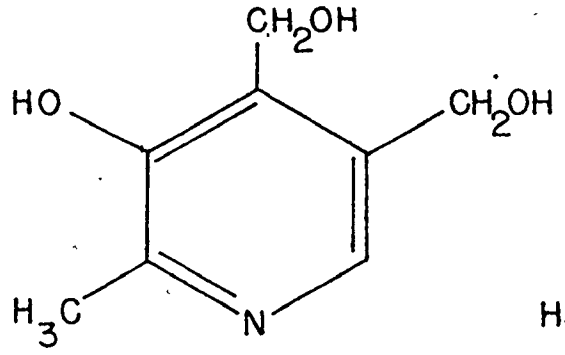
of pyrimidine pyrophosphate and thiazole phosphate (Figure 12).[173]

Mutants of E. coli which cannot synthesize thiamin show a greatly reduced rate of both growth and pyridoxal production when deprived of thiamin. Addition of thiamin to such a culture initiates growth and pyridoxal synthesis. This observation leads to the possible explanation that thiamin pyrophosphate and pyridoxal phosphate are essential cofactors in each others' biosynthetic pathways.[51]

Since thiamin pyrophosphate mediated enzymic reactions usually involve the transfer of activated C_2 units[174] and since pyridoxal biosynthesis is assumed to involve a two-carbon unit, the above interpretation for the interrelationship of thiamin and pyridoxal phosphate is favoured.[51]

The inference from Dempsey's mutant studies, which is in agreement with the evidence obtained from the tracer investigations of Hill and Spenser, is that vitamin B_6 is biosynthesized de novo from C_2 and C_3 compounds. One of the ways such compounds are generated is glycolysis, which yields triose units related to glycerol and pyruvate. Pyruvate serves as the source of several C_2 compounds.

The purpose of the present study was to obtain more information regarding the origin of the C_2 unit, C-2,-2' of pyridoxol in mutant WG2, and to rationalize the mode of incorporation of activity derived from [1-^{14}C]- and [6-^{14}C]-glucose into pyridoxol. Unequivocal evidence has been presented



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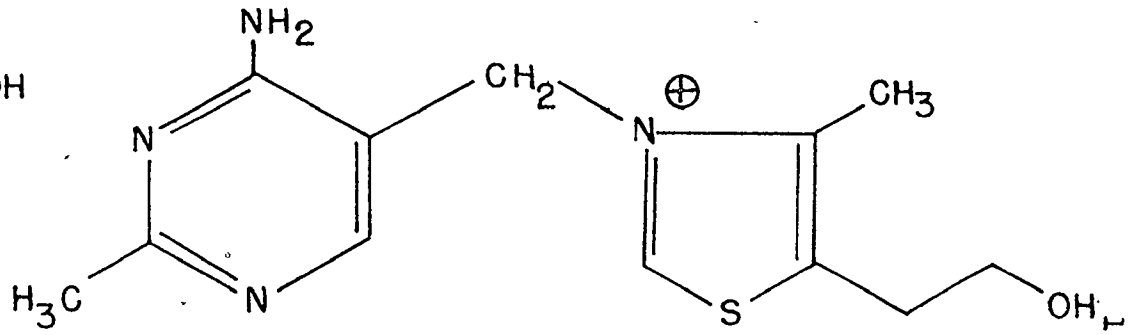


THIAMIN

Figure 12: Structural Similarity between Pyridoxol and the Pyrimidine Moiety of Thiamin.

which shows that the C_2 unit, C-2,-2' of pyridoxol is derived from the methyl and carbonyl moieties of pyruvate. The normal end-products of pyruvate metabolism are excluded as direct progenitors of this two-carbon unit of pyridoxol. On this basis, acylating agents associated with the thiamin-dependent enzyme-catalysed decarboxylation of pyruvate are proposed to play an active role in the biosynthesis of vitamin B_6.

3.2.2 Results

The details of the tracer experiments with E. coli B mutant WG2 are summarized in Table (7). Samples of [1-^{14}C]-acetate (Experiment 9), [1-^{14}C]ribose (Experiment 10), [1-^{3}H,2-^{14}C]glycerol (Experiment 14) and [2-^{14}C]glycerol in the presence of equimolar unlabelled pyruvate (Experiment 11), acetate (Experiment 12), and 3-hydroxypyruvate (Experiment 13) were administered to cultures of E. coli B mutant WG2. A sample of [2-^{14}C]pyruvaldehyde (prepared by selenium dioxide oxidation of [2-^{14}C]acetone - see Table (8)) (Experiment 8) was administered to a culture of E. coli B mutant WG2, in which the glycerol concentration was maintained at 0.2% (21.8 mmol/l). In these experiments, radioactive samples of pyridoxol were isolated from the culture medium by carrier dilution as previously described.[77]

Radioactive pyridoxol samples from all the experiments were converted into appropriate derivatives and then degraded by established reaction sequences (Scheme (5)) to determine

Table 7: INCORPORATION OF LABELLED SUBSTRATES INTO PYRIDOXOL

Substrate Administered				Initial Glycerol Content			Pyridoxol HCℓ	
Expt. No	Substrate(s)	Nominal Specific Activity (mCi/mmol)	Nominal Total Activity (μCi)	Total Volume (ℓ)	Concentration (g/ℓ)	(mmol/ℓ)	Weight of Carrier Added (mg)	Specific Activity (dpm/mmol x 10^{-4})
8	[2-^{14}C]Pyruvaldehyde[a]	0.225	515	2	2	21.8	40	2.00±0.02
9	[1-^{14}C]Acetate[c]	56.9	1000	1	1	10.9	40	0.36±0.01
10	[1-^{14}C]Ribose[c]	53.0	250	1	1	10.9	180	2.50±0.02
11	[2-^{14}C]Glycerol[c] + sodium pyruvate	(16.5)0.036	390	1	1	10.9	230	5.03±0.03
12	[2-^{14}C]Glycerol[c] + sodium acetate	(15.3)0.023	250	1	1	10.9	112	2.91±0.01
13	[2-^{14}C]Glycerol[c] + lithium,3-hydroxy-pyruvate	(16.4)0.023	250	1	1	10.9	101	0.72±0.05
14	[1-^{3}H]Glycerol[b] + [2-^{14}C]Glycerol[b]	(2500)0.230 (40)0.012	5000 250	2	1	10.9	25	5.01±0.02(C^{14})[d]

[a] See Table 8

b Amersham/Seal

[c] New England Nuclear

[d] dpm/mmol x 10^{-5}

Table 8 : SYNTHESIS OF [2-^{14}]PYRUVALDEHYDE FROM [2-^{14}C]ACETONE

Experiment No.	[2-^{14}C]Acetone[a] Nominal Specific Activity (mCi/mmol)	Nominal Total Activity (μCi)	Inactive Acetone Added (mg)	Nominal Specific Activity After Dilution (μCi/mmol)	[2-^{14}C]Pyruvaldehyde Total Activity (μCi)	Weight (mg)
8	7.5	1000	250	225	515	165

[a]New England Nuclear Corp.

the distribution of radioactivity. The results of these degradation experiments are shown in Tables (9) and (10).

In the pyridoxol sample isolated from the experiment with [2-^{14}C]pyruvaldehyde, (Experiment 8, Table (9)), within experimental error, all the activity was found in the C_2 unit, C-2,-2' (Kuhn-Roth acetate) and particularly at C-2. In the experiment in which [1-^{14}C]acetate was administered as the tracer (Experiment 9), the pyridoxol sample was found to contain sixty percent of its total activity at the C_2 unit, C-2,-2' of the vitamin.

Pyridoxol obtained from the experiment with [1-^{14}C]-ribose (Experiment 10, Table (9)) was found to contain approximately one-third of the activity in the C_2 fragment, C-2,-2' (Kuhn-Roth acetate) whereas the C_2 unit, C-5,-5' (N-phthaloylglycine) of this sample accounted for approximately one-eighth of the total activity.

In the three experiments in which [2-^{14}C]glycerol was administered as a tracer in the presence of equimolar unlabelled substrates, only Experiment 11, Table 10, in which pyruvate was added, showed any significant difference in the distribution of label within pyridoxol from that found when glycerol was the sole substrate. In the presence of pyruvate, 19 ± 1% of the total activity derived from [2-^{14}C]glycerol was found to reside at the C_2 unit, C-2,-2' (Kuhn-Roth acetate) of pyridoxol and 40 ± 1% of its label was located at the C_2 unit, C-5,-5'

Table 9: DISTRIBUTION OF RADIOACTIVITY IN PYRIDOXOL DERIVED FROM [2-^{14}C]PYRUVALDEHYDE, [1-^{14}C]ACETATE AND [1-^{14}C]RIBOSE

Experiment No.	(8)		(9)		(10)	
Substrates	[2-^{14}C]Pyruvaldehyde		[1-^{14}C]Acetate		[1-^{14}C]Ribose	
Product (C-atoms of pyridoxol)	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]
Pyridoxol HCℓ(1)(All)	2.00±0.02	100±2	2.16±0.03	100±2	2.50±0.02	100±1
3,4'-O-Isopropylidene-pyridoxol(10)(All)	2.01±0.02	100±2	-	-	2.51±0.02	100±1
3,4'-O-Isopropylidene-5'-N-phthaloyliso-pyridoxamine(20)(All)	-	-	-	-	2.43±0.03	97±1
Kuhn-Roth acetate (as N-acetyl-α-naph-thylamine(12))(2',2)	1.95±0.02	98±1	1.3±0.02	60±1	0.84±0.02	34±1
N-Methylphthalimide(14)(2')	0.018±0.01	1±0.01	-	-	-	-
N-Phthaloylglycine(21)(5',5)	-	-	-	-	0.31±0.01	13±1

[a] Specific activity (dpm/mmol) x 10^{-4}

[b] Relative specific activity (per cent)(pyridoxol HCℓ = 100)

Table 10: INCORPORATION INTO PYRIDOXOL OF [2-^{14}C]GLYCEROL IN THE PRESENCE OF NONRADIOACTIVE SUBSTRATES

Experiment No.		(11)		(12)		(13)	
Nonradioactive Substrates Added		Sodium Pyruvate		Sodium Acetate		Lithium 3-Hydroxy-pyruvate	
Product	(C-Atoms of Pyridoxol)	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]
Pyridoxol HCℓ (1)	(All)	5.03±0.03	100±1	2.90±0.01	100±1	0.72±0.02	100±3
3,4'-O-Isopropylidene-pyridoxol (10)	(All)	5.02±0.06	100±1	2.90±0.02	100±1	0.72±0.01	100±3
3,4'-O-Isopropylidene-5'-N-phthaloylisopyridoxamine (20)	(All)	5.07±0.07	101±1	-	-	0.72±0.01	100±3
Kuhn-Roth acetate (as N-acetyl-α-naphthylamine) (12)	(2',2)	0.96±0.02	19±1	1.00±0.01	34±0.4	0.22±0.01	31±2
N-Phthaloylglycine (21)	(5',5)	2.00±0.02	40±1	-	-	0.24±0.01	34±2

[a] Specific Activity (dpm/mmol) x 10^{-4}

[b] Relative Specific Activity (per cent)(pyridoxol HCℓ = 100)

(N-phthaloylglycine). In the experiments where sodium acetate (Experiment 12, Table (10)) and lithium 3-hydroxypyruvate (Experiment 13, Table (10)) were present as the non-radioactive substrates, the pyridoxol samples in both experiments contained approximately one-third of their respective activities in the C_2 unit, C-2,-2', of the vitamin. Further degradation of the pyridoxol sample from Experiment 13 located additional radioactivity in the carbon fragment C-5,-5', and the amount of activity accounted for approximately one-third of the total pyridoxol activity.

Pyridoxol isolated from a culture of mutant WG2 to which intermolecularly doubly labelled [1-^{3}H,2-^{14}C]glycerol (^{3}H/^{14}C ratio of glycerol tribenzoate 18.7 ± 0.5) had been administered (Experiment 14), contained a ^{3}H/^{14}C ratio 9.4 ± 0.03, indicating the loss of one ^{3}H atom relative to ^{14}C.

3.2.3 Discussion

3.2.3.1 Published Evidence Concerning the Origin of the C_2 Unit, C-2,-2' of Pyridoxol

Evidence that pyridoxol from mutant WG2 is biosynthetically derived from trioses related to glycerol and generated by glycolysis is provided by the mode of incorporation of radioactivity from [2-^{14}C]pyruvate, [3-^{14}C]pyruvate,[75,77] [1-^{14}C]glucose and [6-^{14}C]glucose.[77,78] A plausible hypothesis consistent with the tracer experiments was advanced on the basis of these observations. It is considered that pyridoxol is formed by the condensation of dihydroxyacetone-1-phosphate,

D-glyceraldehyde-3-phosphate and a two-carbon unit derivable from glycerol (Scheme 2).

That this carbon unit can be derived also from pyruvate was shown by the chemical degradation of the pyridoxol samples derived from [2-^{14}C]pyruvate and [3-^{14}C]pyruvate, as all the activity was confined to carbons 2 and 2' of pyridoxol, respectively (Fig. 13A and B). Intermolecularly labelled [1,3-^{14}C]pyruvate, containing 48% of the activity at C-3 and 52% of the activity at C-1 of pyruvate,[75] yielded pyridoxol which contained most of the activity at C-2', derived from the methyl moiety of pyruvate. The carboxyl moiety was not incorporated into the vitamin. Hence, the two-carbon unit which supplies C-2,-2' of pyridoxol is derivable from the carbonyl and methyl moieties of pyruvate, respectively. An earlier experiment with [2-^{14}C]acetate produced pyridoxol in low radiochemical yield and the position of label was not confined to carbons 2 and 2' (Fig. 14A). Hence, it was concluded that the pyruvate-derived two-carbon fragment must enter at the oxidation level of acetaldehyde. Furthermore, radioactivity found at carbons 2 and 2' of pyridoxol, derived from [2-^{14}C]-glycerol and [1-^{14}C]glycerol, respectively, was presumed to be incorporated into pyridoxol as pyruvate by way of glyceraldehyde-3-phosphate.

Subsequent experiments with labelled glucose demonstrated that the terminal carbon atoms of glucose were converted to the 2' methyl moiety of pyridoxol and that pyridoxol derived from [1-^{14}C]glucose contained a higher fraction of label at C-2' than pyridoxol from [6-^{14}C]glucose. In both cases, the other sites within pyridoxol containing radioactivity

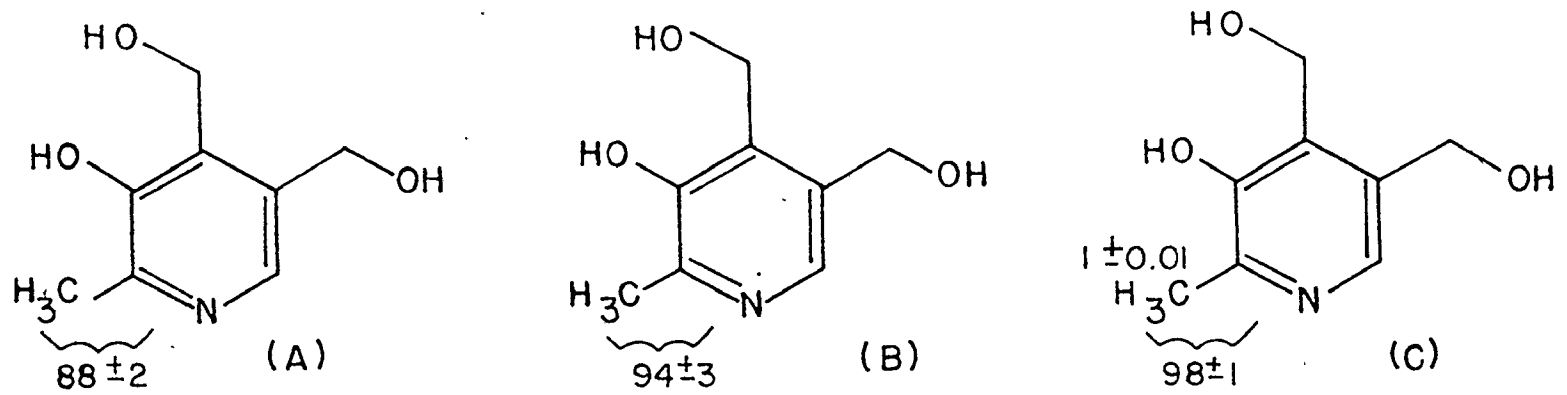


Fig. 13: Incorporation of Label into Pyridoxol Derived from A) [3-^{14}C]Pyruvate,[a] B) [2-^{14}C]Pyruvate[a] and C) [2-^{14}C]Pyruvaldehyde.

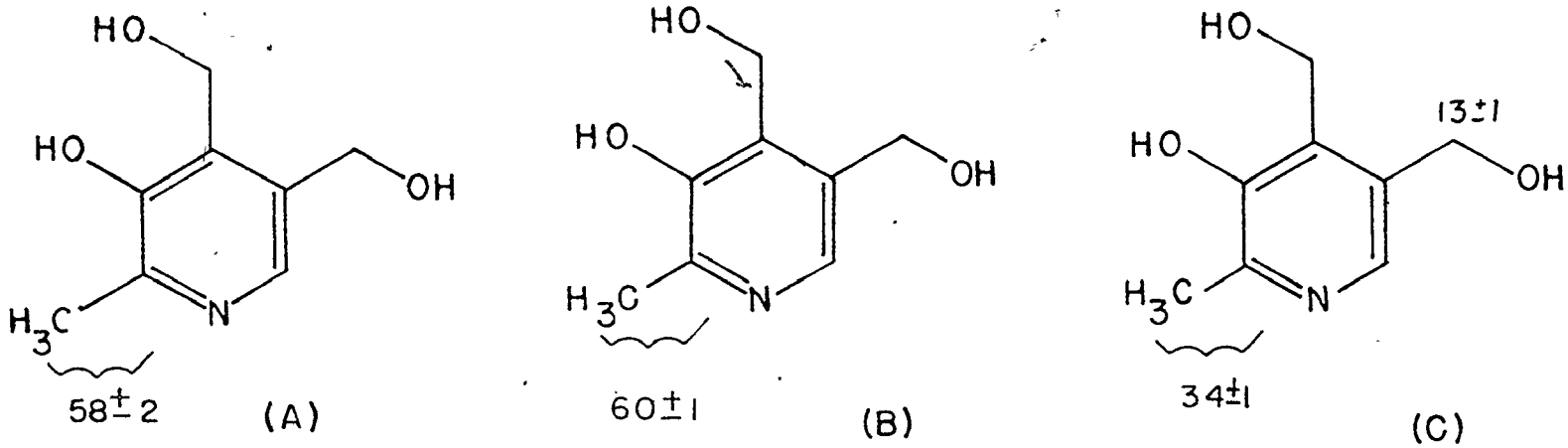


Fig. 14: Distribution of Activity found within Pyridoxol Derived from A) [2-^{14}C]Acetate,[a] B) [1-^{14}C]Acetate and C) [1-^{14}C]Ribose (Activity of Intact Pyridoxol = 100; Per cent of Total Activity at Indicated Carbon Atoms).

from these substrates were C-4' and C-5' and the specific activity at C-2' was identical with that at C-4' and different from that at C-5' (Figure 7). This suggested that the two-carbon unit required for pyridoxol biosynthesis was more closely related to dihydroxyacetone-1-phosphate (the precursor of C-4',-4,-3 of pyridoxol) than to glyceraldehyde-3-phosphate (the precursor of C-5',-5,-6 of pyridoxol). Since the level of activity at C-2' was not identical with that of C-5', pyruvate solely derived glycolytically via glyceraldehyde-3-phosphate could not have served as the C_2 donor for the C-2,-2' unit of pyridoxol.

From the mode of distribution of activity within pyridoxol derived from labelled glucose, it was inferred that the two-carbon unit required for pyridoxol biosynthesis must originate, not only from pyruvate, but also from another source. This alternative route leading from a hexose or dihydroxyacetone-1-phosphate to the C_2 unit C-2,-2' of pyridoxol must converge in an interconvertible metabolic pool with the pyruvate-derived two-carbon unit. Whereas dihydroxyacetone-1-phosphate is not commonly regarded as a precursor of C_2 metabolites, the proposal that the two-carbon unit could be derived from C-1 and C-2 of a hexose, as "active glycolaldehyde", by the action of transketolase on D-fructose-6-phosphate[110] was advanced. The interconvertibility of "active glycolaldehyde" to "active acetaldehyde" in the metabolic pool had to be assumed (Scheme 4).

This interpretation of the results obtained from the labelled glucose experiments was self-consistent and predicted

that the carbon atoms of glycolaldehyde should be incorporated into the C_2 unit, C-2,-2' of pyridoxol. Contrary to this prediction, activity from [2-^{14}C]- and [1,2-^{14}C]glycolaldehyde was confined to carbons 5 and 5' of pyridoxol, biosynthesized by mutant WG3,[86,87] and WG2.[147] Less than 3% of the total activity derived from labelled glycolaldehyde was found within the two-carbon fragment C-2,-2' of pyridoxol.

It was, therefore, necessary to reconsider the interpretation of the results of the experiments with labelled glucose and to examine which substrate would best serve as a direct precursor of the two-carbon unit, C-2,-2' of pyridoxol.

3.2.3.2 New Data Concerning the C_2 Unit, C-2,-2' of Pyridoxol

Sufficient evidence has been obtained which indicates that the two-carbon fragment used in the biosynthesis of pyridoxol by mutant WG2 can be derived from both glycerol and pyruvate. Glycerol can enter the path of glycolysis via the triose phosphates[175] and can serve as a source of pyruvate, whereas the reverse process is unlikely due to the irreversible dephosphorylation of phosphoenolpyruvate to pyruvate. Hence, to examine whether or not glycerol is incorporated into C-2,-2' of pyridoxol by way of pyruvate, a competition experiment was carried out (Experiment 11, Table (10), Fig. (15A)). [2-^{14}C]-Glycerol, together with an equimolar amount of non-radioactive pyruvate was administered to a culture of mutant WG2. Pyruvate had partially spared the incorporation of label derived from

[2-^{14}C]glycerol into C-2,-2' of pyridoxol. The incorporation of unlabelled pyruvate as seen by the differences in the distribution of radioactivity within pyridoxol from this experiment and in that of pyridoxol derived from an experiment in which 2-^{14}C glycerol was the sole substrate (Scheme (9), Fig. (A1)), suggests that pyruvate is more closely related to the two-carbon intermediate than glycerol. The sequence glycerol → pyruvate → pyridoxol, and not pyruvate → glycerol → pyridoxol, must be operative since radioactivity from [2-^{14}C]glycerol is incorporated into three sites of pyridoxol (C-2,C-4,C-5) whereas label from [2-^{14}C]pyruvate enters only at C-2 of the vitamin.

Pyruvate arises from glycerol and from glucose by the Embden-Meyerhof pathway via glyceraldehyde-3-phosphate. However, the mode of incorporation of radioactivity from [1-^{14}C]- and [6-^{14}C]glucose into pyridoxol suggests that the triose glyceraldehyde-3-phosphate does not give rise to the pyruvate which enters pyridoxol in *E. coli* mutant WG2.

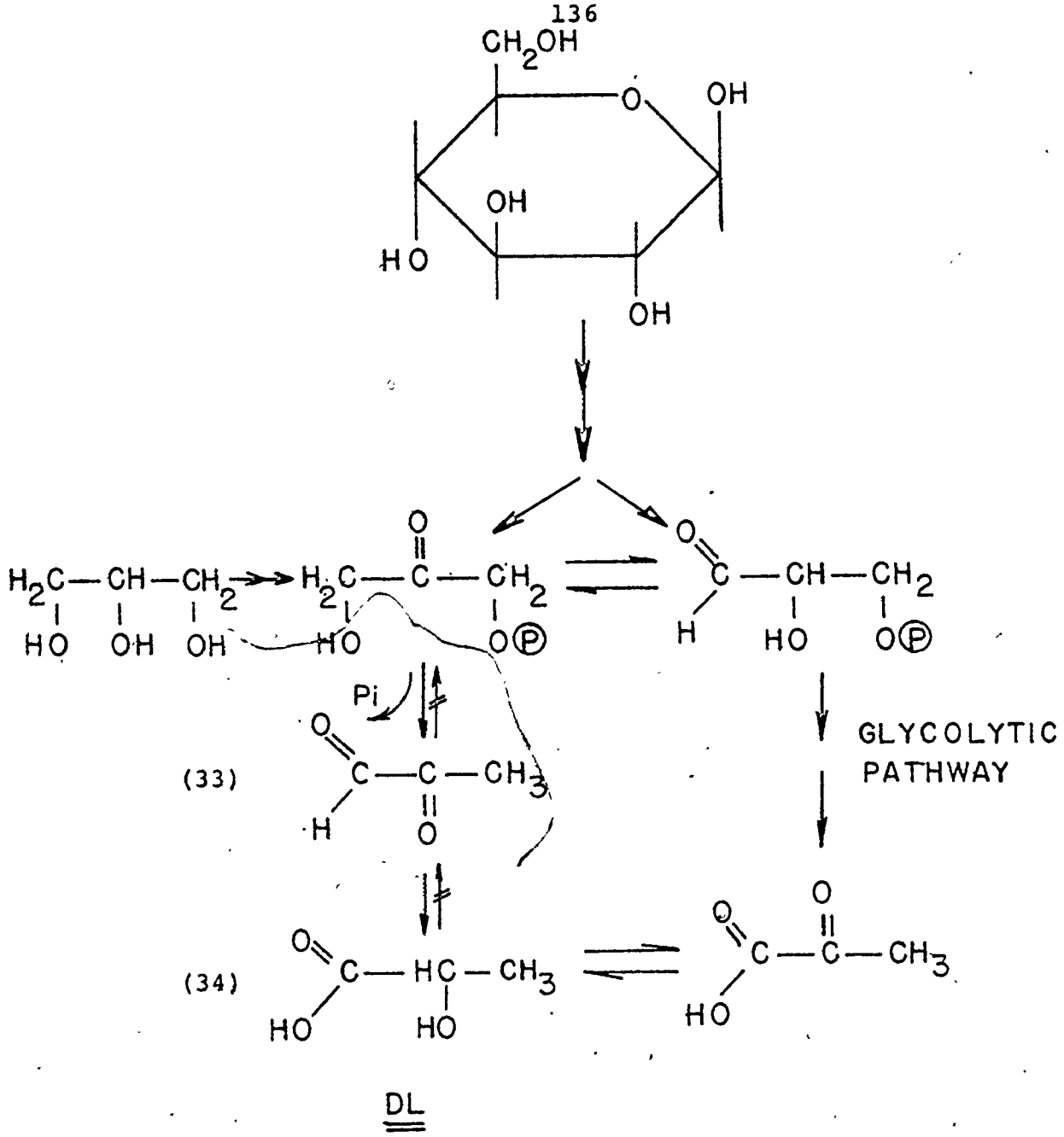
Recently, a route to pyruvate from dihydroxyacetone-1-phosphate and which does not pass through glyceraldehyde-3-phosphate was reported to be very active in *E. coli*,[176-178] Scheme (20). Cooper and coworkers have demonstrated that dihydroxyacetone-1-phosphate can be converted to pyruvaldehyde (33) (methylglyoxal) by methylglyoxal synthase *in vitro*, and that the combined action of the enzymes S-lactoylglutathione methylglyoxallyase (EC 4.4.1.5) and S-2-hydroxyacylglutathione-hydrolase (EC 3.1.2.6) transforms pyruvaldehyde (33) to

lactic acid (34).[179] Conversion of lactic acid (34) to pyruvate completes a "glycolytic shunt" whose function is yet to be determined.[177]

This alternative route to pyruvic acid in mutant WG2 would partially explain the observed distribution of radioactivity within pyridoxol derived from labelled glucose. The path of carbon from dihydroxyacetone-1-phosphate to pyruvate via pyruvaldehyde is in complete agreement with all the tracer evidence obtained thus far and strengthens the hypothesis that a truncated pyruvate supplies pyridoxol with its two-carbon unit, C-2,-2'.

It was for these reasons that [2-^{14}C]pyruvaldehyde was synthesized and administered to a culture of mutant WG2 (Experiment 8). On the basis of the "glycolytic shunt", all the incorporated radioactivity should reside at C-2 of pyridoxol; this was, in fact, what was found (Table 9) (Fig. 13C).

The incorporation of radioactivity derived from [2-^{14}C]-pyruvaldehyde must be rationalized by way of pyruvate to be consistent with the other tracer data because in the reaction sequence, pyruvaldehyde → lactate ⇄ pyruvate, the first step to lactate is irreversible,[180] Scheme (20). Thus, pyruvaldehyde must be converted to pyruvate, rather than pyruvate to pyruvaldehyde, before it can serve as the C_2 donor in pyridoxol biosynthesis. Although lactate is an intermediate in this conversion, it has been dismissed as a direct precursor of the two-carbon unit of pyridoxol on mechanistic grounds, however.



Scheme 20: The "Glycolytic Shunt" - The Pyruvaldehyde By-Pass to Pyruvate from Dihydroxyacetone-1-Phosphate in E. coli.[174]

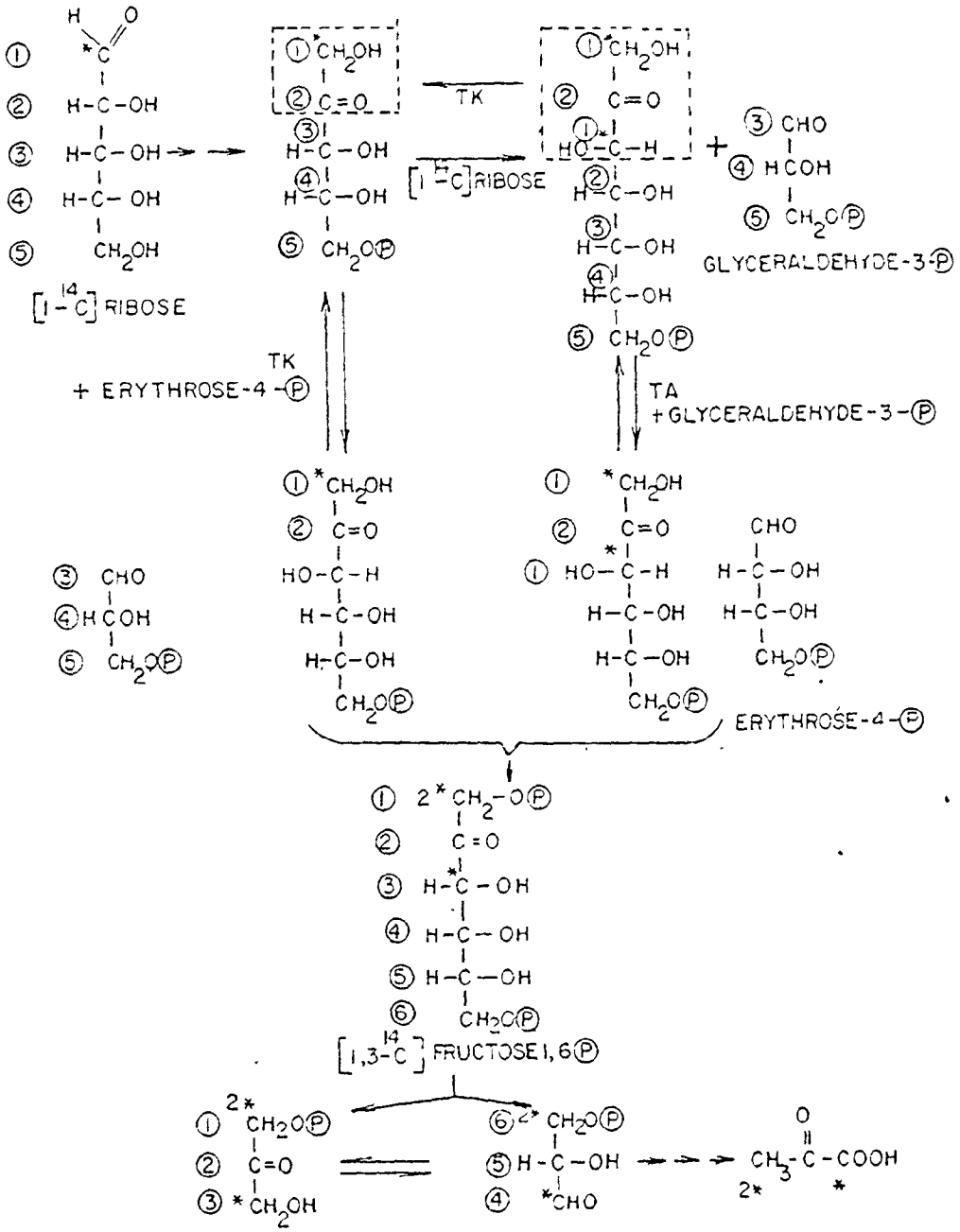
On the basis of this alternative pathway to pyruvate, the glucose data may be interpreted in the following way: Radioactivity derived from [1-^{14}C]glucose would preferentially label the dihydroxyacetone-1-phosphate pool, which can serve as a source of pyruvate via pyruvaldehyde. Pyruvate (the precursor of C-2,-2' of pyridoxol) derived by this route must have the same specific activity as dihydroxyacetone-1-phosphate (the precursor of C-3,-4,-4' of pyridoxol). However, dihydroxyacetone-1-phosphate is interconvertible with D-glyceraldehyde-3-phosphate (the precursor of C-5',-5,-6 of pyridoxol) which is derived from carbons 4, 5 and 6 of glucose and which is initially devoid of label. D-Glyceraldehyde-3-phosphate can also serve as a source of pyruvate by way of glycolysis. Since the latter triose phosphate must depend on isomerase activity for its label, the pyruvate derived from glycolysis must initially be unlabelled. Therefore, incorporation of pyruvate derived from [1-^{14}C]glucose via these two pathways would result in a C_2 fragment with a specific activity lower than that of dihydroxyacetone-1-phosphate (and hence of C-4' of pyridoxol) and higher than that of glyceraldehyde-3-phosphate (and hence of C-5' of pyridoxol). In fact, pyridoxol derived from [1-^{14}C]glucose, C-2' (36%) and C-4' (38%) of pyridoxol were equally labelled and contained more radioactivity than C-5' (26%). Pyridoxol derived from [6-^{14}C]glucose, carbons 2' (26%) and 4' (26%) were again

equally labelled but contained less radioactivity than C-5' (48%) of pyridoxol (Figure 7).

Since the level of activity at C-2' was identical with that at C-4' and different from that at C-5' of pyridoxol in the two experiments, it appears that glycolytic pyruvate (i.e., pyruvate formed by way of glyceraldehyde-3-phosphate) is not used for pyridoxol biosynthesis. Whether glycolytic pyruvate is compartmentalized[181] and used separately from pyruvate derived by way of the "glycolytic shunt" or whether glycolysis ceases entirely during pyridoxal starvation of the cells (and hence during the administration of tracer to the culture medium), so that the only route to pyruvate is the "glycolytic shunt", cannot be answered with the available data.

The inference that pyruvate derived from glyceraldehyde-3-phosphate is not used in pyridoxol biosynthesis, whereas pyruvate derived by way of pyruvaldehyde is, is supported by the results of an experiment in which [1-^{14}C]ribose was used as a tracer (Experiment 10).

To enter the path of glycolysis, the labelled pentose must first be converted to fructose-1,6-diphosphate. This is accomplished by a series of transaldolase- and transketolase-catalysed reactions in the non-oxidative pentose pathway[182] (Scheme (21)). By this route, [1-^{14}C]ribose yields [1,3-^{14}C]-fructose-1,6-diphosphate in which activity at C-1 is twice that at C-3.[183,184] Aldolase cleavage of [1,3-^{14}C]fructose-



Scheme 21: Distribution of Label within Fructose-1,6-Diphosphate Derived from [1-^{14}C]Ribose via the Nonoxidative Pentose Pathway.

1,6-diphosphate leads in the first instance to [1,3-^{14}C]dihydroxyacetone-1-phosphate, in which activity at C-1 is twice that at C-3, and to unlabelled glyceraldehyde-3-phosphate. Partial equilibrium by isomerase activity leads to D-[1,3-^{14}C]glyceraldehyde-3-phosphate whose activity at C-3 is twice that at C-1, but whose specific activity is lower than that of [1,3-^{14}C]-dihydroxyacetone-1-phosphate. Conversion of the labelled triose phosphates, by the two pathways, yield [1,3-^{14}C]pyruvate, in which the activity at C-3 is twice that at C-1.

Assuming that both sources of pyruvate converge in a common pool, the molar specific activity of the pyruvate pool would be expected to be lower than that of dihydroxyacetone-1-phosphate yet higher than that of glyceraldehyde-3-phosphate.

Incorporation of the fragments derived from [1-^{14}C]-ribose in this manner, into the pyridoxol carbon skeleton would place activity at C-3 and C-4' (via dihydroxyacetone-1-phosphate), at C-5' and C-6 (via glyceraldehyde-3-phosphate) and at C-2' (via a truncated pyruvate). The activity at C-4' would be twice that at C-3, and the activity at C-5' would be expected to be twice that at C-6, yet lower than that at C-4'. Activity at C-2' would be expected to be lower than that at C-4', yet higher than that at C-5' of pyridoxol.

If such a distribution of activity derived from [1-^{14}C]-ribose was observed within pyridoxol, it may be concluded that ribose enters pyridoxol in a nonspecific manner. Thus, the observation would suggest that the transketolase intermediate, "active glycolaldehyde", does not supply the two-carbon unit required for pyridoxol biosynthesis. Furthermore, it may be

inferred that the pentoses normally formed by way of the non-oxidative pentose pathway do not serve as obligatory precursors of pyridoxol.

Specific incorporation of activity derived from [1-^{14}C]-ribose into pyridoxol, on the other hand, would place high activity at a specific carbon atom of pyridoxol and should this carbon atom be C-2 or C-2' of pyridoxol, then ketolase intermediates including "active glycolaldehyde" must be postulated as mandatory precursors, giving rise to the C_2 unit, C-2,-2' of pyridoxol.

Radioactive pyridoxol derived from [1-^{14}C]ribose (Experiment 10) was degraded and was found to contain 34 $\pm$ 1% and 13 $\pm$ 1% of the entire pyridoxol activity at C-2,-2' and C-5,-5', respectively (Table (9), Fig. (14C)). The distribution of the remaining activity was not determined due to insufficient sample.

On this basis, it can be concluded that ribose enters pyridoxol in a nonspecific manner since less than half (47 $\pm$ 2%) of the entire pyridoxol activity could be accounted for among four carbon atoms isolated. Yet, assuming that [1-^{14}C]ribose gives rise to [1,3-^{14}C]fructose-1,6-diphosphate as stated by Woods and Katz,[183] the remaining activity within pyridoxol should be predictable.

On the basis of the predicted distribution of activity within the labelled triose phosphates generated by aldolase

cleavage, it is inferred that C-4' of pyridoxol would contain approximately one-third of the total pyridoxol activity, that carbon 3 should contain half the activity of that of C-4', i.e., 16%, and that activity at C-6 should be half of that of C-5', i.e., 6%. Such a distribution of label within pyridoxol from [1-^{14}C]ribose suggests, solely by inference and on the basis of incomplete recovery of label, that C-2' and C-4' contrary to prediction would contain the same amount of activity. This suggestion, however, would be in complete agreement with the distribution of label found within pyridoxol derived from [1-^{14}C]glucose and would imply that pyruvate from dihydroxyacetone-1-phosphate, by way of pyruvaldehyde, is used for pyridoxol biosynthesis in mutant WG2.

3.2.3.3 Substrates Dismissed as Progenitors of C-2,-2' of Pyridoxol

Unequivocal evidence has now been acquired which places pyruvate on the path to pyridoxol as the progenitor of the C_2 unit, C-2,-2' of the vitamin biosynthesized by E. coli B mutant WG2. However, the identity of the two-carbon compound derivable from pyruvate, which is the direct precursor of the C_2 unit, C-2,-2' of the pyridoxol carbon skeleton, remains to be established. Acetyl Coenzyme A, acetaldehyde and acetate are the major two-carbon end-products which are formed by the decarboxylation of pyruvate.

The formation of free acetaldehyde by the decarboxylation of pyruvate has been shown to occur only by pyruvate decarboxylase isolated from wheat germ and yeast. It could not be demonstrated in other enzymic reactions which catalyse the decarboxylation of pyruvate.[174] However, acetaldehyde, bound to thiamin pyrophosphate, i.e., α-hydroxyethylthiamin pyrophosphate (or "active acetaldehyde"), has been shown to be the initial intermediate formed after the decarboxylation of pyruvate in all the reactions investigated.[110] Thiamin-bound acetaldehyde does not readily dissociate from the co-factor unless an acceptor is present[174] as in the formation of acetolactate. Thus, thiamin-bound acetaldehyde ("active acetaldehyde") rather than free acetaldehyde will be discussed later in this chapter in context with acetyl CoA formation catalysed by pyruvate dehydrogenase complex isolated from E. coli.

Since acetyl CoA can be derived in E. coli not only from pyruvate but also from acetate,[186] presumably by way of the acetyl kinase product, acetylphosphate (35),[187] followed by phosphotransacetylase reaction, the possible role of acetate as a source of the C_2 unit required for pyridoxol biosynthesis must be examined.

In an earlier experiment, in which [2-^{14}C]acetate was administered as a tracer to a culture of mutant WG2[75] ap-

proximately sixty percent of the total activity of the isolated pyridoxol was found to reside at the C_2 unit, C-2,-2' (Fig. 14A). Since activity into pyridoxol was low and entry into C-2,-2' was not quantitative, it was concluded that acetate did not serve as a direct precursor of the C_2 unit and that the observed distribution of activity within pyridoxol could have arisen if acetate entered pyridoxol by way of the reversal of glycolysis.

Another experiment with acetate, this time with [1-^{14}C]acetate has now been carried out (Experiment 9). The isolated pyridoxol again contained sixty percent of its total activity in the C_2 fragment, C-2,-2' (Table (9), Fig. (14B)).

The observed distribution of label within pyridoxol derived from radioactive acetate, in the two experiments is consistent with the idea that the C_2 unit, C-2,-2' of pyridoxol is acetate derived. The evidence from two other experiments demonstrates that this is not so, however.

The presence of equimolar inactive acetate in the culture medium did not alter the distribution of label within pyridoxol derived from [2-^{14}C]glycerol (Experiment 12, Table (10), Fig. (15B)) from that observed in the absence of acetate. This result contrasts with that of the earlier experiment (Experiment 11) where addition of pyruvate altered the distribution of label from [2-^{14}C]glycerol. It must be concluded that pyruvate serves as C_2 donor in pyridoxol biosynthesis, whereas acetate does not. The low level of incorporation of radioactivity into pyridoxol derived from [1-^{14}C]- and [2-^{14}C]-

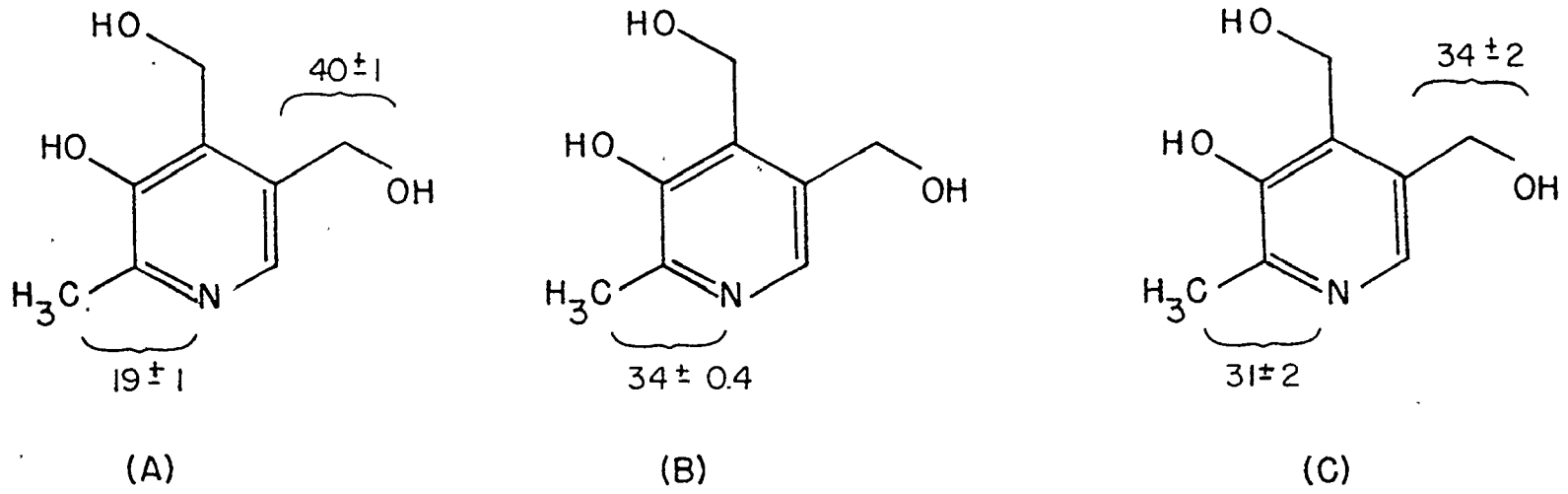
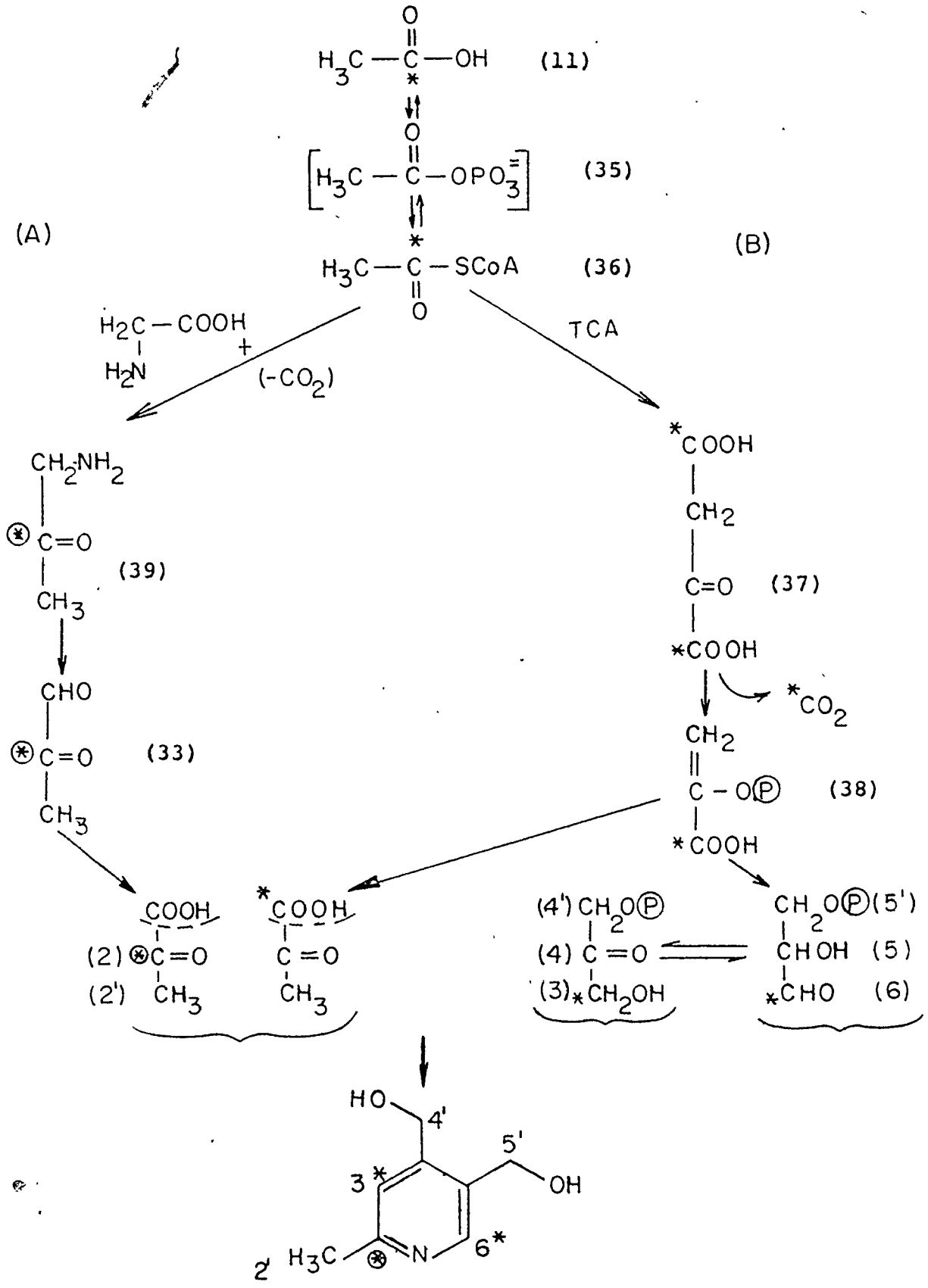


Figure 15: Distribution of Activity found within Pyridoxol Derived from [2-^{14}C]Glycerol in the Presence of A) Sodium Pyruvate, B) Sodium Acetate and C) Lithium-3-Hydroxypyruvate (Activity of Intact Pyridoxol = 100; Per cent of Total Activity at Indicated Carbon Atoms).

acetate indicated that acetate added to the culture medium does not reach the site of pyridoxol biosynthesis in the cell. However, E. coli B mutant WG2 can be adapted to grow on acetate as a carbon source, and thus must reach the site of pyridoxol biosynthesis. Furthermore, acetate in the form of acetyl CoA (36), formed in situ by the enzymic degradation of DL-[3,3',4,5-$^{14}C_4$]-isoleucine[188] which was administered to mutant WG2, produced pyridoxol which was devoid of activity.[77] This result suggests that even an internal source of acetyl CoA is not involved in the biosynthesis of pyridoxol.

The observed distribution of activity, within pyridoxol, derived from labelled acetate may be explained by the indirect transformation of acetate to glycolytic intermediates prior to its incorporation into pyridoxol (Scheme (22)). The conversion of acetate to acetyl CoA (36) permits acetate to enter the citric acid cycle which generates oxaloacetate (37).[186] Conversion of oxaloacetate (37) to phosphoenolpyruvate (38) by phosphoenolpyruvatecarboxykinase followed by dephosphorylation yields pyruvate. The reversal of glycolysis from phosphoenolpyruvate generates the triose phosphates, which are incorporated into pyridoxol, thereby permitting activity derived from labelled acetate to enter the pyridoxol carbon skeleton (Scheme (22B)).

An alternative route from acetyl CoA to pyruvate, by way of pyruvaldehyde (33) has been described.[189] Reaction of glycine and acetyl CoA catalysed by aminoacetone synthetase, followed by decarboxylation yields 1-aminoacetone (39), which on oxidation



Scheme 22: Incorporation of Activity into Pyridoxol Derived from [1-^{14}C]Acetate - Conversion of Acetate to Glycolytic Intermediates via Two Pathways.

or transamination, renders pyruvaldehyde. Entry of pyruvaldehyde into pyridoxol via pyruvate has already been shown to occur (Experiment 8) (Scheme (22)A).

The path of carbon and thus also the distribution of label from either [1-^{14}C]- or [2-^{14}C]-acetate to oxaloacetate (37)[190] and then from oxaloacetate to the triose phosphates and to pyruvate (and therefore into pyridoxol) can be predicted. On the basis of the amount of activity found in the C_2 fragment, C-2,-2' of pyridoxol (60 $\pm$ 1%) derived from [1-^{14}C]acetate, it can be predicted that carbons 3 and 6 of pyridoxol would be equally labelled and would account for the remaining activity (40%) which was not recovered. Similarly, on the basis of the incomplete recovery of label, it can be predicted that the remaining radioactivity within pyridoxol, derived from [2-^{14}C]acetate, would be found on carbons 3, 4, 4', 5, 5' and 6 so that each of carbons 4, 4', 5, and 5' would account for approximately 8% of the total activity and that each of carbons 3 and 6 would account for 4% of the total pyridoxol activity.[191]

This distribution of radioactivity within pyridoxol, derived from labelled acetate, still awaits experimental confirmation. However, the results from experiments (9) and (12) make it doubtful that acetate, acetyl CoA(36) or acetylphosphate (35) are directly involved with the origin of the two-carbon unit, C-2,-2' of pyridoxol.

Several reports in the literature have alluded to the possibility that the C-2' methyl moiety of pyridoxol arises by a reduction of a hydroxymethyl group in the course of pyridoxol biosynthesis. On this basis, several investigators proposed that 3-hydroxy-2,4,5-trihydroxymethylpyridine (9) might lie on the route to pyridoxol. When tested, this compound was demonstrated to support the growth of two vitamin B_6 dependent yeast strains, Saccharomyces carlsbergensis[68] and Kloechera apiculata.[70] The latter was shown to convert [G-^{3}H]-3-hydroxy-2,4,5-trihydroxymethylpyridine to ^{3}H-labelled pyridoxol and pyridoxal-5'-phosphate. However, when tested with vitamin B_6 auxotrophs of E. coli K12 CR63[50] and E. coli K12,[84] no growth or stimulation of growth was observed.

Schroer and Frieden[69] envisaged the intermediate, 3-hydroxy-2,4,5-trihydroxymethylpyridine (9), arising from glycolaldehyde (22), D-xylulose (28), and glyceraldehyde so that C-2 of glycolaldehyde (hydroxy moiety) would enter either at C-2' or at C-4' (Scheme (12)). However, both these notions were disproven when it was shown that glycolaldehyde was incorporated exclusively into carbons 5 and 5' of pyridoxol.[80] Alternatively, the putative intermediate, 3-hydroxy-2,4,5-trihydroxymethylpyridine (9), could arise by the incorporation of 3-hydroxypyruvate into the C-2,-2' fragment, in an analogous manner to the known incorporation of pyruvate.

Evidence that 3-hydroxypyruvate is associated with pyridoxol biosynthesis emerges from Dempsey's extensive mutant

studies. Shimizu and Dempsey[39,89] have shown that pyridoxine-less mutants of E. coli K12, designated as serC mutants, lacking the enzyme 3-phosphoserine:2-ketoglutarate transaminase[138] and thereby unable to convert 3-phosphohydroxypyruvate to 3-phosphoserine, will synthesize normal amounts of pyridoxol when 3-hydroxypyruvate is present in the culture fluid of mutants, deprived of serine and pyridoxal.

To test the intermediacy of 3-hydroxypyruvate in pyridoxol biosynthesis by E. coli, mutant WG2, a preliminary experiment in which non-radioactive 3-hydroxypyruvate and [2-^{14}C]glycerol were used as competitive substrates (Experiment 13) was carried out. The distribution of activity within the isolated pyridoxol sample from Experiment 13, derived from labelled glycerol, did not differ from that within a pyridoxol sample derived from an incubation in the absence of 3-hydroxypyruvate (Table (10), Fig. (15C)). The fact that the presence of 3-hydroxypyruvate did not spare the incorporation of label into pyridoxol derived from [2-^{14}C]glycerol, while the presence of pyruvate did (Experiment 11), indicates that 3-hydroxypyruvate is not associated with the origin of the C_2 unit C-2,-2' of pyridoxol in WG2. However, it may be argued that 3-hydroxypyruvate administered to the culture medium may not reach the site of pyridoxol biosynthesis in the cell. This behaviour of 3-hydroxypyruvate in the serC mutants and in WG2 resembles that of glycolaldehyde in mutants

WG3 and WG2 (Scheme (9), Fig. (C4)) and requires further experimental testing since direct tracer evidence bearing on the intermediacy of 3-hydroxypyruvate is, as yet, not available.

3.2.3.4 Plausible precursors for the C_2 unit, C-2,-2' of Pyridoxol

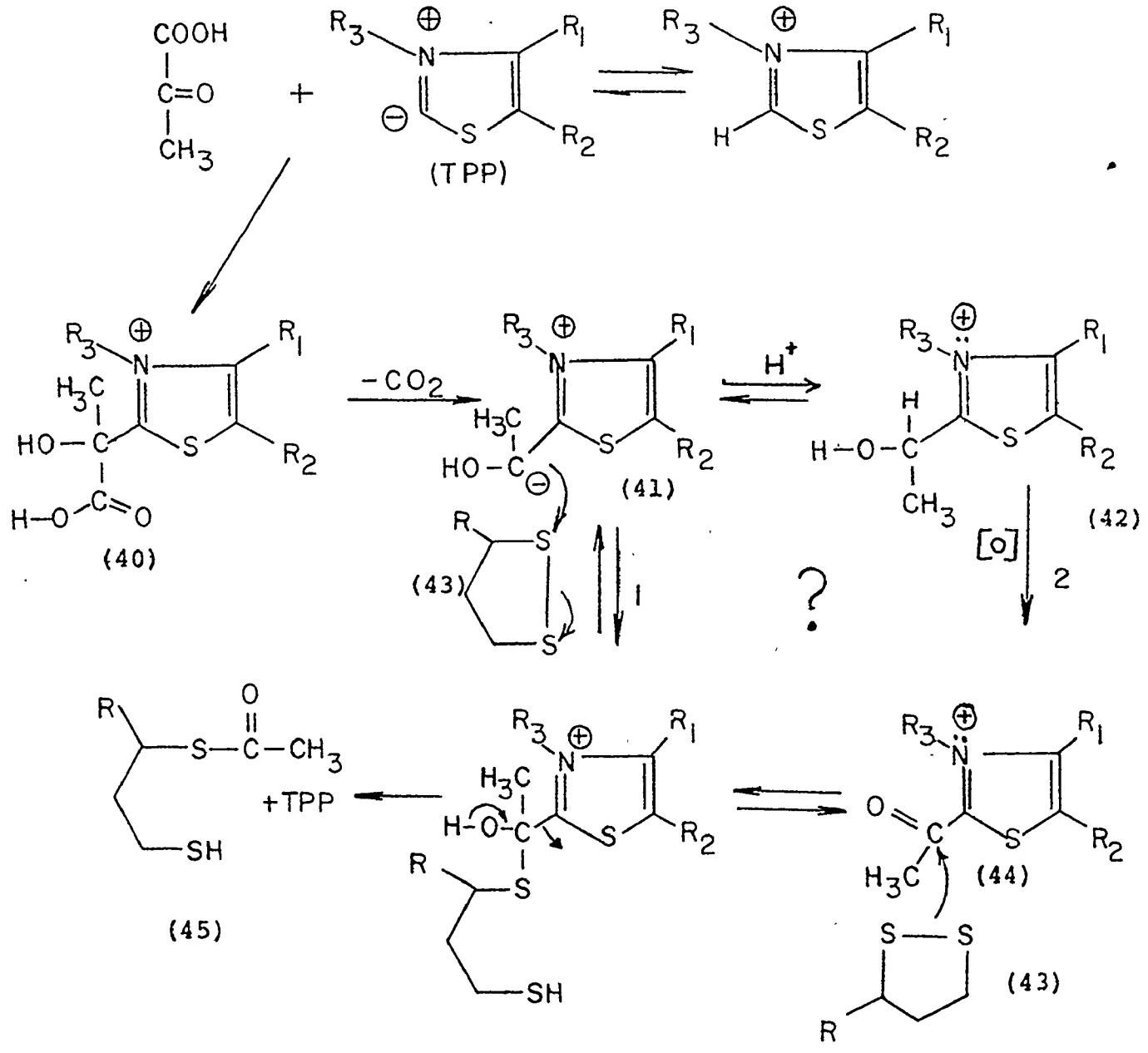
Evidence has now been presented which ensures that pyruvate can effectively supply the two-carbon unit required for pyridoxol biosynthesis. Only the methyl and carbonyl moieties of pyruvate are incorporated into the pyridoxol skeleton and yet the end-products of pyruvate catabolism are ineffective as C_2 donors for the C_2 unit, C-2,-2' of pyridoxol. The mechanism of the enzymic decarboxylation of pyruvate must, therefore, be examined.

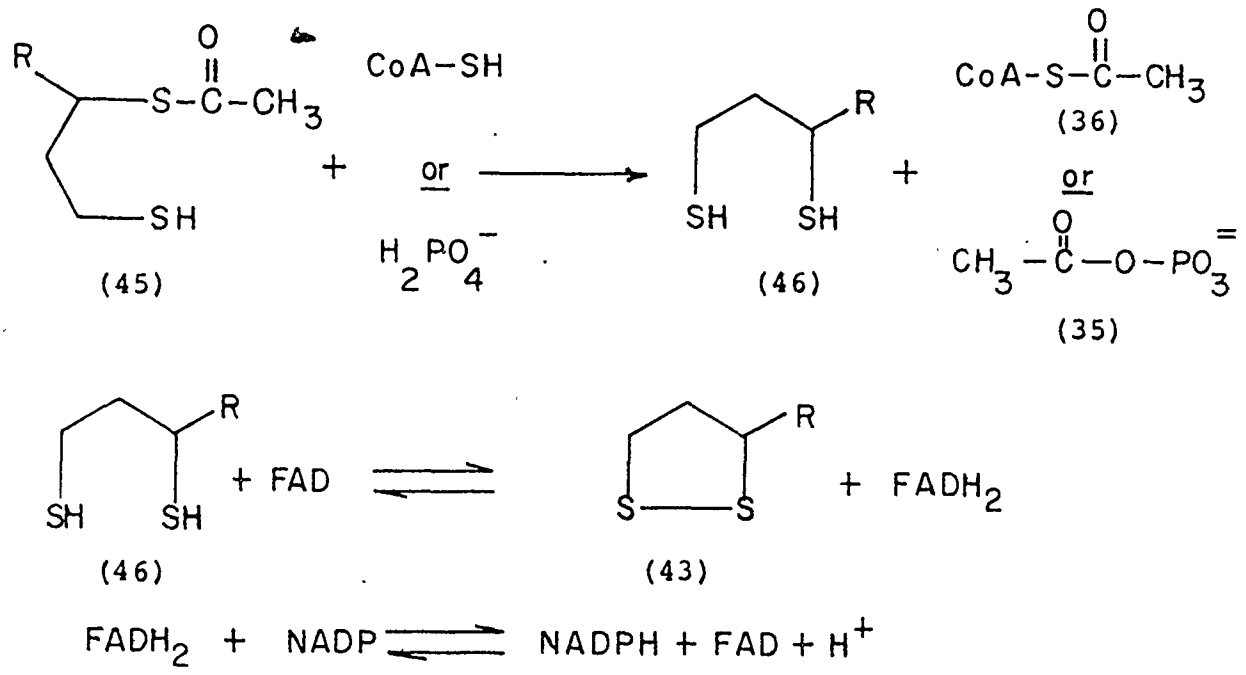
There are several enzymic reactions catalysing the decarboxylation of pyruvate which are thiamin pyrophosphate dependent. These reactions differ in the end-products which are produced by the catabolism of pyruvate, e.g., pyruvate dehydrogenase yields acetyl CoA, pyruvate oxidase yields acetate, pyruvate decarboxylase yields acetaldehyde, and acetolactate synthase yields acetolactate. Although these reactions produce different end-products, similarities do exist. All these enzymic reactions require thiamin pyrophosphate as a cofactor, thus it is assumed that the initial condensation of thiamin pyrophosphate and pyruvate proceeds according to the mechanism which was originally proposed by

Breslow,[116] i.e., nucleophilic attack of the ylide of thiamin pyrophosphate with the carbonyl moiety of pyruvate. The resulting lactylthiamin pyrophosphate adduct ("active pyruvate") (40) rapidly undergoes decarboxylation to yield a second carbanion ("active acetaldehyde") (41). This second carbanion is resonance stabilized with the corresponding enamine form (42), Scheme (23). Reaction of "active acetaldehyde" adduct with a suitable substrate or acceptor produces the respective end-product, and regenerates the ylide of thiamin pyrophosphate The mechanism for acetyl CoA formation by the pyruvate dehydrogenase complex in E. coli has been studied extensively. A brief review of this reaction mechanism will follow.

It is generally accepted that the aerobic oxidation of pyruvate in E. coli proceeds mainly by the reactions catalysed by the multienzyme complex, pyruvate dehydrogenase, to yield the product acetyl CoA via an enzyme-bound "active acetaldehyde" (41) intermediate. The work of Reed et al.[192-194] has demonstrated that the dehydrogenase complex from E. coli consists of three enzymes: Pyruvate decarboxylase (E.C. 1.2.4.1), lipoate acetyltransferase (E.C. 2.3.1.12) and lipoamide dehydrogenase (E.C. 1.6.4.3). Together these components effect the oxidation of pyruvate to acetyl CoA (Scheme (23)).

The reaction sequence begins with the addition of pyruvate to C-2 of the thiazolium ring of thiamin pyrophosphate (ylide) to form an enzyme-bound lactylthiamin pyrophosphate adduct (40), also known as "active pyruvate".[110,116] Rapid


COOH
C=O
CH3
+
R3
N
R1
S
R2
(TPP)
H
(40)
-CO2
H3C
HO-C
(41)
H+
H-O-C
CH3
(42)
[O]
2
(43)
1
?
+TPP
SH
(45)
O
(44)



Scheme 23: Reaction Sequence for Decarboxylation of Pyruvate by Pyruvate Dehydrogenase Complex.

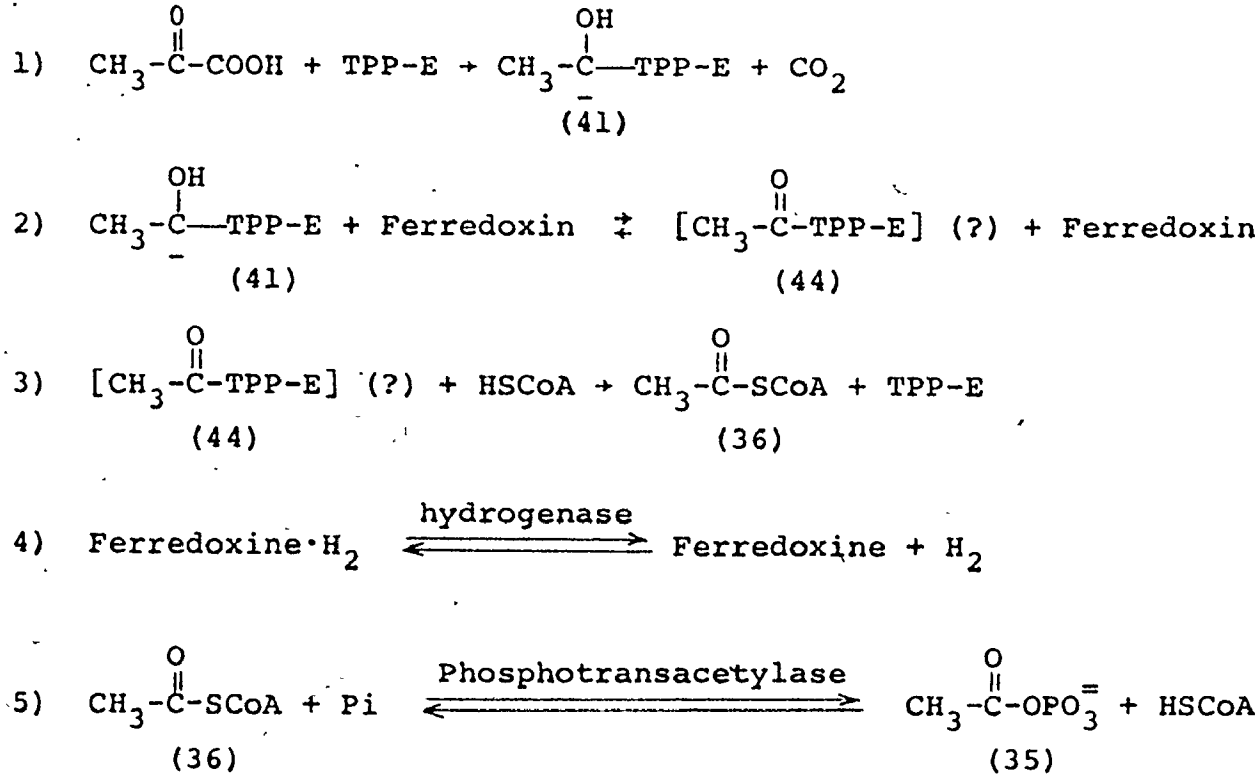
decarboxylation of the lactyl moiety yields α-hydroxyethylthiamin pyrophosphate or "active acetaldehyde" (41), which has been isolated[110,174] from in vitro experiments[195,196] and can be envisaged as a stabilized thiamin-bound acetyl carbanion (41). The mechanism for the conversion of the acetyl carbanion to an acetyl moiety has not been fully elucidated, however. This oxidation can be envisaged to occur either by transfer of the acetyl carbanion from the thiamin pyrophosphate adduct to the protein-bound lipoic acid (43)[194] with concomitant oxidation of the hydroxyethyl moiety to an acetyl moiety (Scheme (23), path 1), or by prior oxidation of the thiamin-bound α-hydroxyethyl moiety (42) by lipoic acid to yield a 2-acetylthiamin pyrophosphate (44) intermediate which, in turn, acetylates the reduced lipoic acid (Scheme (22), path 2). Data which support the latter mechanism have been reported by Reed et al.[197] By either mechanism, 6-S-acetyldihydrolipoic acid (45) is ultimately formed and can function as an acetyl donor to an acceptor such as coenzyme A, in a reaction catalyzed by lipoateacetyltransferase.

A dehydrogenase specific for dihydrolipoic acid (46) yields the oxidation product lipoic acid and ultimately transfers electrons to nicotinamide adenine dinucleotide phosphate (NADP) (Scheme (23)).

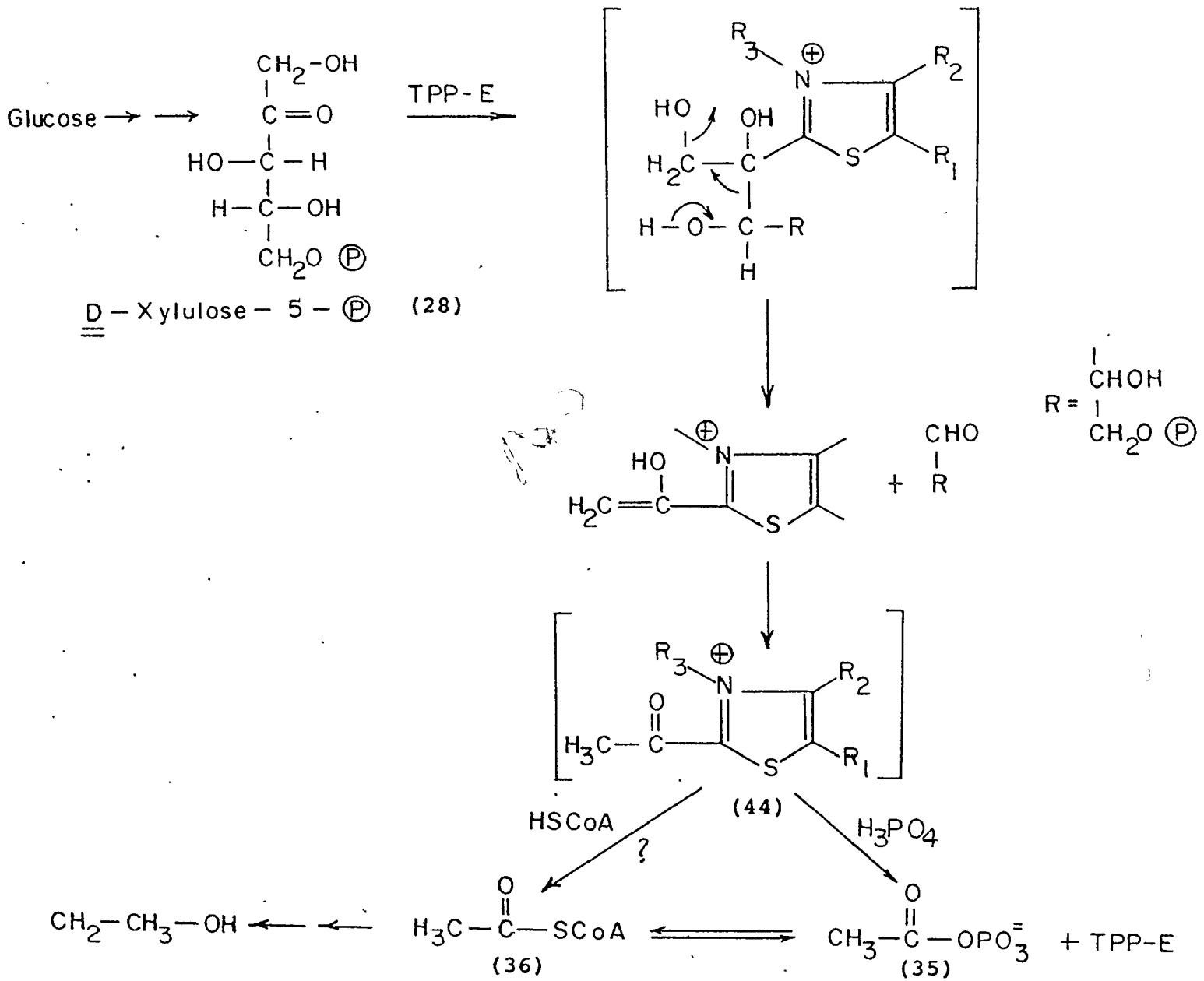
Since it is the same two carbon atoms of pyruvate which give rise to the two-carbon atom adduct of thiamin and to the C_2 unit, C-2,-2' of pyridoxol, it is conceivable that

one of the intermediates associated with the pyruvate dehydrogenase complex might function as an acyl donor in pyridoxol biosynthesis. The first intermediate encountered after the decarboxylation of pyruvate is the "active acetaldehyde" (41) adduct. It can serve as an aldehyde donor in enzymic processes such as the production of acetoin, acetolactate and α-aceto-α-hydroxybutyrate. If the adduct is to serve as an acyl donor, reaction must be accompanied by an oxidation. On the other hand, oxidation of the "active acetaldehyde" adduct by lipoic acid (43) (Scheme (23), path 2) or by ferredoxin (in the phosphoroclastic reaction in anaerobic fermentative bacteria, catalysed by pyruvate ferredoxin oxidoreductase) would yield the intermediate, 2-acetylthiamin (44), Scheme (24). The 2-acetylthiamin adduct could also be envisaged as a product of rearrangement of dihydroxyethylthiamin pyrophosphate during the conversion of xylulose-5-phosphate to acetylphosphate and glyceraldehyde-3-phosphate in the phosphoketolase reaction (Scheme (25)) in anaerobic bacteria.[114]

Although the high energy intermediate, 2-acetylthiamin-pyrophosphate (44), has yet to be shown as a stable intermediate formed by any enzymic reaction,[174] model studies with pyruvate decarboxylase from E. coli in the presence of an artificial electron acceptor such as ferricyanide have demonstrated the oxidation of pyruvate to acetylphosphate (35) (presumably via 2-acetylthiamin adduct) (44) and CO_2.[197] Other investigations have



Scheme (24): Intermediacy of 2-Acetylthiamin in Phosphoroclastic Reaction in Anaerobic Bacteria.



Scheme 25: 2-Acetylthiamin Formation from Pentose Phosphate by Phosphoketolase Reaction in Anaerobic Bacteria.

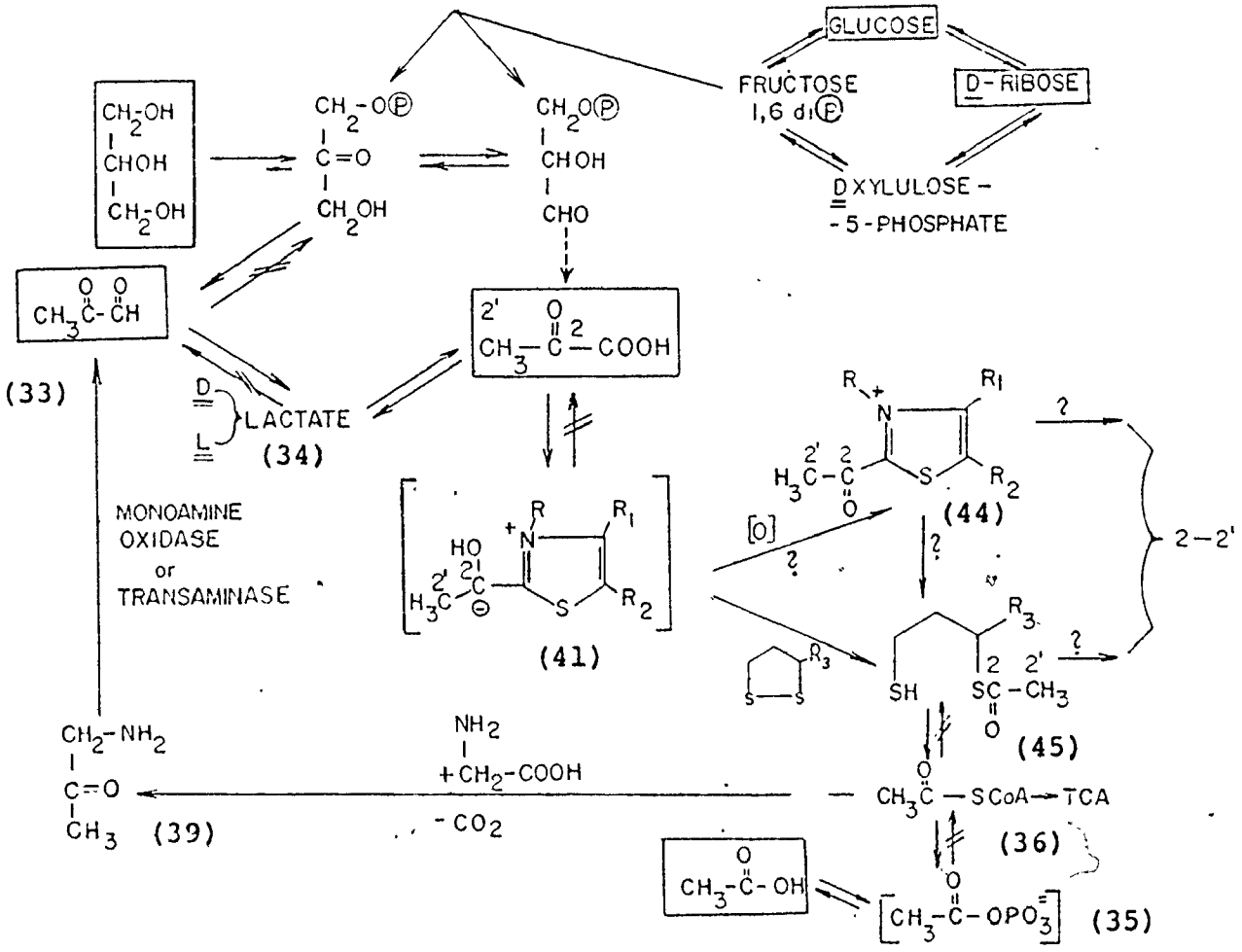
indicated that acyl thiazolium ions are quite labile and would be effective as acyl donors in biological systems.[198-201]

Another acyl donor present in the pyruvate dehydrogenase complex is 6-S-acetyldihydrolipoic acid (45). Lipoic acid (43), necessary for the decarboxylation of α-ketoacids, is regarded as an acyl-generating acyl-transferring agent in this complex. Its function is to transfer an acyl moiety to Coenzyme A. However, a broader specificity of this enzyme could produce a general acylating agent in biological systems.[174]

According to the working hypothesis,[75-78] to serve as a building block of the pyridoxol carbon skeleton, the acyl donor must react with C-3 of dihydroxyacetone-1-phosphate (or its enol form). This would lead to 1-phospho-3-hydroxypentane-2,4-dione as a possible precursor of the carbon atoms 2', 2, 3, 4 and 4' of pyridoxol. An intermediate formed in this fashion would be in complete agreement with the tracer evidence regarding the origin of the C_2 unit, C-2,-2' of pyridoxol, and would satisfy the requirement proposed by Dempsey[51] that pyridoxol biosynthesis is thiamin-dependent. A sequence of reactions for the origin of the C_2 unit, C-2,-2' of pyridoxol, which is based on known biochemical transformations is presented in Scheme (26).

3.3 Revised Hypothesis for the Biosynthesis of Vitamin B_6

The mode of incorporation into pyridoxol of radioactivity from several labelled substrates has been examined



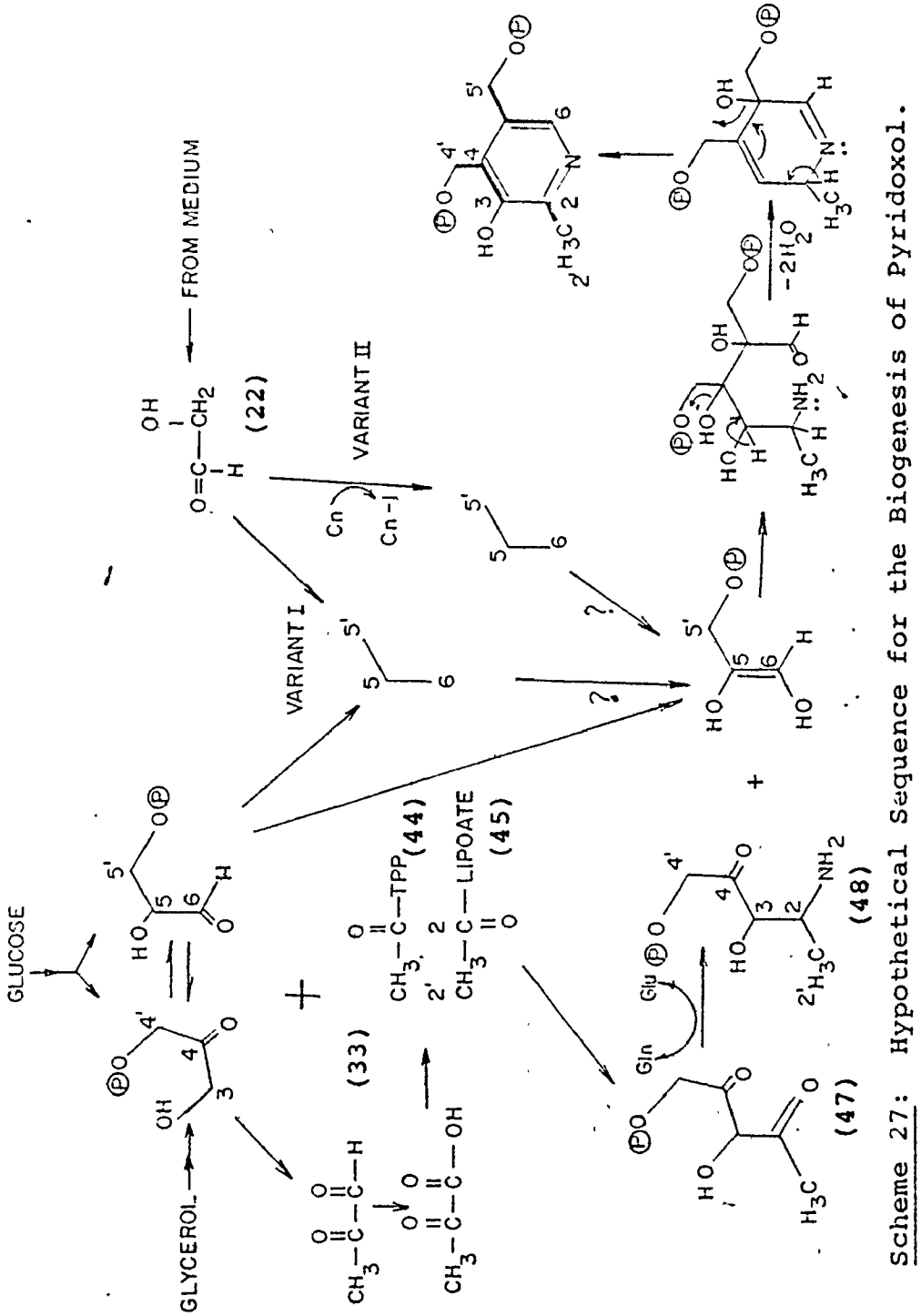
Scheme 26: Postulated Origin for the C_2 Unit C-2,-2' of Pyridoxol. Boxed Substrates have been Tested as Precursors.

in pyridoxineless mutants of E. coli B. Of these substrates, glycerol, pyruvate and glucose have been demonstrated to be specifically incorporated into the carbon skeleton of the vitamin.[75-79] On this basis, pyridoxol is derived from triose units generated by glycolysis: Dihydroxyacetone-1-phosphate and a two-carbon unit, derivable from pyruvate, undergo an aldolase-catalysed reaction to yield 5-deoxy-D-pentulose-1-phosphate which then condenses with glyceraldehyde-3-phosphate and a nitrogen source to ultimately yield pyridoxol (Scheme (2)).

The results reported in this thesis are in agreement with the hypothesis that glycolysis-derived triose units can serve as a source of the structural framework of pyridoxol. However, these results are interpreted to indicate that a 4-amino-4,5-dideoxypentulose (48) rather than 5-deoxypentulose, might serve as the progenitor of C-2',-2,-3,-4,-4'. Furthermore, it is now shown that in a mutant which closely resembles the wild type strain, glycolaldehyde is not an obligatory intermediate on route to pyridoxol. Hence, glycolaldehyde is not considered as an intermediate in the biosynthesis of the vitamin in the latter organism. These interpretations are consistent with all tracer and mutant evidence obtained and are based on several known biochemical reactions (Scheme (27)).

3.3.1 The Origin of C-2,-2' of Pyridoxol

It has been shown that pyruvate can be derived from



Scheme 27: Hypothetical Sequence for the Biogenesis of Pyridoxol.

dihydroxyacetone-1-phosphate via pyruvaldehyde (33) by way of a "glycolytic shunt".[176-178] On this basis, the incorporation of radioactivity into pyridoxol derived from [2-^{14}C]pyruvaldehyde must occur by way of pyruvate (Experiment 8). Furthermore, the existence of the "glycolytic shunt" explains the results of the experiments with labelled glucose[78] and [1-^{14}C]-ribose (Experiment 10) and supports the notion that pyruvate is the C-2 donor required for pyridoxol biosynthesis.

More evidence has been acquired which indicates that pyruvate is the intermediate which gives rise to the C_2 unit, C-2,-2' of pyridoxol. Of all the substrates tested in isotope competition experiments with [2-^{14}C]glycerol, only pyruvate was observed to alter the distribution of radioactivity within pyridoxol (Experiment 11).

The normal catabolic products of pyruvate (acetate, acetyl CoA and acetylphosphate) have been eliminated as possible precursors of the two-carbon unit (Experiments 9 and 12). Thus, a high energy pyruvate derivative, generated by the pyruvate dehydrogenase complex and inferred to be bound to thiamin pyrophosphate or to lipoate, is proposed to provide this carbon fragment of pyridoxol by an acetyl transfer reaction.

On this basis, a hypothetical scheme, which is consistent with all tracer evidence and which supports Hill's postulate of triose intermediacy, is presented (Scheme 27).

Acetylation of dihydroxyacetone-1-phosphate to yield 3-hydroxy-1-phosphopentane-2,4-dione (47), followed by transamination with glutamine:2-ketoglutarate, is proposed to give rise to an amino sugar, 4-amino-4,5-dideoxypentulose-1-phosphate (48). Condensation with glyceraldehyde phosphate would ultimately lead to pyridoxol.

There is no direct evidence that 4-amino-4,5-dideoxy pentuloses exist in nature. However, several other 4-amino-4-deoxypentoses and hexoses have been isolated from bacteria[202] and/or have been synthetically prepared and are well characterized.[203] Of the pentoses, 4-amino-4-deoxy-L-xylose and its 5-deoxy analogue have been synthesized.[204,205] Furthermore, the six-membered sugars, 4-amino-4,6-dideoxy-D-glucose and its 4-epimer have been synthesized[206,207] and have been isolated from extracts of E. coli B,[208] E. coli Y-10[209] and Chromobacterium violaceum.[210] In E. coli, these amino sugars are thought to arise from thymidine-5'-pyrophosphate-bound 6-deoxy-4-ketoglucose by transamination with glutamate which serves as a nitrogen source.[211,212]

The biosynthesis of L-rhamnose (6-deoxyarabinohexulose) is also thought to occur in this manner in Strepomyces griseus.[213] E. coli, adapted to grow on a medium containing L-rhamnose, convert it to 6-deoxy-4-uloarabinohexose-1-phosphate.[214] Hence, it is not unreasonable to propose that 4-amino-4,5-dideoxypentulose-1-phosphate, formed via the diketone intermediate (47), is also present in E. coli, even though it has

not yet been isolated. The difficulty in the isolation of 4-amino-4-deoxysugars is due to their decomposition during acid hydrolysis of polysaccharides. The six-carbon amino sugars decompose to acid-labile pyrrole derivatives, presumably by dehydration.[206,215]

The hypothetical sequence (Scheme 27) accomodates Dempsey's postulates that thiamin and 2-ketoglutarate (glutamate) are required for pyridoxol biosynthesis and assigns possible roles for their function.

3.3.2 Incorporation of Glycolaldehyde

The specific incorporation of the carbon atoms of glycolaldehyde into vitamin B_6 by two mutants of E. coli, WG3 and WG2, is suggested to occur by a minor route leading to pyridoxal and pyridoxol, respectively. Whether this route is induced when glycolaldehyde is a nutritional supplement or whether it is normally operative is a question which cannot be answered on the basis of the available data.

In mutant WG2, which is blocked at the oxidase step of pyridoxol biosynthesis and hence is more representative of the wild-type strain, carbons 1 and 2 of glycerol can supply C-5',-5 of pyridoxol,[78,80] respectively. The carbon atoms of glycolaldehyde (C-2,-1, respectively) can also supply these carbon atoms of pyridoxol (Experiment 2) but at a reduced efficiency (Experiments 5 and 6). Yet, the presence of glycolaldehyde failed to alter the distribution within pyridoxol

of radioactivity derived from [2-^{14}C]glycerol (Experiment 4). On this basis, glycolaldehyde was considered not to be an obligatory intermediate required for pyridoxol biosynthesis in this mutant.

An interpretation for the apparent utilization of glycolaldehyde in pyridoxol biosynthesis in mutant WG2 is that a second source of the C_3 unit, C-5',-5,-6, which can be used for pyridoxol biosynthesis, exists. Whether the glycolaldehyde-derived intermediate is the same or similar (Varient I and II) (Scheme (11)) to that derived from glycerol is not known, however.

In mutant WG3, a glycolaldehyde-requiring organism, carbons 5 and 5' are solely derived from C-1,-2 of glycolaldehyde,[80] respectively. Activity from [2-^{14}C]glycerol is not incorporated into this two-carbon fragment of pyridoxol (Experiment 3). On the basis that two sources of the C_3 unit exist, mutant WG3 must contain a genetic lesion which prevents the synthesis of the glycerol-derived intermediate which normally supplies C-5',-5,-6 of pyridoxol, so that the course, using glycolaldehyde, usually the minor source, becomes the sole source for this three-carbon fragment.

The genetic lesions in mutant WG3 must prevent the synthesis of the intermediate which is normally used for pyridoxol biosynthesis by the major route; hence, the mutant utilizes the minor route to synthesize this intermediate from glycolaldehyde. In the major pathway; D-glyceraldehyde-3-

phosphate is the proposed precursor for the three-carbon fragment of pyridoxol. Thus, mutant WG3 is unable to synthesize either D-glyceraldehyde-3-phosphate or the intermediate used for pyridoxol biosynthesis. The minor pathway must convert glycolaldehyde to either D-glyceraldehyde-3-phosphate or an unidentified intermediate also derivable from D-glyceraldehyde-3-phosphate (Variant I, Scheme (10)).

The conversion of glycolaldehyde to D-glyceraldehyde-3-phosphate can be envisioned to occur by the carboligase reaction as shown in Scheme 16 and may be considered as the minor route for pyridoxal biosynthesis. This possibility can be eliminated on the basis of the evidence obtained from both tracer and mutant investigations, however.

If [2-^{14}C]glycolaldehyde were converted to [3-^{14}C]-glyceraldehyde-3-phosphate, label would be expected to reside at C-5' of pyridoxal, as was found. However, isomerase activity should convert [3-^{14}C]glyceraldehyde-3-phosphate to [1-^{14}C]dihydroxyacetone-1-phosphate. Hence, in pyridoxol derived from [2-^{14}C]glycolaldehyde label would be expected not only at C-5', but also at C-4' and C-2'. This was not found.

Lack of D-glyceraldehyde-3-phosphate is unlikely since the triose is formed from several sources: From glycerol via dihydroxyacetone-1-phosphate, from glucose via fructose-1,6-diphosphate and from pentoses by transketolase reaction.

Thus, the genetic lesion in mutant WG3 would have to affect at least three different enzymes.

Alternatively, if D-glyceraldehyde-3-phosphate were not the direct intermediate as proposed by Hill et al.,[77] but is converted to a related triose which is also formed by the minor route, glycolaldehyde utilization for pyridoxol biosynthesis in mutant WG3 might be explained (Scheme 10). That is, in mutant WG3, the major pathway from D-glyceraldehyde-3-phosphate to a triose intermediate normally used for pyridoxol biosynthesis is blocked and hence the glycolaldehyde route becomes the major source of this intermediate. If this proposal is correct, then the intermediate cannot be directly derivable from glucose or glycerol, otherwise mutant WG3 would produce pyridoxal from these compounds as sole carbon sources.

The mode of incorporation of radioactivity from [2-^{14}C]-glycerol into pyridoxal (Experiment 3) in WG3 leads to the inference that C-6 of pyridoxal is derived from C-2 of glycerol by the glycolaldehyde route. Thus, glycolaldehyde must react with a compound containing C-2 of glycerol to form the required intermediate for pyridoxal synthesis. This implies the formation of a different intermediate than that formed by the major route (Variant II) since C-6 of pyridoxol from mutant WG2 is derived from C-1 of glycerol.[79] On this basis, Variant II (two separate sources to the C_3 unit) is favoured for the explanation of glycolaldehyde utilization.

Recently, there has been some question regarding the location and number of genetic lesions in the pdxB mutants[39] as well as the role of glycolaldehyde in pyridoxol biosynthesis.[135] These observations support the inference that the minor route is induced when glycolaldehyde is added to the culture and evidence is now available which negates the obligatory intermediacy of this two-carbon compound in the biosynthesis of vitamin B_6.

3.3.3 Incorporation of Acetate and Aspartate

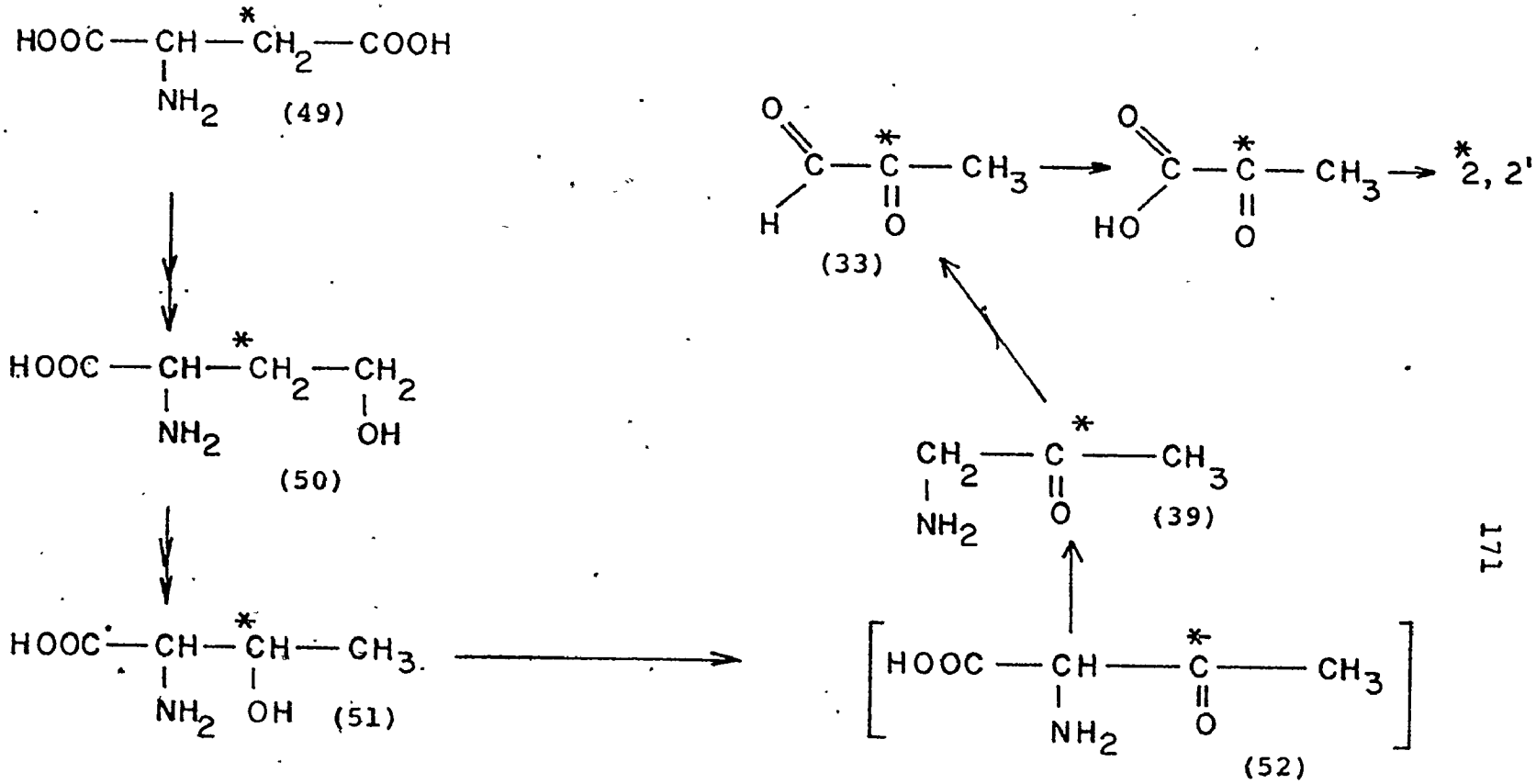
Experiments 9 and 12 clearly demonstrate that acetate is not the C_2 donor which gives rise to the C_2 unit, C-2,-2' of pyridoxol. These results (Tables 9 and 10) support Hill's notion that acetate is not an intermediate on route to the vitamin. The incorporation of radioactivity derived from [2-^{14}C]acetate[77] and [1-^{14}C]acetate (Experiment 9) has been interpreted to suggest that acetate is converted to pyruvate and to the triose intermediates which make up the pyridoxol carbon skeleton. Formation of pyruvate from acetate (acetyl CoA) can occur by two routes: 1) The condensation of acetyl CoA and glycine followed by decarboxylation and transamination[189] yields pyruvaldehyde (Scheme (22)), which can lead to pyruvate; and, 2) Acetyl CoA enters the Krebs' cycle and forms oxaloacetate which is converted to phosphoenolpyruvate and to pyruvate. The latter route also permits the triose phosphates to be labelled by reversal of glycolysis

from phosphoenolpyruvate (Scheme (22)). Entry of labelled pyruvate from glycolytic triose units into pyridoxol permits activity from ^{14}C-acetate to be incorporated into the vitamin.

The incorporation of radioactivity derived from labelled aspartate (49)[69,77] can also be rationalized by conversion to pyruvate[77,191] in a similar manner. Conversion of aspartate to pyruvate and the glycolytic intermediates via the Krebs' cycle has been described.[191] Conversion to pyruvate can also occur by the catabolism of threonine (51) via the aminoacetone cycle[189,216] (Scheme (28)).

Aspartate (49) is the primary source of homoserine (50) and threonine (51) in E. coli and other bacteria.[217,218] All enzymes and cofactors in this biosynthetic pathway have been isolated and purified.[219] The conversion of threonine (51) to aminoacetone (39) in bacteria was first reported by Elliot[220] and presumably involves the spontaneous decarboxylation of the β-ketoacid, 2-amino-3-ketobutyrate (52).[189] Transamination or oxidation by monoamine oxidase[220] yields pyruvaldehyde which may then be converted to pyruvate.

The mode of incorporation into pyridoxol of pyruvate derived from [3-^{14}C]aspartate via the aminoacetone cycle and via phosphoenolpyruvate should place label at C-2 of the vitamin.[77] Thus, the observed distribution (59% at C-2,-2') of activity from [3-^{14}C]aspartate can be rationalized by these two pathways to pyruvate. The remaining 41% of activity must be randomly distributed within pyridoxol and arises by entry



Scheme 28: Pyruvate Formation from Aspartate (49) via Homoserine (50), Threonine (51) and 1-Aminoacetone (39).

of the labelled triose units via the reversal of glycolysis.

3.3.4 The Role of 3-Hydroxypyruvate

From the preliminary results obtained from Experiment 13 (Table (10)), it is inferred that 3-hydroxypyruvate is not an intermediate on route to pyridoxol as implicated in mutant studies.[89] Its behaviour in the isotope competition experiment (Experiment 13) is similar to that of glycolaldehyde (Experiment 4) and demonstrates no change in the distribution of label within pyridoxol derived from [2-^{14}C]glycerol. Two models may be constructed on this basis.

The first model suggests that 3-hydroxypyruvate is associated with the minor route, giving rise to the same intermediate that is derived from glycolaldehyde.[221] The change in distribution by the incorporation of this intermediate would be too small to be detected with confidence. This model is also based on the earlier work of Morris[86] who showed that growth of *E. coli* mutants was stimulated by 3-hydroxypyruvate as well as glycolaldehyde.[86]

The second model proposes that 3-hydroxypyruvate enters pyridoxol via the glycolytic intermediates and spares the incorporation of [2-^{14}C]glycerol at all sites thereby leaving the distribution of activity within pyridoxol unchanged. 3-Hydroxypyruvate may enter the glycolytic pathway via glyceric acid. The enzyme, 3-phosphoglycerate dehydrogenase, which catalyzes the conversion of 3-phosphohydroxypyruvate to 3-

phosphoglyceric acid, has been purified from E. coli B.[222]

These models can be adequately tested by tracer techniques.

3.3.5 Incorporation of [1-^{3}H,2-^{14}C]Glycerol

The distribution of tracer within pyridoxol derived from ^{14}C and ^{13}C labelled glycerol has been investigated[75-77,79] and forms the basis for the postulated biosynthetic sequence of pyridoxol. The fate of the hydrogen atoms of glycerol was examined by an experiment with doubly labelled glycerol (Experiment 14), to gain more insight into the pathway leading from glycerol to pyridoxol.

The pyridoxol molecule contains eight hydrogen atoms. Seven of these should be derived from the $-CH_2O-$ group of glycerol. The eighth (one of the hydrogen atoms of the methyl moiety which is derived from the primary carbon of glycerol) must be introduced by a subsequent step if the proposed Scheme (27) is correct. Three carbon atoms of pyridoxol (C-2,-4,-5) are known to be derived from C-2 of glycerol. Thus, the maximum ratio of hydrogen atoms at the primary positions of glycerol to secondary carbon atoms of glycerol which could be incorporated into pyridoxol is 7:3 (Fig. (16)).

The sample of [1-^{3}H]glycerol which was used in Experiment 14 contained a molar specific activity of 18.7 $\pm$ 0.5 times greater than that of [2-^{14}C]glycerol as measured by radio assay of [1-^{3}H,2-^{14}C]glycerol tribenzoate, prepared

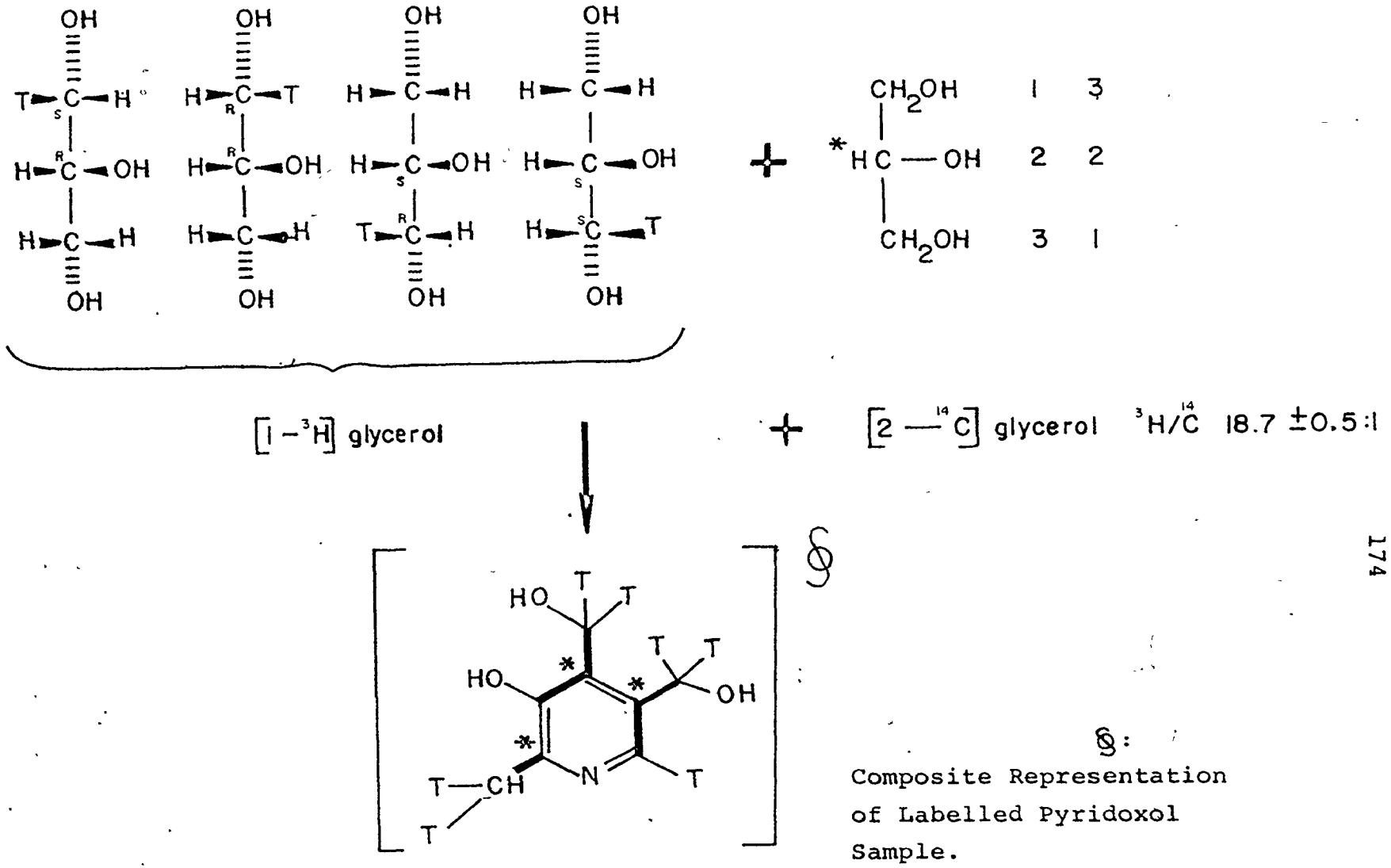


Figure 16: Predicted Sites of Incorporation within Pyridoxol of Activity Derived from [1-^{3}H,2-^{14}C]Glycerol.

from a sample of the mixture [1-^{3}H]- and [2-^{14}C]glycerol. Due to the introduction of tritium at four possible sites on the primary carbon atoms of glycerol, two diasteriomeric enantiotopic pairs of isomers or four "radiostereoisotopemers" are generated and make up the [1-^{3}H]glycerol sample (Fig. (16)). Thus, the average molar specific activity for each tritiated glycerol species containing one tritium atom must be 4.7 times greater than that of [2-^{14}C]glycerol.

If all seven hydrogen atoms of pyridoxol are derived from glycerol, then the composite pyridoxol sample (Fig. (16)) derived from [1-^{3}H,2-^{14}C]glycerol should contain a ratio 32.7:3 (or 10.9:1 after normalizing) of ^{3}H activity relative to ^{14}C. Incorporation of less than seven tritium atoms would decrease this ratio by 4.7 (or 1.5 after normalizing) for each tritium lost. The isolated pyridoxol sample contained 9.4 $\pm$ 0.03 more ^{3}H activity relative to ^{14}C, indicating that six of the possible seven hydrogen atoms are incorporated into pyridoxol.

It has not yet been determined which one of the carbon atoms of pyridoxol is deficient in tritium. Nevertheless, the results of Experiment 14 strengthen the hypothesis that two intact triose units derivable from glycerol construct part of the carbon skeleton of pyridoxol. On this basis, the C_2 unit, derivable from glycerol and pyruvate, required to complete the structure of pyridoxol, is anticipated to have lost a tritium atom. This may occur via enolization

of pyruvate or any methyl ketone intermediate formed along the route to pyridoxol. Alternatively, if carbon 6 contains no tritium, then the intermediacy of D-glyceraldehyde-3-phosphate becomes questionable. Degradation methods for the isolation of carbon 6 are currently being investigated.

3.4 Future Investigations

If the interpretation is correct that two sources exist from which the C_3 unit, C-5',-5,-6, of vitamin B_6 originates, then any future investigation of the minor source must be carried out with mutant WG3. To complete Experiment 3, the remaining activity within pyridoxol derived from [2-^{14}C]glycerol in mutant WG3 must be located. In addition to the activity found at C-2,-2' (34% ± 1, Experiment 3), it is anticipated that C-4 and C-6 should be equally labelled (33% of total activity at each site) and carbons 2',-3,-4',-5 and -5' should be devoid of activity. If this distribution were to be found, a complementary experiment using [1-^{14}C]glycerol would yield pyridoxal labelled equally at C-2', C-3 and C-4' (33% of total activity). Similarly, experiments with [1-^{14}C]- and [6-^{14}C]glucose would yield the same distribution at C-2', C-3 and C-4' and the remaining carbon atoms of pyridoxal should be unlabelled in all three cases.

If C-2 of glycerol gives rise to C-6 of pyridoxal in WG3, the presence of glycine should spare the incorporation of activity, derived from [2-^{14}C]glycerol, into C-6 and hence the distribution of activity at C-2 and C-4 should approach

50% in the limiting case (from 33% to 50%). Furthermore, this experiment would test the intermediacy of glycine (or N-acetylglycine) as proposed in Scheme (17). Degradation leading to the isolation of C-6 of pyridoxal in all the experiments suggested above would confirm or refute the notion that C-6 arises from C-2 of glycerol.

An additional experiment using [3-^{14}C]pyruvate would label pyridoxal only at C-2', if pyruvate were to be the C_2 donor of the unit, C-2,-2' in the minor pathway.

The distribution of activity within pyridoxol derived from these substrates in mutant WG3 should provide evidence which will ratify or disprove the notion that the origin of C-2',-2,-3,-4 and -4' by the minor route is the same as that of the major route in mutant WG2.

An experiment with ^{14}C labelled 3-hydroxypyruvate in WG2 and a serC mutant would demonstrate whether or not activity from this substrate is incorporated into pyridoxol. If labelled, the sites and mode of incorporation of label within pyridoxol must be determined by the isolation of the appropriate degradation products. Together, these experiments would provide evidence for the possible intermediacy of 3-hydroxypyruvate. Although labelled 3-hydroxypyruvate is not commercially available, a method for its synthesis has been described.[223-226]

To test the pathway from aspartate to pyruvate via

the catabolism of threonine, an experiment with [3,4-^{14}C]-homoserine would yield pyridoxol predominantly labelled at C-2,-2'. Activity from this substrate should not be as randomly distributed as that from aspartate since homoserine cannot enter the Krebs' cycle directly. Thus, incorporation of label via pyruvate which is formed by way of aminoacetone and pyruvaldehyde should yield the carbon fragment, C-2,-2' of pyridoxol with more activity than that derived from aspartate (59% at C-2,-2').[77]

Doubly labelled pyridoxol derived from [1-^{3}H,2-^{14}C]-glycerol contained a ratio 9.4 $\pm$ 0.03 of ^{3}H activity relative to ^{14}C (Experiment 14) indicating that a protium atom was incorporated into a site within pyridoxol instead of a tritium atom. There are four possible sites in which this could have occurred (Fig. (16)). To determine with confidence the site which contains less than the maximum tritium label, a systematic degradation method whose mechanism does not favour exchange of protium and tritium atoms must be employed.

Kuhn-Roth oxidation conditions do not exchange the tritium atoms of [2-^{3}H]acetic acid with protium atoms of solvent.[227] On this basis, isolation of the C-2,-2' fragment of pyridoxol as acetic acid[77] followed by radioassay would determine whether C-2' is the site deficient in tritium.

If the Kuhn-Roth acetate contains a 9.4:1 ^{3}H/^{14}C ratio, then no loss of tritium has occurred at this site. However, if tritium is lost, then a 4.7:1 ^{3}H/^{14}C ratio would

be expected.

Alternatively, carbon 6 of pyridoxol may be deficient of tritium. Chemical modification of the C-6 position by oxidation to a carbonyl group would change the $^{3}H/^{14}C$ ratio of pyridoxol to 7.8 if tritium were originally present. Otherwise, the $^{3}H/^{14}C$ ratio of the oxidized product should remain 9.4:1, the same as that of pyridoxol.

The ultimate objective of these tracer experiments is to identify precursors on route to vitamin B_6. Identification of intermediates followed by the isolation of enzymes which catalyse the steps in the biosynthetic pathway would bring experimental progress to the stage where in vitro studies would ascertain the definitive sequence.

It has been suggested that pyruvate dehydrogenase complex may be involved in the origin of the C_2 unit C-2,-2' of pyridoxol. Acetylthiamin or acetyl lipoate is also thought to be involved. An experiment with propionylthiamin, an analogue of acetylthiamin, in a cell-free system might generate 2-ethylpyridoxol. Alternatively, conducting tracer experiments in the presence of known specific enzyme inhibitors may also provide insight into the biosynthetic sequence. Unfortunately, this increases the number of variables in a complex system which still requires much more fundamental research.

CHAPTER IV

SUMMARY

The biosynthesis of vitamin B_6 has been investigated by employing tracer methods in two pyridoxineless mutants of Escherichia coli B, WG2 and WG3. Adaptation of published methods for the culturing conditions for the growth of the mutants and for the isolation and purification of pyridoxol and pyridoxal synthesized de novo by the two mutants, respectively, were employed.

Adaptation of chemical degradation methods permitted the isolation of C-2', C-2 plus -2', C-5' and C-5 plus -5' of pyridoxol as the degradation products methylamine, acetic acid, benzoic acid and N-phthaloylglycine. Methylamine and acetic acid were converted to solid derivatives for radioassay.

Non-random incorporation of activity into pyridoxol derived from labelled glycolaldehyde, glycerol, pyruvaldehyde, ribose and acetate was observed when these substrates were administered to cultures of mutant WG2. Isotope competition experiments with [2-^{14}C]glycerol in the presence of unlabelled glycolaldehyde, pyruvate, acetate or 3-hydroxypyruvate demonstrated that only pyruvate could partially spare the incorporation of activity within pyridoxol. The incorporation into pyridoxol in mutant WG2 of activity derived from doubly

labelled glycolaldehyde, indicated complete retention of tritium relative to ^{14}C, whereas activity derived from doubly labelled glycerol demonstrated the loss of one tritium atom relative to ^{14}C. In mutant WG3, labelled glycerol yielded non-randomly labelled pyridoxal.

These results are consistent with the hypothetical scheme that vitamin B_6 arises from two triose units and a C_2 unit all of which can be derived from glycolysis. The incorporation of activity into pyridoxol and pyridoxal derived from glycolaldehyde could not be rationalized via the proposed glycolitic intermediates and hence it is postulated that a second minor route involving glycolaldehyde, must also be operative in mutant WG2. In WG3, the minor route is the sole pathway to pyridoxal (Scheme (27)).

It is now shown that pyruvate is the C_2 donor in the major pathway and further, that the normal end-products of pyruvate catabolism are precluded as progenitors of C-2,-2' of pyridoxol. The role of 3-hydroxypyruvate in the major pathway was briefly examined and on this basis, is not considered as an obligatory intermediate in pyridoxol biosynthesis. It may, however, serve as a precursor in the minor pathway which is thought to be induced in the presence of glycolaldehyde.

REFERENCES

1. Gyorgy, P. (1934) Nature 133, 498.
2. Birch, T.W., and Gyorgy, P. (1936) Biochem. J. 30, 304-315.
3. Keresztesy, J.C., and Stevens, J.R. (1938) Proc. Soc. Exp. Biol. Med. 38, 64-65.
4. Lepkovsky, S. (1938) Science 87, 169-170.
5. Ichiba, A. and Michi, K. (1938) Sci. Papers Inst. Phys. Chem. Res., Tokyo 34, 623.
6. Kuhn, R., and Wendt, G. (1938) Ber. Dtsch. Chem. Ges. 71, 780-782.
7. Gyorgy, P. (1938) J. Am. Chem. Soc. 60, 983-984.
8. Stiller, E.T., Keresztesy, J.C., and Stevens, J.R. (1939) J. Am. Chem. Soc. 61, 1237-1242.
9. Kuhn, R., Wendt, G. and Westphal, K. (1939) Ber. Dtsch. Chem. Ges. 72B, 310-311.
10. Harris, S.A. and Folkers, K. (1939) Science 89, 347.
11. Harris, S.A., Stiller, E.T. and Folkers, K. (1939) J. Am. Chem. Soc. 61, 1242-1244.
12. Harris, S.A. and Folkers, K. (1939) J. Am. Chem. Soc. 61, 3307-3310.
13. Snell, E.E., Guirard, B.M. and Williams, R.J. (1942) J. Biol. Chem. 143, 519-530.
14. Snell, E.E. (1944) J. Biol. Chem. 154, 313-314.
15. Snell, E.E. (1944) J. Am. Chem. Soc. 66, 2082-2088.

16. Harris, S.A., Heyl, D. and Folkers, K. (1944) J. Am. Chem. Soc. 66, 2088-2092.

17. Harris, S.A., Heyl and Folkers, K. (1944) J. Biol. Chem. 154, 315-316.

18. McCormick, D.B., Gregory, M.R. and Snell, E.E. (1961) J. Biol. Chem. 236, 2076-2084.

19. Wada, H. and Snell, E.E. (1961) J. Biol. Chem. 236, 2089-2095.

20. Rabinowitz, J.C. and Snell, E.E. (1947) J. Biol. Chem. 169, 643-650.

21. Gunsalus, I.G., Bellamy, W.D. and Umbreit, W.W. (1944) J. Biol. Chem. 155, 685-686.

22. Umbreit, W.W., Bellamy, W.D. and Gunsalus, I.C. (1945) Arch. Biochem. 7, 185-199.

23. Umbreit, W.W. and Gunsalus, I.C. (1945) J. Biol. Chem. 159, 333-341.

24. Baddiley, J. and Gale, E.F. (1945) Nature 155, 727-728.

25. Guirard, B.M. and Snell, E.E. (1964) in "Comprehensive Biochemistry", Ed. M. Florkin and E.H. Stotz, Elsevier, Amsterdam, 15, 138.

26. Snell, E.E., Burg, R.W., Dempsey, W.B., Nyns, E.J., Sundarum, T.K. and Zach, D. (1963) in "Chemical and Biological Aspects of Pyridoxal Catalysis", Ed. E.E. Snell, P.M. Fasella, A. Braunstein and A.R. Fanelli, MacMillan, New York, p. 563-577.

27. Goodwin, T.W. (1963) in "The Biosynthesis of Vitamins and Related Compounds", Academic Press, London, p. 159-166.

28. Wagner, A.F. and Folkers, K. (1964) in "Vitamins and Coenzymes", Interscience Pub., New York, p. 160-193.

29. Robinson, F.A. (1966) in "The Vitamin Co-Factors of Enzyme Systems", Pergamon Press, Oxford, p. 328-405.

30. Sauberlich, H.E. (1968) in "The Vitamins", Ed. W.H. Sebrell, Jr. and R.S. Harris, Academic Press, New York, p. 31-33.

31. Snell, E.E. and Haskell, B.E. (1971) in "Comprehensive Biochemistry", Ed. M. Florkin and E.H. Stotz, Elsevier, Amsterdam, 21, 47-71.

32. Rodwell, V.W. (1970) in "Metabolic Pathways", Ed. D.M. Greenberg, Academic Press, New York, 4, p. 411.

33. Nutritional Reviews (1972) 30, 238-240.

34. Leete, E. (1973) in "Biosynthesis" Specialist Periodical Report, Ed. T.A. Geissman, The Chemical Society, London 3, 123-128.

35. Plaut, G.W.E., Smith, C.M. and Alworth, W.L. (1974) Ann. Rev. Biochem. 43, 899-922.

36. Murakami, M. and Takahashi, K. (1977) in "Methodicum Chimicum", Ed. F. Korte and M. Goto, Academic Press, New York, 11, Part 2, 96-101.

37. Tani, Y. (1977) Vitamin 51, 437-448.

38. Tani, Y. and Ogata, K. (1978) in "Memoirs of the College of Agriculture", No. 111 (Chem. Ser. No. 34).

39. Dempsey, W.B. (1980) in "Vitamin B_6 Metabolism and Role in Growth", Ed. G.P. Tryfiates, Food and Nutrition Press, Conn., p. 93-111.

40. Dempsey, W.B. and Pachler, P.F. (1966) J. Bact. 91, 642-645.

41. Dempsey, W.B. (1966) J. Bact. 92, 333-337.

42. Garrod, A.E. (1923) in "Inborn Errors of Metabolism", 2nd Ed., Oxford.

43. Dempsey, W.B. (1969) J. Bact. 97, 1403-1410.

44. Dempsey, W.B. (1969) J. Bact. 100, 295-300.

45. Guirard, B.M., Ames, B. and Snell, E.E. (1971) J. Bact. 108, 359-363.

46. Grimminger, H. and Lingens, F. (1969) Febs Lett. 5, 225-226.

47. Bukhari, A.I. and Taylor, A.L. (1971) J. Bact. 105, 988-998.

48. Henderson, G.B. and Snell, E.E. (1971) Proc. Natl. Acad. Sci. 68, 2903-2907.

49. Falkinham, J.O. (1977) J. Bact. 130, 566-568.

50. Hockney, R.C. and Scott, T.A. (1979) J. Gen. Microbiol. 110, 275-283.

51. Dempsey, W.B. (1971) J. Bact. 108, 1001-1007.

52. Dempsey, W.B. (1965) J. Bact. 90, 431-437.

53. Dempsey, W.B. (1971) J. Bact. 108, 415-421.

54. McIlwain, H. (1946) Nature 158, 898-902.

55. Bachman, B.J., Low, K.B. and Taylor, A.L. (1976) Bact. Rev. 40, 116-167, Quoted from ref. 39.

56. Hurwitz, J. (1953) J. Biol. Chem. 205, 935-947.

57. Kenny, K.V. and Dempsey, W.B. (1967) Nature 213, 830.

58. Grutzner, J.B. (1972) Lloydia 35, 375-398.

59. Simpson, T.J. (1975) Chem. Soc. Rev. 4, 497-522.

60. Floss, H.G. (1972) Lloydia 35, 399-417.

61. McInnes, A.G., Smith, D.G., Vining, L.C. and Johnson, L.F. (1971) J. Chem. Soc. London, Section D (Chem. Comm.) 325-327.

62. Stothers, J.B. (1974) in "Topics of Carbon-13 NMR Spectroscopy", Ed. G.C. Levy, Wiley-Interscience, New York 1, p. 238.

63. Perlin, A. (1977) in "Isotopes in Organic Chemistry", Ed. E. Buncel and C.C. Lee, Elsevier, Amsterdam 3, p. 171-235.

64. Brown, S.A. (1972) in "Biosynthesis" Specialist Periodical Report, Ed. T.A. Geissman, The Chem. Soc. London 1, p. 9.

65. Catch, J.R. (1961) "Carbon-14 Compounds", Butterworth, London, p. 82.

66. Umbreit, W.W. and Waddell, J.G. (1949) Proc. Soc. Expl. Biol. Med. 70, 293-299.

67. Pflug, W. and Lingens, F. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 559-570.

68. Argoudelis, C.J. (1968) Fed. Proc. Amer. Soc. Exp. Biol. 27, 553.

69. Schroer, R.A. and Frieden, E.H. (1973) Proc. Soc. Expt. Biol. Med. 142, 369-373.

70. Scott, T.A. and Picton, C. (1976) Biochem. J. 154, 35-41.

71. Ikawa, M. and Snell, E.E. (1954) J. Amer. Chem. Soc. 76, 637-638.

72. Vatsala, T.M., Shanmugasundaram, R.K. and Shanmugasundaram, E.R.B. (1976) Current Science 45, 617-619.

73. Vatsala, T.M., Shanmugasundaram, R.K. and Shanmugasundaram, E.R.B. (1976) Biochem. Biophys. Res. Comm. 72, 1570-1575.

74. Ishida, M., Nagayama, H., Shimura, K. (1973) Agri. Biol. Chem. 37, 1277-1283.

75. Hill, R.E. and Spenser, I.D. (1970) Science 169, 773-775.

76. Hill, R.E., Gupta, R.N., Rowell, F.J. and Spenser, I.D. (1971) J. Amer. Chem. Soc. 93, 518-520.

77. Hill, R.E., Rowell, F.J., Gupta, R.N. and Spenser, I.D. (1972) J. Biol. Chem. 247, 1869-1882.

78. Hill, R.E. and Spenser, I.D. (1973) Can. J. Biochem. 51, 1412-1416; 52, 271.

79. Hill, R.E., Muira, I. and Spenser, I.D. (1977) J. Amer. Chem. Soc. 99, 4179-4181.

80. Hill, R.E., Horsewood, P., Spenser, I.D. and Tani, Y. (1975) J. Chem. Soc. Perkin I, 1622-1627.

81. Suzue, R. and Haruna, Y. (1970) J. Vitamin. 16, 150-159.

82. Ishida, M. and Shimura, K. (1970) Agri. Biol. Chem. 34, 327-334.

83. Ishida, M., Nagayama, H., and Shimura, K. (1973) Agri. Biol. Chem. 37, 1881-1892.

84. Scott, T.A. and Hockney, R.C. (1979) J. Gen. Microbiol. 110, 285-289.

85. Morris, J.G. and Woods, D.D. (1959) J. Gen. Microbiol. 20, 576-596.

86. Morris, J.G. (1959) J. Gen. Microbiol. 20, 597-604.

87. Tani, Y. and Dempsey, W.B. (1973) J. Bact. 116, 341-345.

88. Dempsey, W.B. (1969) Biochem. Biophys. Res. Comm. 37, 89-93.

89. Shimizu, S. and Dempsey, W.B. (1978) J. Bact. 134, 944-949.

90. Dempsey, W.B. (1972) Biochim. Biophys. Acta 264, 344-353.

91. Rickenberg, H.V., Yanofsky, C. and Bonner, D.M. (1953) J. Bact. 66, 683-687.

92. Friedmann, B., Levin, H.W. and Weinhouse, S. (1956) J. Biol. Chem. 221, 665-672.

93. Brum, V.C. (1966) J. Pharm. Sci. 55, 351-352.

94. Murry, A. and Williams, D.L. (1958) in "Organic Syntheses with Isotopes", Interscience, New York, 1, p. 46-47.

95. Goepfert, G.J. and Nord, F.F. (1943) Arch. Biochem. 1, 289-301.

96. Vogel, A.I. (1956) in "Practical Organic Chemistry", Longmans-Green and Co., London, p. 447-448.

97. Leete, E., Gregory, H. and Gros, E.G. (1965) J. Amer. Chem. Soc. 87, 3475-3479.

98. Nefkens, G.H.L., Tesser, G.I. and Nivard, R.J.F. (1960) Recl. Trav. Chim. Pays-Bas 79, 688-698.

99. Willard, M.L. and Jones, M.Z. (1940) J. Amer. Chem. Soc. 62, 2876-2877.

100. Wanag, G. and Veinbergs, A. (1942) Ber. Dtsch. Chem. Gres. 75B, 1558-1569.

101. Neuberg, G. and Rosenthal, P. (1914) Biochem. Z. 161, 171.

102. Fred, E.B., Peterson, W.H. and Davenport, A. (1920) J. Biol. Chem. 42, 175-189.

103. Challenger, F., Flein, L. and Walker, T.K. (1929) J. Chem. Soc. 1498-1505.

104. Fischler, F. and Boettner, R. (1928) Z. Physiol. Chem. 177, 264.

105. Dickens, F. (1938) Biochem. J. 32, 1645-1653.

106. Horecker, B.L. and Smyrniotis, P.Z. (1953) J. Amer. Chem. Soc. 75, 1009-1010.

107. Horecker, B.L., Smyrniotis, P.Z. and Klenow, H. (1953) J. Biol. Chem. 205, 661-682.

108. Horecker, B.L., Smyrniotis, P.Z. and Hurwitz, J. (1956) J. Biol. Chem. 223, 1009-1019.

109. Racker, E., de la Haba, G. and Leder, I.G. (1953) J. Amer. Chem. Soc. 75, 1010-1011.

110. Holzer, H. Da Fonseca-Wollheim, F., Kohlhaw, G. and Woenckhaus, C.W., Ann. New York Acad. Sci. 98, 453-465.

111. Horecker, B.L. (1959) J. Cell. and Comp. Physiol. 54, 89-107.

112. Datta, A.G. and Racker, E. (1961) J. Biol. Chem. 236, 624-628.

113. Heath, E.C., Hurwitz, J., Horecker, B.L. and Ginsberg, A. (1958) J. Biol. Chem. 231, 1009-1029.

114. Schramm, M. and Racker, E. (1957) Nature 179, 1349-1350.

115. Goldberg, M.L. and Racker, E. (1962) J. Biol. Chem. 237, 3841-3842.

116. Breslow, R. (1958) J. Amer. Chem. Soc. 80, 3719-3726.

117. Breslow, R. (1959) J. Cell and Comp. Physiol. 54, 100.

118. Krumdieck, C.L., Shaw, E. and Baugh, C.M. (1966) J. Biol. Chem. 241, 383-387.

119. Jones, T.H.D. and Brown, G.M. (1967) J. Biol. Chem. 242, 3989-3997.

120. Richey, D.P. and Brown, G.M. (1969) J. Biol. Chem. 244, 1582-1592.

121. Narrod, S.A. and Jakoby, W.B. (1964) J. Biol. Chem. 239, 2189-2193.

122. Miedema, E. and Richardson, K.E. (1966) Plant Physiol. 41, 1026-1030.

123. Richardson, K.E., Miedema, E. and Vandor, S.L. (1966) C.J. Biochem. 44, 1271-1275.

124. Kulkarni, A.P. and Hodgson, G. (1973) Comp. Biochem. Physiol. 44B, 407-422.

125. Fleshood, H.L. and Pitot, H.C. (1970) J. Biol. Chem. 245, 4414-4420.

126. Richardson, K.E. and Tolbert, N.E. (1961) J. Biol. Chem. 236, 1285-1290.

127. Weissbach, A. and Sprinson, D.B. (1953) J. Biol. Chem. 203, 1031-1037.

128. Taylor, R.J. and Richardson, K.E. (1967) Proc. Soc. Expt. Biol. Med. 124, 247-252.

129. Richardson, K.E. and Tolbert, N.E. (1961) J. Biol. Chem. 236, 1280-1284.

130. Tani, Y., Morita, H. and Ogata, K. (1977) Agri. Biol. Chem. 41, 1749-1754.

131. Tani, Y., Morita, H. and Ogata, K. (1978) Agri. Biol. Chem. 42, 63-68.

132. Morita, H., Tani, Y. and Ogata, K. (1978) Agri. Biol. Chem. 42, 69-73.

133. Morita, H., Nishimura, T., Tani, Y., Ogata, K. and Yamada, H. (1979) Agri. Biol. Chem. 43, 185-186.

134. Morita, H., Yamamoto, K., Tani, Y., Ogata, K. and Yamada, H. (1979) Agri. Biol. Chem. 43, 317-323.

135. Shimizu, S. and Dempsey, W.B. (1978) J. Bact. 133, 1273-1277.

136. Shimizu, S. and Dempsey, W.B. (1976) J. Bact. 127, 1593-1594.

137. Merck Index (1978), Ed. M. Windholz, 9th Edition, Merck and Co., U.S.A., p. 1035.

138. Dempsey, W.B. and Itoh, H. (1970) J. Bact. 104, 658-667.

139. Calculated from Table 2, on the assumption that yield of pyridoxol is 100 µg/ℓ of culture.[77]

140. Morita, H., Tani, Y., Ogata, K. and Yamada, H. (1978) Agri. Biol. Chem. 42, 2077-2081.

141. Tani, Y., Morita, H. and Ogata, K. (1974) Agri. Biol. Chem. 38, 2057-2058.

142. Hough, L. and Jones, J.K.N. (1952) J. Chem. Soc. 4052-4055.

143. Dempsey, W.B. and Sims, K.R. (1972) J. Bact. 112, 726-735.

144. Krakow, G. and Barkulis, S.S. (1956) Biochim. Biophys. Acta 21, 593-594.

145. Jaenicke, L. and Koch, I. (1962) Biochem. Z. 336, 432-443.

146. Kohlhaw, G., Deus, B. and Holzer, H. (1965) J. Biol. Chem. 240, 2135-2141.

147. This thesis.

148. Hahn, G., Barwald, L., Schales, O. and Werner, H. (1935) Justus Liebigs Ann. Chem. 520, 107-123.

149. Battersby, A.R., Binks, R., Huxtable, R. (1967) Tetrahedron Lett. 563-565.

150. Battersby, A.R., Binks, R., Huxtable, R. (1968) Tetrahedron Lett. 6111-6115.

151. Leete, E. and Braunstein, J.D. (1969) Tetrahedron Lett. 451-452.

152. Kapadia, G.J., Rao, G.S., Leete, E., Fayez, M.B.E., Vaishnav, Y.N., and Fales, H.M. (1970) J. Amer. Chem. Soc. 92, 6943-6951.

153. Stolle, K. and Groger, D. (1968) Arch. Pharm. 301, 561-571.

154. O'Donovan, D.G., Buckley, L. and Geary, P. (1976) Proc. Royal. Irish Acad. 76B, 187-190.

155. Wilson, M.L. and Coscia, C.J. (1975) J. Amer. Chem. Soc. 97, 431-432.

156. Pizer, L.I. (1963) J. Biol. Chem. 238, 3934-3944.

157. Pizer, L.I. and Potochny, M.L. (1964) J. Bact. 88, 611-619.

158. Umbarger, H.E., Umbarger, M.A. and Siu, P.M.L. (1963) J. Bact. 85, 1431-1439.

159. Blatt, L., Dorer, F.E. and Sallach, H.J. (1966) J. Bact. 92, 668-675.

160. Dempsey, W.B. (1969) J. Bact. 100, 1114-1115.

161. Clarke, S.J., Low, B. and Konigsberg, W.H. (1973) J. Bact. 113, 1091-1095. Quoted from ref. 39.

162. Clarke, S.J., Low, B. and Konigsberg, W.H. (1973) J. Bact. 113, 1096-1103. Quoted from ref. 39.

163. Dempsey, W.B. (1970) Biochim. Biophys. Acta. 222, 686-687.

164. Wood, W.A. and Gunsalus, I.C. (1949) J. Biol. Chem. 181, 171-182.

165. Metzler, D.E. and Snell, E.E. (1952) J. Biol. Chem. 198, 353-362.

166. Metzler, D.E. and Snell, E.E. (1952) J. Biol. Chem. 198, 363-373.

167. Ashworth, J.M., Kornberg, H.L. and Nothamm, D.L. (1965) J. Mol. Biol. 11, 654-657. Quoted from ref. 51.

168. Schultz, A.S., Atkin, L. and Frey, C.N. (1940) J. Bact. 40, 339-346.

169. Rabinowitz, J.C. and Snell, E.E. (1951) Arch. Biochem. Biophys. 33, 472-481.

170. Morris, J.G., Hughes, D.T.D. and Mulder, C. (1959) J. Gen. Microbiol 20, 566-575.

171. Koser, S.A. (1968) in "Vitamin Requirements of Bacteria and Yeast", Charles C. Thomas (Publ.), Springfield, Ill., p. 187.

172. Harris, D.L. (1956) Arch. Biochem. Biophys. 60, 35-43.

173. Lewin, L.M. and Brown, G.M. (1963) Arch. Biochem. Biophys. 101, 197-203.

174. Krampitz, O.L. (1969) Ann. Rev. Biochem. 38, 213-240.

175. Hayashi, S. and Lin, E.C.C. (1965) Biochim. Biophys. Acta 94, 479-487.

176. Cooper, R.A. and Anderson, A. (1970) Febs. Lett. 11, 273-276.

177. Hopper, D.J. and Cooper, R.A. (1971) Febs. Lett. 13, 213-216.

178. Hopper, D.J. and Cooper, R.A. (1972) Biochem. J. 128, 321-329.

179. Hall, S.S., Doweyko, A.M. and Jordan, F. (1976) J. Amer. Chem. Soc. 98, 7460-7461 and references therein.

180. Racker, E. (1951) J. Biol. Chem. 190, 685-696.

181. Oaks, A. and Bidwell, R.G.S. (1970) Ann. Rev. Plant. Phys. 21, 43-66.

182. Lanning, M.C. and Cohen, S.S. (1954) J. Biol. Chem. 207, 193-199.

183. Wood, H.G. and Katz, J. (1958) J. Biol. Chem. 233, 1279-1282.

184. Horecker, B.L. (1964) in "Comprehensive Biochemistry", Ed. M. Florkin and E.M. Stotz, Elsevier, Amsterdam 15, 52-55.

185. Mahler, H.R. and Cordes, E.H. (1971) in "Biological Chemistry", Harper and Row, New York, p. 455.

186. Kornberg, W.L., Phizackerley, P.J.R. and Sadler, J.R. (1960) Biochem. J. 77, 438-445.

187. Spector, L.B. (1980) Proc. Natl. Acad. Sci. USA, 77, 2626-2630.

188. Robinson, W.G., Bachhawat, B.K. and Coon, M.J. (1956) J. Biol. Chem. 218, 391-400.

189. Urata, G. and Granick, S. (1963) J. Biol. Chem. 238, 811-820.

190. Spenser, I.D. (1968) in "Comprehensive Biochemistry", Ed. M. Florkin and E.M. Stotz, Elsevier, Amsterdam, 20, 365.

191. Hill, R.E. (1970), Ph.D. Thesis (McMaster University).

192. Koike, M., Reed, L.J. and Carrol, W.R. (1963) J. Biol. Chem. 238, 30-39.

193. Reed, L.J. and Cox, D.J. (1966) Ann. Rev. Biochem., 35, 57-84.

194. Reed, L.J. (1974) Acc. Chem. Res. 7, 40-46.

195. Holzer, H. and Beaucamp, K. (1959) Angen. Chem. 71, 776.

196. Holzer, H. and Beaucamp, K. (1961) Biochim. Biophys. Acta 46, 225-243.

197. Das, M.L., Koike, M. and Reed, L.J. (1961) Proc. Natl. Acad. Sci USA, 47, 753-759.

198. Breslow, R. and McNelis, E. (1960) J. Amer. Chem. Soc. 82, 2394-2395.

199. White, F.G. and Ingraham, L.L. (1960) J. Amer. Chem. Soc. 82, 4114-4115.

200. Nash, C.P., Olson, C.W., White, F.G. and Ingraham, L.L. (1961) J. Amer. Chem. Soc. 83, 4106-4107.

201. Daigo, K. and Reed, L.J. (1962) J. Amer. Chem. Soc. 84, 659-662.

202. Hough, L. and Richardson, A.C. (1967) in "Rodd's Chemistry of Carbon Compounds", Ed. S. Coffey, Elsevier, Amsterdam, p. 500-529.

203. Horton, D. (1969) in "The Amino Sugars", Ed. R.W. Jeanloz, Academic Press, New York, 1A, p. 176.

204. El-Ashmawy, A.E. and Horton, D. (1965) Carbohydr. Res. 1, 164. Quoted from reference 203.

205. El-Ashmawy, A.E. and Horton, D. (1966) Carbohydr. Res. 3, 1966. Quoted from reference 203, C.A. (1967) 66, 65772K.

206. Stevens, C.L., Blumbergs, P., Daniher, F.A., Wheat, R.W., Kujomoto, A. and Rollins, E.L. (1963) J. Amer. Chem. Soc. 85, 3061.

207. Stevens, C.L., Blumbergs, P., Otterbach, D.H., Strominger, J.L., Matsuhashi, M. and Dietzler, D.N. (1964) J. Amer. Chem. Soc. 86, 2937-2938.

208. Okazaki, R., Okazaki, T. and Kuriki, Y. (1960) Biochim. Biophys. Acta 38, 384-386.

209. Strominger, J.L. and Scott, S.S. (1959) Biochim. Biophys. Acta 35, 552-553.

210. Wheat, R.W., Rollins, E.L. and Leatherwood, J.M. (1962) Biochem. Biophys. Res. Comm. 9, 120-125.

211. Matsuhashi, M. and Strominger, J.L. (1964) J. Biol. Chem. 239, 2454-2463.

212. Matsuhashi, M. and Strominger, J.L. (1966) J. Biol. Chem. 241, 4738-4744.

213. Blumson, N.L. and Baddiley, J. (1961) Biochem. J. 81, 114-124.

214. Wilson, D.M. and Ajl, S. (1955) Biochim. Biophys. Acta 17, 289.

215. Reist, E.J., Spencer, R.R., Baker, B.R. and Goodman, L. (1962) Chem. Ind. 1794-1795.

216. Green, M.L. and Elliot, W.H. (1964) Biochem. J. 92, 537.

217. Cohen, G.N. and Hirsch, M.L. (1954) J. Bact. 67, 182-190.

218. Datta, P. and Prakash, L. (1966) J. Biol. Chem. 241, 5827-5835.

219. Greenberg, D.M. (1969) in "Metabolic Pathways", Ed. D.M. Greenberg, Academic Press, New York, 3, p. 253.

220. Elliot, W.H. (1959) Nature 183, 1051-1052.

221. Tani, Y., Matsuura, M., Negoro, T. and Yamada, H. (1980) Agri. Biol. Chem. 44, 849-854.

222. Rosenbloom, J., Sugimoto, E. and Pizer, L.I. (1968) J. Biol. Chem. 243, 2099-2107.

223. Sprinson, D.B. and Chargaff, E. (1946) J. Biol. Chem. 164, 417-432.

224. Michaud, G., Genson, A.A. and Calvin, M. (1956) J. Biol. Chem. 218, 599-606.

225. Schröter, W. and Holzer, H. (1963) Biochim. Biophys. Acta 77, 474-481.

226. Holzer, H. and Schröter, W. (1962) Biochim. Biophys. Acta 65, 271-288.

227. Holland, H.L., Castille, M., MacLean, D.B. and Spenser, I.D. (1974) Can. J. Chem. 52, 2818-2831.