BIOSYNTHESIS OF VITAMIN B_6

By

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The biosynthesis of vitamin B_6 was studied by administering radioactive putative precursors to a mutant of Escherichia coli B., WG2. A new method for the isolation and purification of the vitamin congener, pyridoxol, is described. Partial degradation of the radioactive pyridoxol revealed non-random incorporation of a number of precursors into pyridoxol. On the basis of these results a biosynthetic scheme was constructed which envisages that pyridoxol is derived from three glycerol units. One of these is incorporated via pyruvic acid as a two carbon fragment at the oxidation level of acetaldehyde. The other two are incorporated intact, possibly by way of triose phosphate (Fig. 33).

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I INTRODUCTION

Vitamin B_6 is the name given to a family of compounds, structurally related to pyridoxal phosphate (the 5'-phosphate ester of 4-formyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine) (1.1), a cofactor of many enzymes which catalyse transformations of amino acids.

Mammalian tissues do not sustain pyridoxal biosynthesis, and it was on the basis of nutritional studies, in the late twenties and early thirties, that the vitamin was first inferred to exist (György, 1934; Birch & György, 1936).

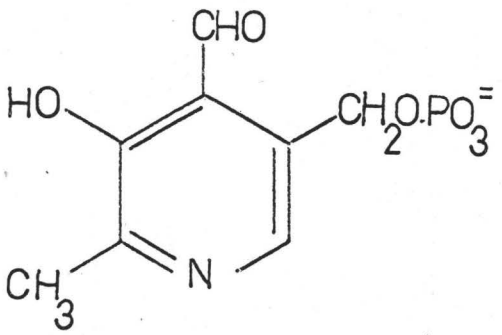
Isolation of the first member of the B_6 group of compounds, pyridoxine (1.2) (4,5-di(hydroxymethyl)-3-hydroxy-2-methylpyridine), was achieved by several independent research groups in 1938 (Keresztesy & Stevens, 1938; Lepkovsky, 1938; György, 1938), and structural elucidation (Stiller, Keresztesy & Stevens, 1939) and synthesis (Harris, Stiller & Folkers, 1939) followed promptly. Other members of the B_6 group were recognised during the forties (Snell, Guirard & Williams, 1942; Snell, 1944 a, b, Harris & Folkers, 1944 a, b).

It is now accepted that the physiologically active members of the B_6 group are the 5' phosphate esters of pyridoxal (1.1) and pyridoxamine (1.3).

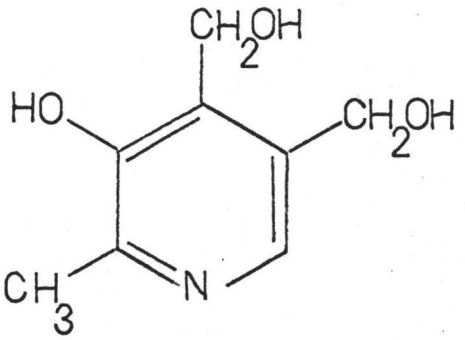
The interconversion of the various members of the B_6 group and the essential part which these substances play in transamination and

FIGURE 1

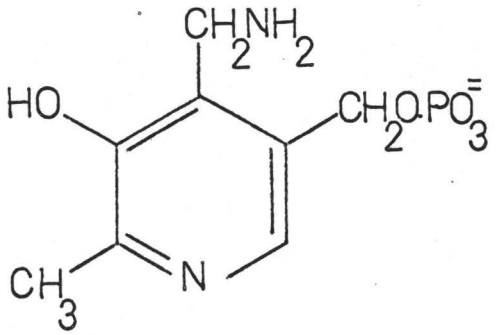
Some vitamin B_6 congeners



Pyridoxal Phosphate (1.1)



Pyridoxol (1.2)



Pyridoxamine Phosphate (1.3)

other biochemical transformations of amino acids as well as in other enzymic processes was intensely studied in the fifties and the mechanism of many of the processes is now well understood (Snell et al., 1963).

Another aspect of the biochemistry of vitamin B_6, on the other hand, has been almost entirely neglected: little is known of the early stages of its biosynthesis. The present thesis is an attempt to rectify this situation.

II METHODS USED IN THE STUDY OF BIOSYNTHESIS

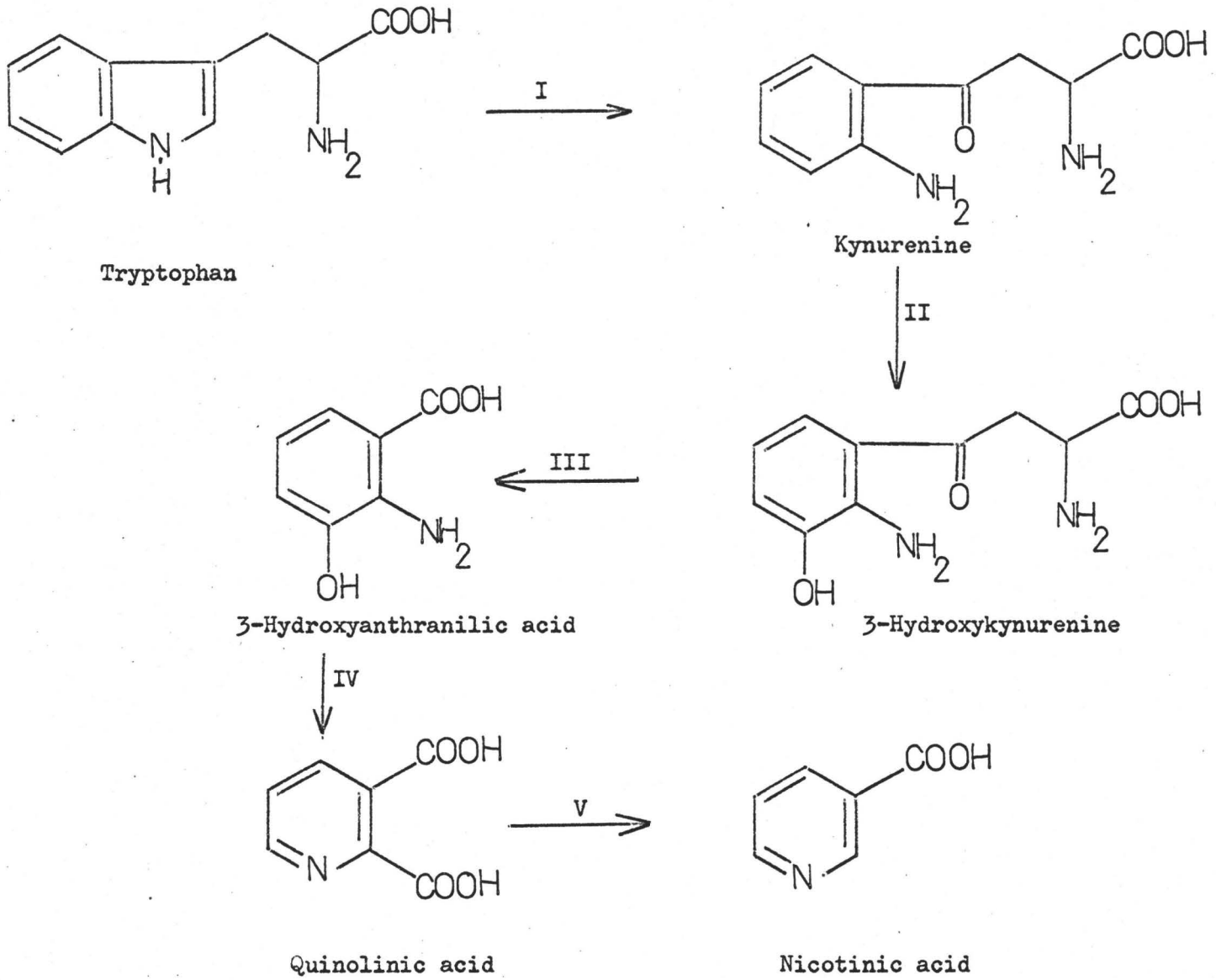
Three general approaches have been employed to obtain evidence concerning biosynthetic precursors and intermediates: the study of intact mutant organisms with spontaneous or induced metabolic blocks, the use of isotopic tracers and the investigation of enzyme systems *in vitro*.

(1) Metabolic blocks

Significant biochemical findings in mammalian and microbiological systems result from studies of intact organisms with spontaneous or induced genetic abnormalities. The study of hereditary metabolic abnormalities in humans, the so-called "inborn errors of metabolism", is an early instance of the contribution of biochemical genetics to the understanding of metabolic processes. The examination of strains of micro-organisms, whose genetic make-up had been altered by treatment with ionizing radiation or chemical mutagens, has proved a particularly fruitful source of biochemical knowledge. These studies which led to the fundamental discoveries in the field of biochemical genetics, yielded information on the identity of intermediates of numerous primary metabolic pathways. An example is supplied by the demonstration of the sequence tryptophan, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, quinolinic acid, nicotinic acid (fig. 2) in *Neurospora*. This pathway requires at least five enzymes.

FIGURE 2

The route from tryptophan to nicotinic acid



The "one gene one enzyme" hypothesis of Beadle & Tatum (1941) postulates that the function of genes is to determine synthesis of the enzymes which catalyse metabolic reactions, each gene being concerned with the synthesis of a single enzyme. On the basis of this hypothesis the five enzymes of the pathway leading from tryptophan to nicotinic acid are biochemical products of five genes (I-V fig. 2). A mutation in any one of these genes affects the normal synthesis of its corresponding enzyme. A mutation at gene IV prevents the conversion of 3-hydroxyanthranilic acid into quinolinic acid. Lack of this intermediate in turn prevents the formation of nicotinic acid. Since nicotinic acid is an essential product, growth will stop unless the organism is supplied with exogenous nicotinic acid or quinolinic acid. If either is supplied, growth will continue. Since there is no block in the preceding steps, 3-hydroxyanthranilic acid will be synthesized normally, will accumulate, and may pass through the cell wall into the environment. If the mutation is at gene III growth will again cease due to lack of niacin. However, in this case it can be maintained by exogenous nicotinic acid, quinolinic acid or 3-hydroxyanthranilic acid. Here 3-hydroxykynurenine will accumulate.

These observations illustrate a fundamental point: A mutant organism can be maintained by an exogenous supply of any metabolite that is normally synthesized by a reaction distal to the metabolic block caused by mutation. When this is the case, normal metabolism is restored and the intermediate immediately preceding the step

TABLE I

The response of some nicotinic acid auxotrophs to intermediates of the tryptophan - niacin pathway

MUTANT	SUPPLEMENTS (+ = growth - = no growth)					
	Tryptophan	Kynurenine	3 OH - Kynurenine	3 OH-Anthranilic Acid	Quinolinic Acid	Nicotinic Acid
65001[a]	+	+		+		+
3416[a]	-	-	-	-		+
4540[a]	-	-	-	-		+
44008[a]	+	+		+		+
39401[a]	+	+	+	+		+
C 86[b]	+	+	+	+		+
E.5029[b]	-	-	+	+		+

[a] (Beadle, Mitchell & Nyc, 1947, 1948)

[b] (Haskins & Mitchell, 1949)

affected by the mutation accumulates.

Investigations using the mutants listed in Table I permitted the sequence of the tryptophan - nicotinic acid pathway to be established.

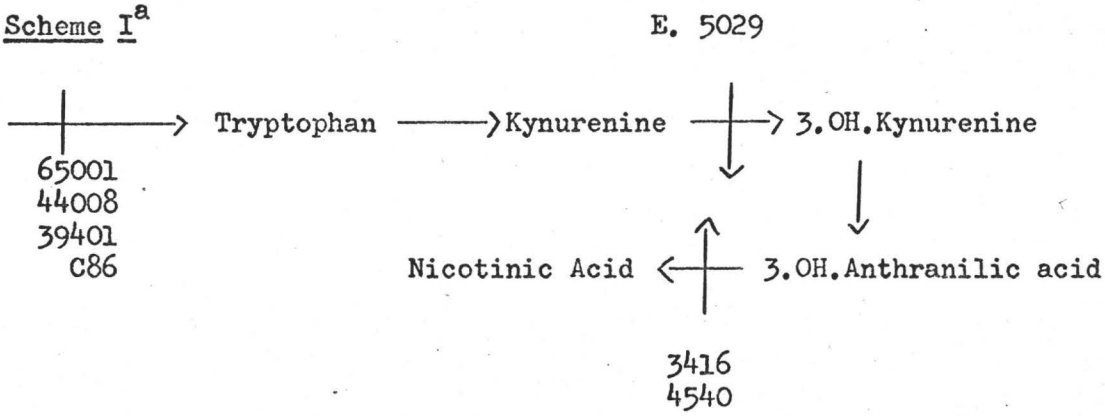
Mutants 65001, 44008 and 39401 are maintained equally well by either tryptophan, kynurenine, 3-hydroxyanthranilic acid or nicotinic acid. 3-Hydroxykynurenine can replace any of these compounds to maintain 39401.

Furthermore, another mutant, 3416, which is highly specific for nicotinic acid, is maintained by the exhausted growth medium of 65001 when this medium is initially supplemented with l-kynurenine. A fifth mutant, 4540, remains specific for nicotinic acid, (Beadle, Mitchell & Nyc, 1947, 1948). Haskins & Mitchell (1949) corroborated these results with work on two further mutants, C86 and E 5029, which are maintained by 3-hydroxykynurenine. The results of these studies, summarized in Table I, are consistent with Scheme I (fig. 3). Consideration of this scheme shows that two strains, 3416 and 4540, grow only in the presence of nicotinic acid. However, the fact that these mutations involve different genes indicates that there is more than one enzyme catalysed step between 3-hydroxyanthranilic acid and nicotinic acid. Studies with small mammals (Henderson & Hirsch, 1949) suggested quinolinic acid as an intermediate. Mutant 4540 was subsequently seen to be maintained by this compound and 3416 was shown to accumulate it when grown on a medium containing nicotinic acid,

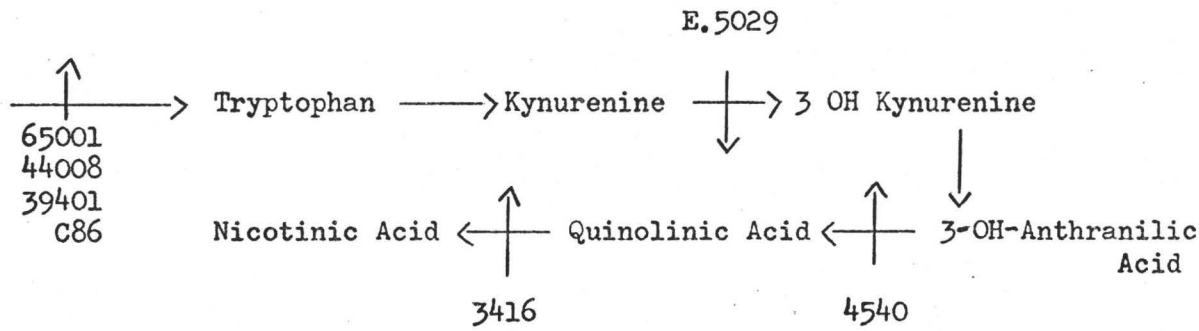
FIGURE 3

The sequence of intermediates between tryptophan and nicotinic acid in Neurospora, showing the positions of some metabolic blocks.

Scheme I[a]



Scheme II[b]



[a] (Beadle, Mitchell & Nyc, 1947, 1948)
(Haskins & Mitchell, 1949)

[b] (Beadle, Mitchell & Nyc, 1947, 1948)
(Haskins & Mitchell, 1949)
(Henderson, 1949) (Yanofsky & Bonner, 1951)

(Henderson, 1949). Further work (Yanofsky & Bonner, 1951) showed that 39401 converts kynurenine, 3-hydroxyanthranilic acid or quinolinic acid to nicotinic acid and that 3416 converts kynurenine or 3-hydroxyanthranilic acid to quinolinic acid but not to nicotinic acid. These results are consistent with Scheme II (fig. 3).

The pathway shown in Scheme II represents the total knowledge gained from nutritional studies with these mutants. In retrospect, the work was simplified because the relationship between tryptophan and the end product, nicotinic acid, had already been suggested by studies on a mammalian system (Krehl, Tepley, Sarma & Elvehjem, 1945). Kynurenine was also known to be closely associated with tryptophan matabolism (Kotake & Iwao, 1931). On the basis of structure 3-hydroxykynurenine, 3-hydroxyanthranilic acid and quinolinic acid must have seemed likely intermediates.

In the absence of such prior knowledge identification of the intermediates leading to a required growth factor becomes more difficult. Even so, the mutants can be assigned in a functional order corresponding to the sequence of synthetic steps blocked in them. Two techniques are involved, one known as cross-feeding or syntropism; the other, a purely genetic technique called the complementation test. Syntropism relies on the fact that if restricted growth of any mutant is allowed by a minimal supply of end product, the intermediate produced prior to the block will accumulate and will be secreted into the surrounding medium. This intermediate will then support the growth of all mutants blocked at a preceding step

but not of mutants blocked at the same point or any distal types. In such experiments the mutants are grown separately on complete medium. The cells or mycelium are harvested, washed and arranged on plates of minimal agar containing limiting amounts of end product. After incubation the mutual influence of the mutants on each other's growth can be assessed and a functional sequence constructed. Clearcut results depend on care in arranging the mutants on the minimal plates. Such factors as the correct amount of end product used to supplement the minimal medium and the time of incubation must be precisely controlled since they may affect the result.

The complementation test is a more sophisticated version of this technique. Briefly, if mutual syntropism can operate across the barriers which separate individual cells, it will operate with greater efficiency and precision if the genomes of the two mutants are present together in the same cell. Diploid cells arising from the union of the two mutant parental cells are phenotypically wild-type provided the mutations are in different functional loci. On the other hand the diploid displays the mutant phenotype if the mutations in the two parents involve the same synthetic step. In diploid organisms with a sexual cycle complementation tests present little difficulty. However, in fungi and bacteria, the sources of most biochemical mutants, the vegetative stage is haploid or a sexual cycle is lacking. Fortunately there are techniques designed to produce diploids in these species which permit complementation tests to be carried out. (For a full discussion of the methods see Hayes, 1964).

The experiments of W. B. Dempsey (1966, 1968) on the biosynthesis of pyridoxal phosphate in Escherichia coli provide good examples of the use of syntropism and the complementation test.

Using a modified penicillin enrichment technique, Dempsey isolated a series of pyridoxine auxotrophs from the wild type E. coli B. The different phenotypes within this group were distinguished by their responses to feeding with various members of the Vitamin B_6 group, (Table II) and by cross feeding experiments (Table III). The results of these studies are consistent with scheme I (fig. 4). This shows that seven of the phenotypes feed in linear sequence. This primary characterisation of the auxotrophs was later supported by complementation tests. The results allow division of the mutants into five unlinked groups (I-V scheme II, fig. 4). Further nutritional experiments indicated that group II mutations are subdivided into two cistrons and that group I mutations may represent three cistrons. These five, together with pyridoxal kinase and the other three loci suggest that nine enzymes may be required to biosynthesize pyridoxal phosphate from a metabolite common to several pathways (scheme II, fig. 4). The existence of pyridoxal kinase and the oxidase step represented by genotype H (II, fig. 4) was demonstrated in an independent study (Dempsey, 1966a)on mutant 2 (Table II). This strain grows only on pyridoxal and appears blocked at the oxidase step. The nature of the intermediates leading to pyridoxol are still unknown however.

TABLE II

Response of some pyridoxine auxotrophs to different members of the pyridoxine family.*

Mutant	Feeding Response			
	Pyridoxal	Pyridoxol	Pyridoxamine	Pyridoxal-5'-Phosphate
3	+4	+4	+1	-
15	+3	+3	+1	-
	+4	+4	+2	-
1B	+3	+5	+1	trace
53	+3	+3	+2	+2
2	+4	-	+1	-
Z	+3	+4	+1	-
5	+3	+4	+2	-

Each + represents approximately a 1-cm diameter of growth under the point of application of the test compound.

* (Dempsey & Pachler, 1966)

TABLE III

Results of cross feeding between different pyridoxine auxotrophs of *E. coli**

(+ = growth, o = no growth)

Fed	Feeders										
	3	μ	1B	15	73	Ɛ	Z	5	25	53	2
3	o	+	+	o	+	+	+	+	o	+	+
μ	o	o	o	+	+	+	+	+	+	o	+
1B	o	o	o	o	+	+	+	+	+	+	+
15	o	o	+	o	+	+	+	+	+	+	+
73	o	o	o	o	o	+	+	+	o	+	+
Ɛ	o	o	o	o	o	o	o	+	+	+	+
Z	o	o	o	o	o	o	o	o	+	+	+
5	o	o	o	o	o	o	o	o	+	+	+
25	o	o	o	o	o	o	o	o	o	o	+
53	o	o	o	o	o	o	o	o	o	o	+
2	o	o	o	o	o	o	o	o	o	o	o

* (Dempsey & Pachler, 1966)

FIGURE 4

Scheme I[a] [a] (Dempsey & Pachler, 1966)

Proposed sequence of some Pyridoxine auxotrophs in E. coli B.

Common metabolite ⟶ A ⟶ B ⟶ C ⟶ D ⟶

mutant 3 μ 15 1B 73

E ⟶ F ⟶ G ⟶ Pyridoxol ⟶ Pyridoxal

mutant ε Z,5 53,25 2

Scheme II[b] [b] (Dempsey, 1968)

Proposed sequence of alleles of some pyridoxine auxotrophs of E. coli B.

Genotype: A B C D

Common metabolite ⟶ ⟶ ⟶ ⟶

Linkage group V I I I

E F G

⟶ ⟶ ⟶ Pyridoxol

II III II

Pyridoxol kinase

H

Pyridoxal 5' Phosphate ⟵ Pyridoxol 5' Phosphate

IV

(2) Isotopic Tracer Experiments

This approach tests a biosynthetic sequence by allowing a viable organism to grow in contact with a putative precursor labelled with an isotope. After an arbitary period of growth the desired end product is isolated, rigorously purified and assayed for isotope content. If the product is labelled it is degraded by chemically unambiguous steps so that the distribution of the label can be deduced.

In most experiments the labelled putative precursor is a multi-carbon substrate specifically labelled with carbon-14. In such cases the degradations must account for the total activity of the product in terms of the activities at individual carbon atoms.

Useful conclusions arise only if non-random incorporation of the label is demonstrated. The mere fact of incorporation of radioactivity will not in itself establish a direct precursor-product relationship since the probability remains that label enters the product indirectly after metabolic breakdown of the precursor. It follows that generally labelled multicarbon precursors are not suitable for this type of study. Consequently the power of the method has risen in direct proportion to the increase in availability of specifically labelled materials.

Successful experiments depend upon the correct choice of labelled precursor, effective incorporation conditions and selective degradation. The latter requirement is of supreme importance and calls for reactions

which proceed cleanly by known mechanisms. The labelled putative precursor must be a compound which reaches the site of biosynthesis. Choice of its labelling pattern must be governed by a knowledge of its metabolism, by its predicted structural relationship to the end product, and by the availability of degradation reactions.

An example of the technique is supplied by the investigations which led to the elucidation of a second pathway of nicotinic acid biosynthesis.

Yanofsky (1954) reported that a series of nicotinic acid auxotrophs of E. coli and Bacillus subtilis utilised neither tryptophan nor any of the intermediates in the Neurospora pathway as a replacement for nicotinic acid. The non-availability of the tryptophan-nicotinic acid pathway was later demonstrated in Mycobacterium tuberculosis (Mothes, Gross, Schütte & Mothes, 1961) and in a number of higher plants, notably corn and tobacco (Henderson et al., 1959).

Radioactive carbon from a variety of 2-, 3-, and 4-carbon compounds is readily incorporated into nicotinic acid in cultures of E. coli (Ortega & Brown, 1960), of M. tuberculosis (Gross, Schütte, Hübner & Mothes, 1963), of Serratia marcescens (Scott & Hussey, 1965) and of Clostridium butylicum (Isquith & Moat, 1966), into ricinine (10.10) in R. communis (Juby & Marion, 1963) and into the pyridine nucleus of anabasine (10.14) (Friedman & Leete, 1963; Leete & Friedman, 1964) and of nicotine (10.12) (Griffith, Byerrum & Hellman, 1960, 1962; Griffith & Byerrum, 1963).

Chemical isolation of the nitrile carbon of ricinine and of the carboxyl carbon of nicotinic acid indicated predominant incorporation of label into these centres from [1-^{14}C] acetic acid, [1,4-$^{14}C_2$] succinic acid and [4-^{14}C] aspartic acid.

Determination of the distribution of activity in the pyridine nucleus of the products was hampered by the relative inaccessibility of individual carbons. Complete degradation sequences have now been worked out for nicotinic acid (Friedman & Leete, 1963, Scott, 1967), and for ricinine (Juby & Marion, 1963). Label from [2-^{14}C] acetic acid and from [2,3-^{14}C] succinate enters predominantly and equally into C_2 and C_3 of the pyridine nucleus. C_4 and C_6 are significantly labelled when [1,3-^{14}C] glycerol serves as the precursor, C_5, on the other hand, derives activity from [2-^{14}C] glycerol.

Most of the evidence (Table IV) is compatible with the view that an early step in the biosynthesis of nicotinic acid is a reaction of a three carbon unit related to glycerol with a four carbon dicarboxylic acid (fig. 5). The actual identity of the two fragments which combine to yield quinolinic acid remains unknown.

Radioactive tracers may be employed to detect possible intermediates of biosynthetic pathways by two techniques which do not require degradation of the product. The first of these is the "carrier dilution" technique: If activity from a labelled precursor is specifically incorporated into a product, then this activity must have passed through every intermediate between precursor and product.

TABLE IV

The incorporation of labelled glycerol, succinic acid and aspartic acid into the pyridine nucleus of nicotinic acid, nicotine, anabasine and ricinine.

Precursor	Product	% activity in carbon atoms						Reference
		2	3	4	5	6	COOH CN	
[1,4-^{14}C] Succinic Acid	Nicotinic Acid						85	a
	Ricinine	←10→		0	0	0	77	b
[4-^{14}C] Aspartic Acid	Nicotinic Acid						100	c
							91	d
	Ricinine	0	0	0	0	0	97	b
			5				93	e
[2,3-^{14}C] Succinic Acid	Nicotinic Acid						17	f
	Ricinine	39	37	0	0		19	g,h
		←47→					16	b
	Nicotine	39	39			7	-	i
[3-^{14}C] Aspartic Acid	Nicotinic Acid						16	d
	Ricinine	0	44	6	1	10	17	j
		38	40	0	1		18	e
	Nicotine	38	57					k
[1,3-^{14}C] Glycerol	Nicotinic Acid						29	f
	Ricinine			40	3	31	14	h
		←43→		18	0	20	14	b
	Nicotine	←34→		36	0	31	-	l
		16	12	32	2	35	-	m
[2-^{14}C] Glycerol	Nicotinic Acid						5	d
	Ricinine			3	53	0	38	h
		←63→		0	33	0	16	b
	Nicotine	←32→		0	73	0	-	l
		2	2	0	99	0	-	m
	Anabasine	29	29		32	4	-	n

a = Waller & Henderson (1961)
b = Yang & Waller (1965)
c = Mothes et al. (1961)
d = Albertson & Moat (1965)
e = Johns & Marion (1966)
f = Ortega & Brown (1960)
g = Juby & Marion (1963)
h = Essery, Juby, Marion & Trumbull (1963)
i = Griffith & Byerrum (1963)
j = Schiedt & Boeckh-Behrens (1962)
k = Jackanicz (1966)
l = Yang, Gholson & Waller (1965)
m = Fleeker & Byerrum (1965)
n = Leete & Friedman (1964)

Even though such intermediates may be present in the system in minute amounts only, and their direct isolation may, therefore, be technically impossible, their radioactivity may be high enough to be detectable. If the chemical identity of such an intermediate can be guessed at on biogenetic grounds or inferred from the chromatographic behaviour of its radioactivity, and a sample of cold, i.e. non-radioactive, material of the inferred structure is added in quantities sufficient for recrystallization, reisolation and rigorous purification, and if this reisolated sample is found to be radioactive, its derivation in the experimental system from the precursor is proven. The compound may then be an intermediate.

Another approach to the tentative identification of possible intermediates in a biosynthetic pathway is the quantitative study of incorporation into a product of activity from a labelled general carbon source (e.g. carbon dioxide or glucose) in the presence of non-radioactive substrates. A compound whose addition causes a reduction in the efficiency of incorporation of radioactivity into the product, may be an intermediate of the pathway of biosynthesis.

Unlike incorporation studies these methods are of limited usefulness when applied to intact organisms and cannot elicit conclusive evidence. However, they may provide useful information when employed in conjunction with incorporation studies.

Carbon-14 has found the widest application in tracer investigations. Other radioactive isotopes e.g. tritium and phosphorus-32, as well as stable isotopes, such as deuterium, carbon-13, nitrogen-15

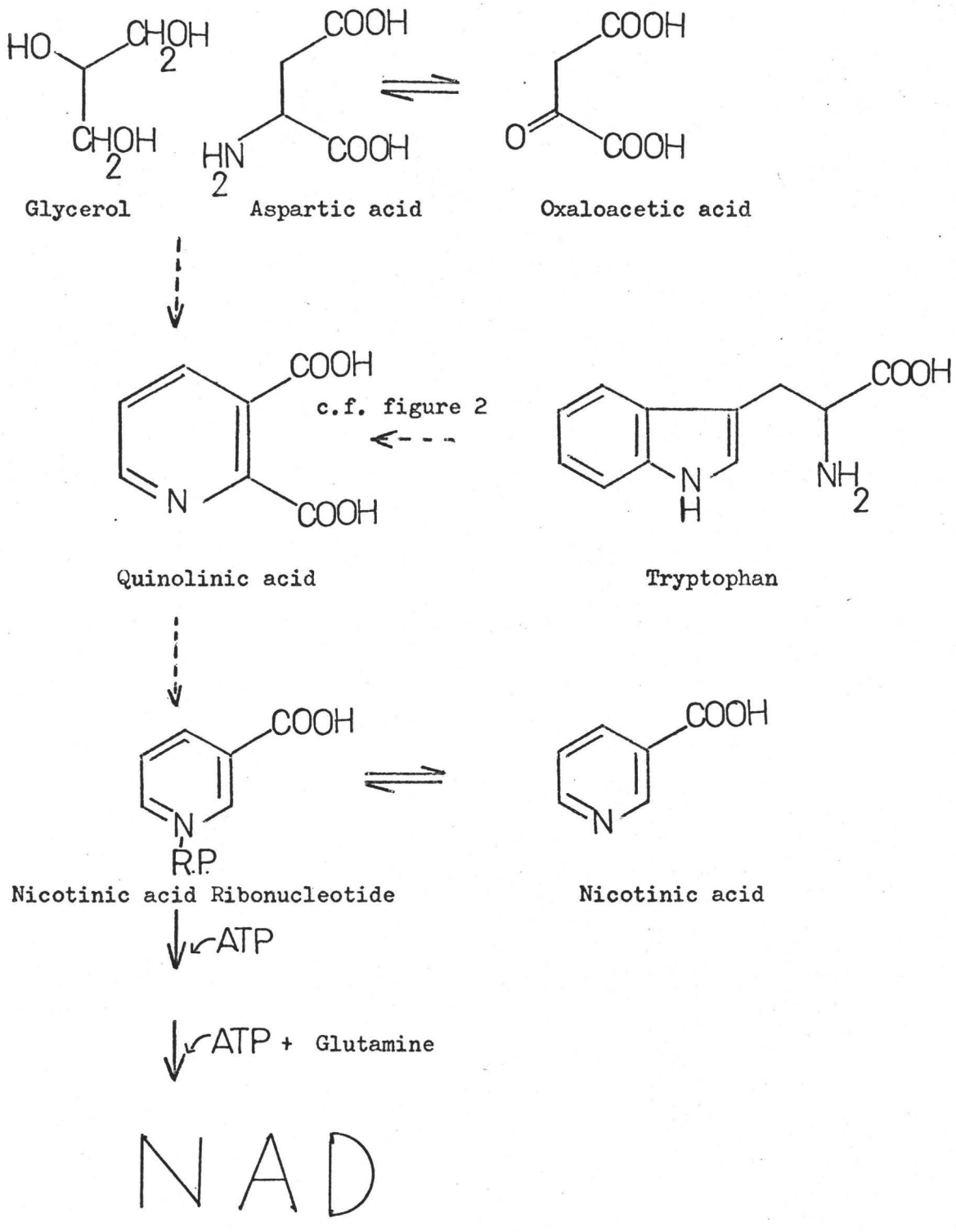
and oxygen-18 have also been employed. Radioactive isotopes are preferred because of the greater sensitivity of the methods of measurement of radioactivity as compared with those available for the determination of heavy isotopes. This is important in *in vivo* experiments as it permits the quantity of labelled precursor which is administered, to be kept to a minimum, a condition required to minimize alterations in the normal steady-state condition of the living organism.

(3) *In vitro studies*

In contrast to the large number of investigations into biosynthetic sequences in micro-organisms and plants using metabolic blocks or tracers, the majority of *in vitro* studies have been carried out with mammalian systems. However, a pertinent exception are the studies of the route from quinolinic acid to nicotinamide adenine dinucleotide. Investigations of this sequence have been performed on *in vitro* systems arising from micro-organisms (Andreoli, Ikeda, Nishizuka & Hayaishi, 1963), green plants (Hadwiger, Badiei, Waller & Gholson, 1963) as well as from mammals (Nakamura, Ikeda et al. 1963). In each of these systems activity of the enzyme *quinolinate transphosphoribosylase*, which catalyses the conversion of quinolinic into nicotinic acid ribonucleotide, the first step of this sequence, is dependent on the presence of phosphoribosylpyrophosphate. Free nicotinic acid, long thought to be an obligatory intermediate, is now known to be a degradation product of NAD, rather than an intermediate in its biosynthesis.

FIGURE 5

Quinolinic acid as a common intermediate in the biosynthesis of NAD



The results of these studies also suggest that quinolinic acid is a common intermediate of NAD biosynthesis from tryptophan and from small molecules (fig. 5).

(4) Limitations of the techniques

The investigation of the biosynthesis of a naturally occurring substance entails three phases. The objective of the first phase is the identification of the primary precursors. The aim of the second phase is the identification of intermediates and the elucidation of the sequence leading from the primary precursors to the product. It remains for the third and final phase to elucidate the mechanism of each step. This is most effectively accomplished by in vitro work using cell free extracts or, optimally, purified, crystalline enzymes.

The "blocked organism" and "tracer" techniques used in the first two phases of a biosynthetic investigation possess certain limitations.

One limitation of studies that make use of induced metabolic blocks is that they are confined to micro-biological systems. Isolation of the induced mutants is a necessary first step and is itself limited by the efficiency of the screening techniques. However, once this has been achieved information is usually forthcoming. The approach has been especially successful when used to investigate sequences that begin with a relatively complex molecule e.g. tryptophan to nicotinic acid. However, it appears to be less successful when the

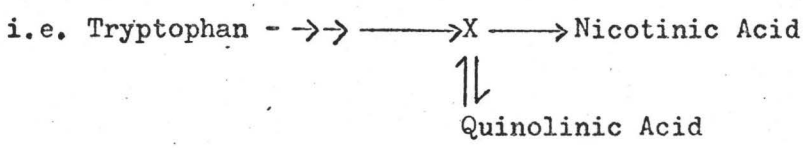
pathway begins with small molecules that are common to several pathways of primary metabolism. The identity of the small molecules is difficult to establish by this method alone. This point is illustrated by the attempts to establish the routes to nicotinic acid and to pyridoxol in micro-organisms. Although the problem of the origin of the latter compound has been the subject of exhaustive investigations using this approach (Snell & Guirard, 1943; Snell, 1944, 1945; Morris & Wood, 1959; Morris, 1959; Sato, 1965a, 1965b, 1966; Dempsey, 1966, 1968,) no definite evidence has been presented concerning the identity of precursors or intermediates.

Evidence elicited by the use of blocked mutants usually falls into one of the following basic categories: (i) absence of a normal end product may be correlated with the accumulation of a new compound; (ii) increased accumulation of a product may be observed as a consequence of the feeding of another compound; and (iii) ability of two or more compounds to maintain growth may be established.

Each of these observations has been used, at one time or another, as evidence for the existence of a biosynthetic relationship between the compounds involved. However, critical examination shows each type of evidence, by itself, to support other, equally tenable interpretations. It follows that any conclusion that is based on one of these observations alone is of doubtful validity.

The following discussion illustrates this situation with actual examples, some taken from the investigations which led to the tryptophan-nicotinic acid pathway.

Early work (Yanofsky & Bonner, 1949) showed that a nicotinic acid deficient mutant of Neurospora accumulates quinolinic acid. Two interpretations were possible. The first placed quinolinic acid as a true intermediate in the biosynthesis of nicotinic acid. The second deemed it to be a side product, in equilibrium with the true intermediate. On the basis that it showed only weak growth promoting activity in other nicotinic acid requiring strains Yanofsky and Bonner (1949) assigned to quinolinic acid the role of a side product.



This conclusion, based on essentially negative data, was later shown to be wrong.

In fact, negative data of any kind are particularly likely to be misleading when one is attempting to establish the role of an accumulated metabolite. A precursor may not, for example, be converted to a later intermediate or to the end product by unblocked strains because of impermeability in experiments in vivo or because of enzyme inactivation or uncoupling in experiments in vitro. The same reasons can account for the lack of incorporation in in vivo or in vitro tracer studies.

Further evidence regarding the role of quinolinic acid in this pathway falls into the second category. Henderson (1949) showed that the amount of N-methylnicotinamide in rat urine is greatly increased when the animal is fed large amounts of quinolinic acid.

Even though other interpretations of this observation were plausible, quinolinic acid was assigned the role of a true precursor of nicotinic acid.

i.e. 3-Hydroxyanthranilate ⟶ Quinolinic acid ⟶ Nicotinic acid

↓

N-Methylnicotinamide ⟵ Nicotinamide

Another interpretation which may be considered in cases of this general type, is that the presence of high concentrations of the fed compound induces formation of enzymes which are normally absent or present in insignificant amounts.

For example, a Neurospora mutant blocked in the conversion of anthranilic acid to tryptophan was found to accumulate anthranilic acid when suspended in a medium containing high concentrations of tryptophan. This observation led Haskins and Mitchell (1949) to the conclusion that Neurospora employs a "tryptophan cycle". Bonner's group, however, showed by tracer experiments that anthranilic acid does not accumulate when tryptophan is being synthesised endogenously in normal low amounts. (Partridge, Bonner & Yanofsky, 1952). It was later suggested that the "cycle" functions only when the medium contains concentrations of tryptophan sufficiently high to induce formation of kynureninase (Adelberg, 1953).

A final interpretation, consistent with this type of evidence suggests that the fed compound is acting as a co-enzyme, which is originally present in a rate limiting concentration.

For example, rats which are fed kynurenine have been shown to excrete much more N-methylnicotinamide if they have a normal intake of vitamin B_2 than if they are deficient for this vitamin (Henderson, Weinstock & Ramasarma, 1951). The interpretation is not that vitamin B_2 is a precursor of nicotinic acid, however, but rather that it is a co-enzyme for a step in its biosynthesis.

Negative results in this category are equally ambiguous: The failure of compound "A" to stimulate accumulation of "B" must be interpreted with caution. A variety of reasons may be responsible for such a failure. These include chemical instability, toxicity and the inability of the compound to penetrate the cell.

Finally, if two or more different compounds restore the growth of a mutant deficient in a growth factor, they are deemed to be precursors of the growth factor. However, once again the results support the dual interpretation that the compounds may be either true intermediates or side products. Bonner's work (1946) on the biosynthesis of arginine in Penicillium notatum exemplifies this problem. It also illustrates how it may be overcome.

He tested fifty independent arginine requiring mutants for growth response to an extended range of presumptive arginine precursors. They were found to fall into five groups as follows:

Group	Growth in Presence of:				
	Arginine	Citrulline	Ornithine	Proline	Glutamate
1	+	-	-	-	-
2	+	+	(±)	-	-
3	+	+	+	-	-
4	+	+	+	+	-
5	+	+	+	+	+

This pattern of response would seem to indicate the synthetic sequence glutamate ⟶ proline ⟶ ornithine ⟶ citrulline ⟶ arginine, and suggests therefore that proline is an intermediate. However, another mutant was found which responded to proline alone, and not to ornithine or any other ornithine precursors. This made it clear that proline cannot precede ornithine directly since, if it did, this prolineless mutant should have grown in the presence not only of proline, but also of ensuing intermediates. This result shows that proline is not involved in the main chain of arginine synthesis but is derived from a precursor of ornithine.

i.e. glutamate ⟶ X ⟶ ornithine ⟶ citrulline

X ⇅ proline

citrulline ↓ arginine

At the same time evidence is presented for a further intermediate compound X.

The examples given above illustrate the problems raised when working with one system alone. They also make it clear that, before any conclusions can be drawn, all results must be substantiated with alternative types of evidence, from tracer and <u>in vitro</u> studies.

Unlike the "mutant" approach radioactive tracers have been used to investigate problems of biosynthesis in all living species. The technique has flourished in recent years and has proved to be the more powerful of the two. As well as substantiating evidence gained from work with mutant organisms the technique has established many relationships between small molecule precursors and end products. An example is the non-tryptophan pathway to nicotinic acid (fig. 5), which has as its initial reaction a union of a three carbon and a four carbon compound. This result illustrates the power of the technique, but, at the same time, serves to express its limitations. Even though the incorporation results are conclusive, the actual identity of the two compounds remains nevertheless unknown, nor is there evidence concerning the identity of the intermediates between the initial reactants and quinolinic acid. A similar uncertainty exists regarding intermediates, following the establishment of relationships between primary metabolites and alkaloid end products in various plant species.

Despite these shortcomings the two techniques have served in the initial phase and part of the second phase of the investigations of many pathways of biosynthesis. However, it is unlikely that they will produce a great deal more information. A limitation of both techniques is that their results do not establish the normal obligatory occurrence of a metabolic sequence. This can only be attained by the use of the <u>in vitro</u> studies of the third phase.

To obtain conclusive evidence it must be shown that substrates which are specifically incorporated into an end product or support growth of a mutant are normally present in the system, that enzymes which catalyse the individual steps of a postulated sequence are usually available or inducable, and that the rate of each enzyme catalysed step under steady state conditions is consistent with the overall rate of formation of the product. Even when all these conditions are satisfied, the possibility cannot be ruled out that the compound in question, instead of being an obligatory intermediate of the product under study, is a side product in enzymic equilibrium with the true intermediate. Not even work with cell extracts can rule out this possibility with absolute certainty. In theory, only investigations with pure single enzymes can establish with finality that a compound is a true intermediate. In this case the strength of the proof is limited solely by the reliability of enzyme purification techniques. Paradoxically, even if absolute purity were attainable the reconstructed sequence of reactions catalysed by these enzymes would be established only as an *in vitro* phenomenon. There would be no assurance that the same sequence took place *in vivo*.

The truly conclusive criterion for establishment of a biosynthetic intermediate then exists in theory only and we are left with the conclusion that present day techniques are unable to establish metabolic intermediates with finality. In practise however the "single enzyme" concept is considered the ultimate in positive evidence. Unfortunately investigations have not yet reached the point

of dealing commonly with purified enzymes and until that time biosynthetic schemes will be based on evidence obtained by the methods reviewed above. Even though this evidence is not entirely conclusive, it provides the foundation of a great deal of biochemical knowledge which has stood the test of time.

III THE BIOSYNTHESIS OF THE PYRIDINE RING

(1) The Biosynthesis of Vitamin B_6

Radioactive tracers, as well as blocked mutants have been used in attempts to elucidate the biosynthesis of vitamin B_6. These investigations have, however, been very limited in their success. What progress has been made is confined to the terminal stages of the pathway.

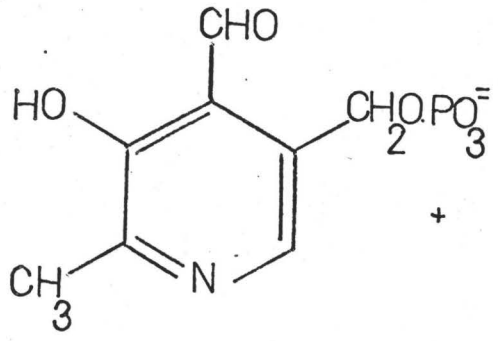
(i) The Terminal stages of the pathway:

Shortly after pyridoxol (1.2) had been isolated in pure form and its structure established, Snell (1942), working with mutant strains of Lactobacilli, produced evidence for a more biologically active form of vitamin B_6. He called this substance "pseudopyridoxine". This material was soon shown to be either of two compounds. One, an oxidation product of pyridoxol, is called pyridoxal (4-formyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine). The other, an amination product, is called pyridoxamine (4-aminomethyl-3-hydroxy-5-hydroxymethyl-2-methylpyridine).

The suspected importance of vitamin B_6 in intermediary metabolism stimulated intensive in vivo and in vitro investigations aimed at defining its role. The results of many studies established the 5'-monophosphate esters of pyridoxal and pyridoxamine as the

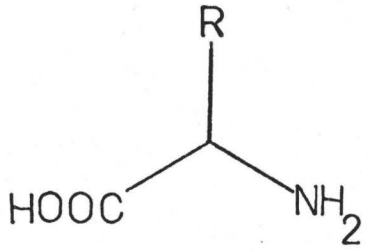
FIGURE 6

The interconversion of pyridoxal 5' phosphate and pyridoxamine 5' phosphate

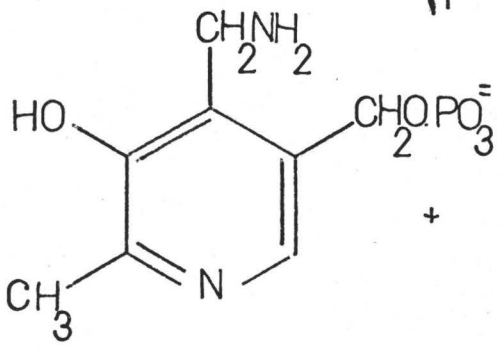


Pyridoxal 5' phosphate

+

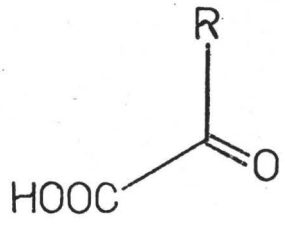


α-amino acid



Pyridoxamine 5' phosphate

+



α-keto acid

biologically active forms of the vitamin. They also illustrated the interconvertibility of these compounds, a reaction catalysed by transaminase enzymes, (fig. 6). Pyridoxol was assumed to be a biological precursor of these compounds. However, it was only recently that this sequence was established (fig. 7), by studies with a pyridoxal auxotroph of E. coli (Dempsey 1966).

This organism was one of many pyridoxine auxotrophs isolated by Dempsey (1965). Originally referred to as E. coli $B\text{-}B_{6\text{-}2}$, it has recently been renamed and is now called E. coli WG2. The organism had characteristics expected of a mutant blocked in the ultimate or penultimate step of the biosynthesis of pyridoxal-5'-phosphate: The mutant grew well in the presence of pyridoxal but did not grow when pyridoxal was replaced by pyridoxol. In cross feeding tests the mutant was found to feed all other pyridoxineless mutants, which had been isolated.

In vivo studies showed the mutant to synthesise pyridoxol-5'-phosphate as well as pyridoxol in a pyridoxal starved culture. Moreover, the rate of this synthesis was found to be four times as fast as the rate shown by the wild type. The bulk of the newly synthesised vitamin was excreted from the cell. This result, together with the requirement of the mutant for pyridoxal, suggested that it was unable to oxidise the 4'-hydroxymethyl substituent of either pyridoxol or pyridoxol-5'-phosphate to the 4'-aldehyde group found in pyridoxal or its 5'-phosphate ester.

In vitro studies followed in which the ability of the mutant and wild-type extracts to convert pyridoxol-5'-phosphate to pyridoxal-5'-phosphate was assayed. These studies showed that extracts of wild type *E. coli* catalysed this conversion, whereas extracts of the mutant did not. These data are consistent with the scheme in figure seven (7), which is also consistent with other findings about the activities of related enzymes in *E. coli*. The following observations can be accommodated:

(a) Extracts of *E. coli* catalyse the phosphorylation of pyridoxal to pyridoxal-5'-phosphate. Under the same conditions pyridoxol remains unphosphorylated. (Quoted from Dempsey, 1966).

(b) Microorganisms, in general, lack the enzyme pyridoxol oxidase (Goodwin, 1963).

(c) The presence of pyridoxal kinase activity has been demonstrated in *E. coli* (Hurwitz, 1953). Presumably it is the presence of this enzyme which allows growth of *E. coli* WG2 in a pyridoxal supplemented medium.

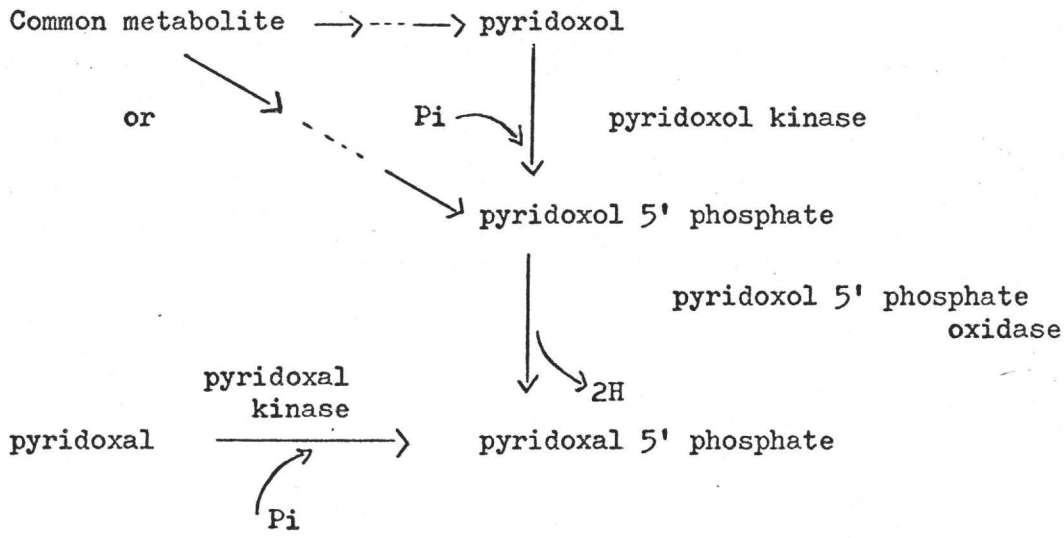
The question still remains whether pyridoxol is a true intermediate of the route to pyridoxal phosphate, as is generally assumed. It is equally feasible, however, that pyridoxol is merely a side product, convertible into the true intermediate by the action of a kinase and derivable from it by a phosphatase catalysed process (fig. 7). This question has not yet been resolved.

(ii) The early steps:

The identification of the primary precursors of pyridoxine has

FIGURE 7

The terminal steps in the biosynthesis of Pyridoxal 5' phosphate*



* (Dempsey, 1966a)

been the objective of many studies. However, no definite conclusions have been arrived at. Both major techniques, the blocked organism approach and the radioactive tracer approach, have been used in these investigations.

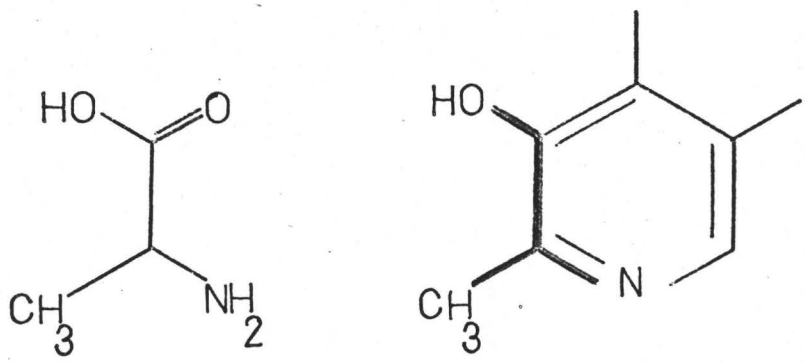
(a) Investigations with organisms containing metabolic blocks

In a series of experiments designed to identify the true nature of "pseudopyridoxine", Snell (1942) observed that large amounts of alanine completely replaced pyridoxine as a growth factor for Streptococus lactis R. On the basis of this evidence he suggested that alanine might be a precursor of pyridoxol. This hypothesis was supported by a structural relationship between alanine and pyridoxol (fig. 8). Later work (Snell, 1945) showed D-alanine to be six times as effective as L-alanine in replacing the vitamin as a growth factor. However, an independent study (Lichstein, Gunsalus & Umbreit, 1945) established that Streptococus faecalis failed to form detectable amounts of pyridoxal phosphate when grown with DL-alanine in place of pyridoxol. On the basis of this evidence, Snell concluded that it was unlikely that either D- or L-alanine was a precursor of pyridoxol, and it was finally shown by the same worker (Holden & Snell, 1949) that D-alanine was itself an essential metabolite of these bacteria and that vitamin B_6 was required for its biosynthesis. Addition of D-alanine to the culture medium did not replace the requirement for the vitamin, but merely reduced the demand to the point where it seemed to be totally replaced.

With the conclusion of this work the problem of the primary precursors of pyridoxine lay dormant for about ten years. In 1959 two E. coli mutants were isolated (Morris, 1959) which required vitamin B_6

FIGURE 8

Structural relationship between alanine and pyridoxine*



*Snell, et al. (1942)

for growth. Both of these auxotrophs were observed to synthesise the vitamin when grown in the presence of serine or glycine, together with glycolaldehyde. On the basis of this evidence it was suggested that these compounds may be involved in the biosynthesis of pyridoxine.

Remaining experiments using this approach are of more recent vintage.

Papers by Sato (1965a, 1965b, 1966a) entitled "Biogenesis of Vitamin B_6 in Candida albicans 4888" describe the effects of various growth media on pyridoxal biosynthesis by this yeast. When a series of α-amino acids were tested as the sole carbon source, aspartate or glutamate induced maximal synthesis of the vitamin. In further work, with dicarboxylic acids of the Krebs' cycle as the carbon source, maximal production resulted when either malate or citrate was used. Finally, it was found that even though glyoxylate alone did not support growth of the yeast, glyoxylate in the presence of acetate supported growth of the yeast and a high rate of pyridoxal synthesis. These results were interpreted to indicate that pyridoxal is produced from organic or amino acids by way of the glyoxylate cycle.

The studies undertaken by Dempsey (1966) which led to the sequence in figure 7, have already been described in Section (i). Work which aligned a series of pyridoxine auxotrophs in functional sequence (Dempsey 1966a, 1968) has also been used in Chapter II to illustrate this technique. None of these investigations are relevant to the present discussion, as they were not primarily aimed at the identification of early precursors. However, more recent experiments (Dempsey, 1969) yielded results which suggested 3-phosphoserine to be a primary precursor.

Three E. coli mutants were used, each lacking a different enzyme of serine biosynthesis. Mutant WG1145, a member of a group of mutants which show an absolute requirement for both serine and pyridoxine, lacks 3-phosphoserine transaminase. Mutants WG1100 and WG1143 lack 3-phosphoserine phosphatase and 3-phosphoglycerate dehydrogenase, respectively.

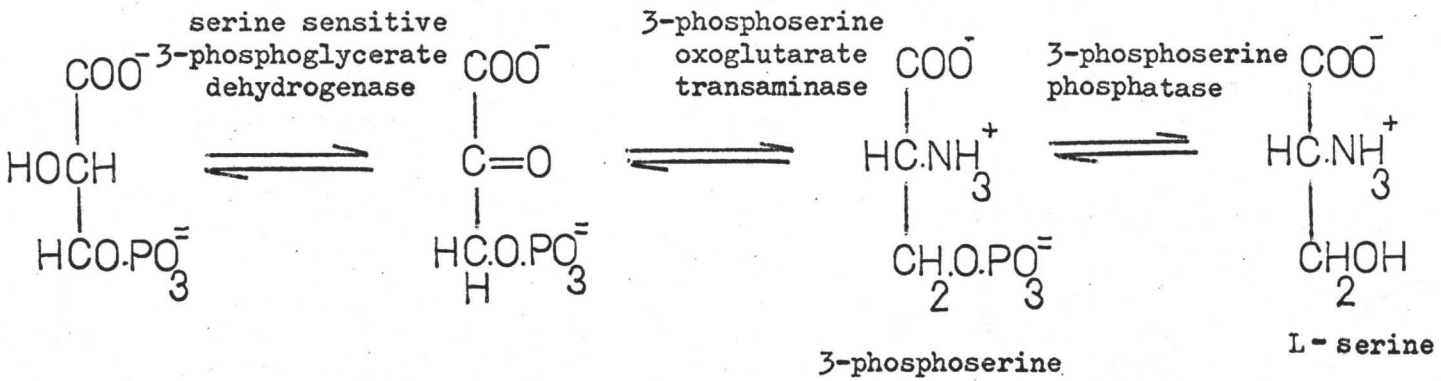
Since mutant WG1145 could not synthesise 3-phosphoserine and required pyridoxine as well as serine for growth, whereas mutant WG1100 could synthesise 3-phosphoserine but not pyridoxine, and did not require pyridoxine for growth, Dempsey suggested that 3-phosphoserine is a primary precursor of pyridoxine. Furthermore, during serine starvation, mutant WG1100 synthesised pyridoxine at twice the normal rate. It is suggested that this enhanced rate of synthesis arose from a breakdown of a feed back inhibition mechanism, and that serine, the normal end product, was responsible for this phenomenon. Under conditions of serine starvation control was lacking, and in mutant WG1100, this led to uncontrolled accumulation of 3-phosphoserine, which, in turn, stimulated rapid pyridoxine synthesis (fig. 9).

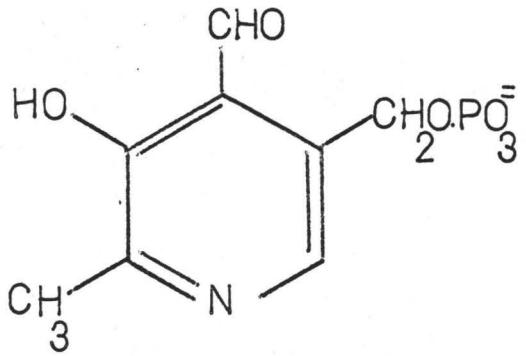
(b) Investigations using radioactive isotopes

Activity from [1-^{14}C] glycerol and [U-^{14}C] leucine was reported to be efficiently incorporated into pyridoxal synthesised by resting cells of two strains of soil bacteria. Label from [1-^{14}C] leucine, on the other hand, was not incorporated. Incubation of the same bacteria with [2-^{14}C] glycerol in the presence of non-radioactive aspartate led to pyridoxal labelled largely in the pyridine ring, (Suzue & Haruna, 1963a, 1963b; Suzue et al. 1964). On the basis of these results it was concluded that, in these bacteria, vitamin B_6 is derived from a

FIGURE 9

Proposed metabolic scheme interrelating vitamin B_6 and serine biosynthesis*





pyridoxal-5'-phosphate

* Dempsey, 1969

three carbon compound related to glycerol and from a five carbon compound related to leucine or aspartate. β-Methyl-β-hydroxyglutaryl CoA was suggested as the five carbon unit in question. Ammonium ion was proposed as the source of the nitrogen in the pyridine ring.

Label from [2,3-^{14}C] succinate was incorporated into vitamin B_6 produced by growing cells of Candida albicans 4888 (Sato, 1966b). This result was used to support earlier evidence which suggested that pyridoxal arises from dicarboxylic acids via the glyoxylate cycle (Sato, 1965a, 1965b, 1966a).

The yeast Candida utilis ATCC 9950 was grown on sucrose as the main carbon source in the presence of various ^{14}C-labelled substrates, (Lunan & West, 1963). Pyridoxamine was isolated from the cells and purified to constant specific activity. However, in each experiment, the level of radioactivity incorporated into pyridoxamine was too low to permit any valid conclusions concerning the role of these substrates in pyridoxamine biosynthesis.

Recently (Stanley, 1969), the same system was used to test the roles of nicotinic acid, quinolinic acid and aspartic acid in pyridoxamine biosynthesis. In each case the pyridoxamine isolated appeared to contain amounts of activity suitable for useful degradation studies. However, when the pyridoxamine was further purified by high vacuum sublimation, all the activity was seen to remain in non-volatile contaminants. As this step was not included in the purification procedure of Lunan and West any conclusion drawn from their results is of doubtful validity.

(iii) Conclusions:

Past investigations aimed at studying the biosynthesis of vitamin B_6 have been haphazard in their approach. Most of the work described above involved the accumulation of data followed by attempts to rationalise the results. Not only do these results fail to point in a specific direction but they tend to confuse the issue of possible pyridoxine precursors.

This haphazard approach is in sharp contrast to work which uses radioactive tracers to investigate biosynthetic sequences leading to other naturally occurring compounds, amongst them plant alkaloids. Long before tracers became available organic chemists were wondering about the biosynthesis of these natural products and speculated about their mode of formation. Hypothetical biogenetic schemes were proposed, which were based on the recognition that families of alkaloids contained common structural features. Thus Pictet suggested (1906), as long ago as 1906, that the indole nucleus is derived from the amino acid tryptophan, and Winterstein and Trier (1910) postulated in 1910 that nicotine was derived from nicotinic acid and proline.

These hypotheses and more recent contributions by Barton (1963), Woodward (1959), Wenkert (1959) and others, established the main outlines of alkaloid biogenesis. Following development of isotope labelling techniques, in the late 1940's, and under the stimulus of these biogenetic postulates, experimental work was begun. Since then, the knowledge accumulated has shown that many of these biogenetic speculations were basically correct.

It would seem therefore, that attempts to employ tracer methods to establish a precursor product relationship between a substrate and the

compound under investigation are facilitated if biogenetic hypotheses are available which can be subjected to critical experimental test.

Such hypotheses may be constructed on the basis of several arbitrary considerations, amongst which analogy and structural relations are perhaps the most usual.

In order to use analogy as a guide to the development of hypotheses of pyridoxine biogenesis which can be examined experimentally, known biosynthetic relationships between precursors and other naturally occurring pyridine derivatives, as established by tracer methods, will be briefly reviewed. Some of this evidence, together with structural considerations, will then be used in the construction of several biogenetic schemes for pyridoxine.

It was hoped that a systematic study of these hypotheses would produce results which would lead to the elucidation of primary precursors. In any event the investigations test, in logical sequence, preconceived ideas, and should serve to narrow the range of possible precursors.

(2) The Biogenesis of some other naturally occurring Pyridine derivatives

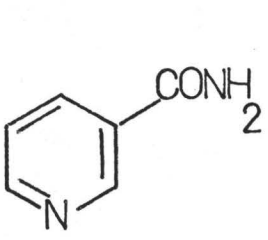
Two derivatives of pyridine, nicotinamide (10.1) and pyridoxal phosphate (1.1) are required by all living tissues and are of fundamental biochemical importance. Other naturally occurring pyridine derivatives are of a more limited distribution. Among these are dipicolinic acid (10.3), formed in sporulating bacteria, fusaric acid (10.4), a plant toxin of fungal origin and desmosine (10.5) and isodesmosine (10.6) isolated from elastin. Piericidin A and B (10.7, 10.8), insecticidal substances, and pyridomycin (10.9), an antibiotic, occur naturally in the genus Streptomyces. Notable amongst the pyridine alkaloids are trigonelline (10.11), nicotine (10.12), anabasine (10.14) and actinidine (10.13). A number of derivatives of 2- and of 4-pyridone, e.g. ricinine (10.10) and mimosine (10.15), also occur in nature. Indicaxanthin (10.16), another alkaloid, contains a substituted hydropyridine moiety.

With the exception of some work on the biosynthesis of pyridoxine and nicotinamide, which used the blocked organism approach, the biosynthetic routes leading to the above compounds have been studied exclusively by the tracer technique. The results of these investigations, discussed briefly below, demonstrate that there is a surprising variety of routes which lead to pyridine derivatives in nature.

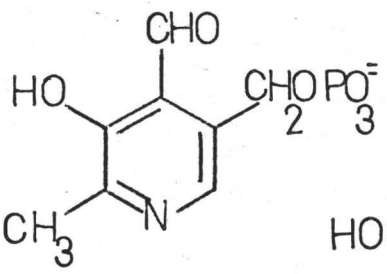
A compound which serves as a precursor of several different pyridine derivatives is aspartic acid. Activity from various radiomers of aspartic acid was shown to enter non-randomly into the pyridine rings at several of the compounds shown in fig. 10. Most fully investigated

FIGURE 10

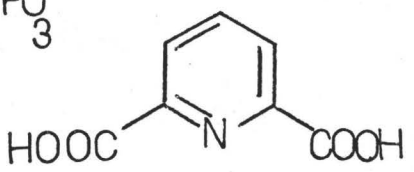
Some naturally occurring pyridine derivatives



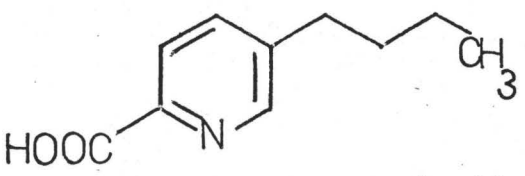
Nicotinamide (10.1)



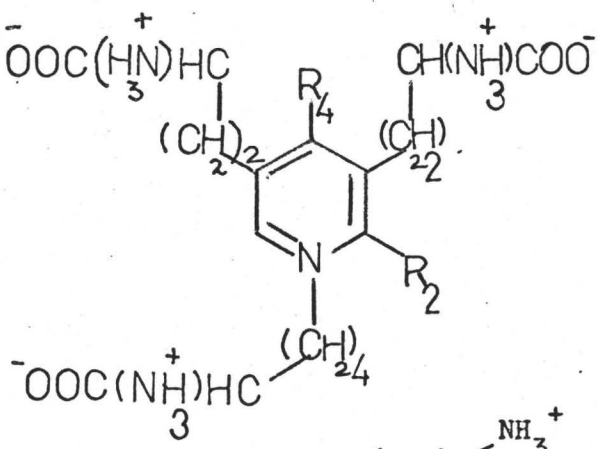
Pyridoxal phosphate (1.1)



Dipicolinic acid (10.3)

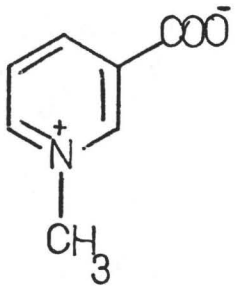


Fusaric acid (10.4)

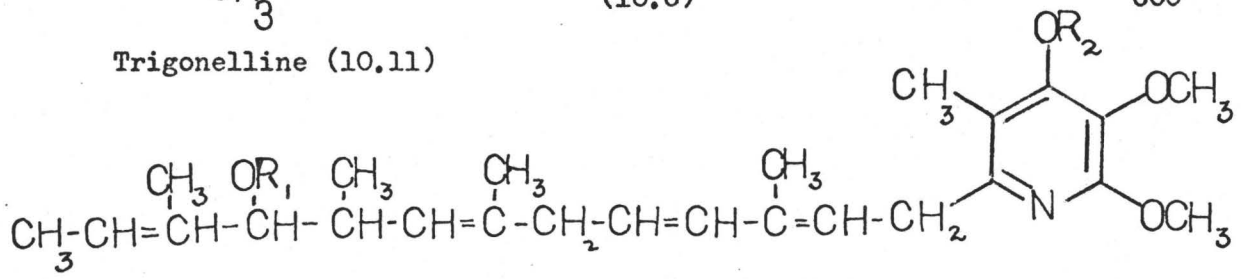


Desmosine (10.5): R_2 = H. R_4 = $-(CH_2)_3-CH(NH_3^+)-COO^-$

Isodesmosine (10.6): R_4 = H. R_2 = $-(CH_2)_3-CH(NH_3^+)-COO^-$

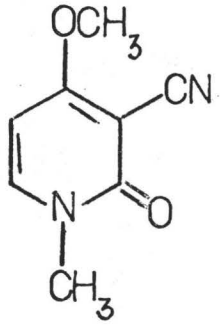


Trigonelline (10.11)

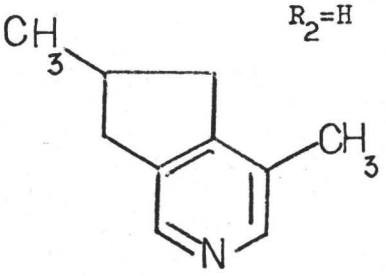


Piericidin A R_1=R_2=H (10.7)

Piericidin B R_1=CH_3, R_2=H

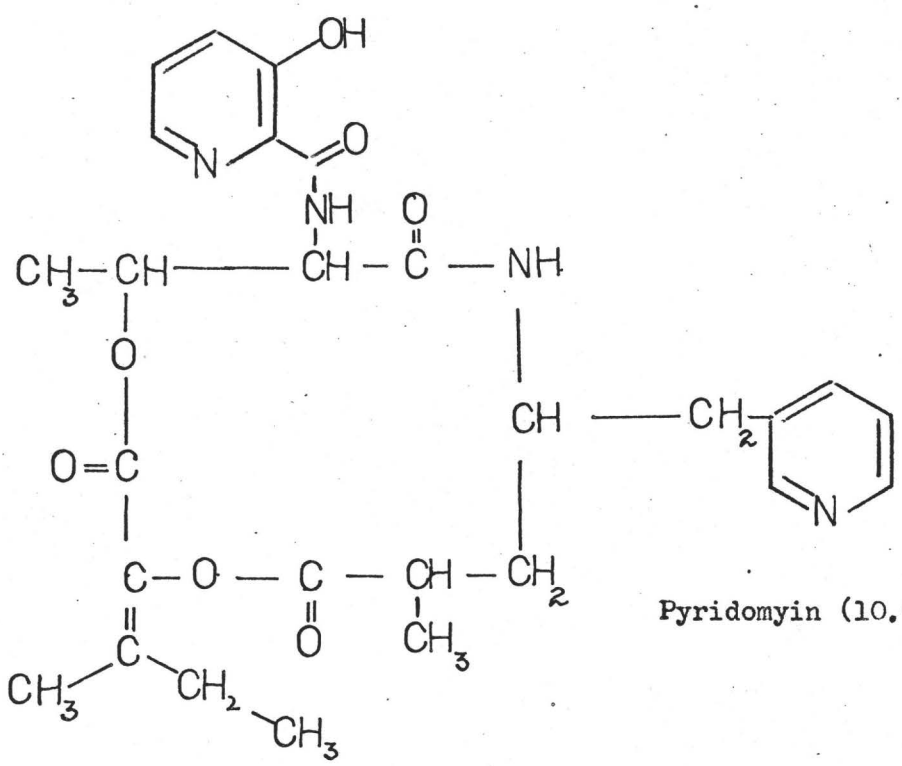


Ricinine (10.10)

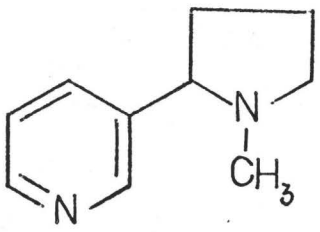


Actinidine (10.13)

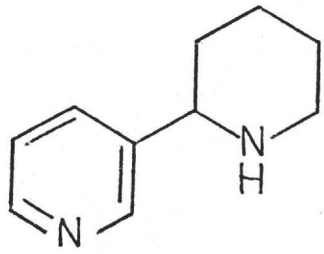
FIGURE 10 (continued)



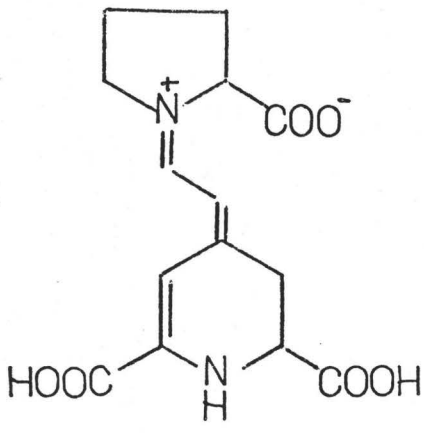
Pyridomyin (10.9)



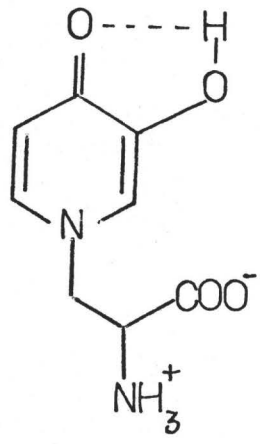
Nicotine (10.12)



Anabasine (10.14)



Indicaxanthin (10.16)



Mimosine (10.15)

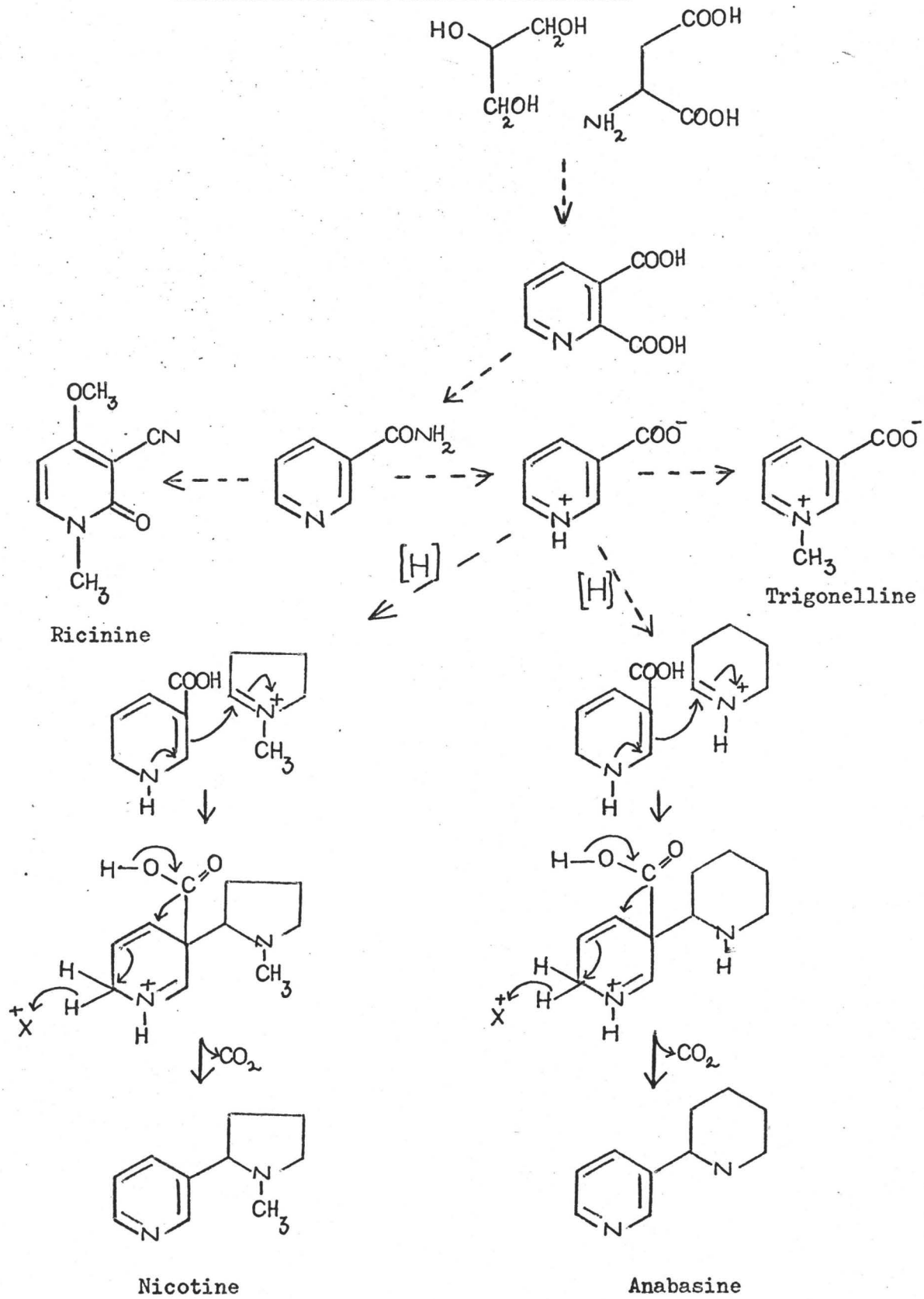
is its mode of incorporation into nicotinic acid in plants and micro-organisms (already discussed in chapter II) and into trigonelline, anabasine, nicotine and ricinine, by way of nicotinic acid, in green plants (fig. 11). Entry of aspartic acid into dipicolinic acid (Martin & Foster, 1958), pyridomycin (Ogawara, Maeda & Umezawa, 1968) and fusaric acid (Dobson, et al., 1967), all products of microbial metabolism, has also been demonstrated.

The sources of the remaining carbons of the pyridine rings of these compounds differ, however.

Activity from labelled three carbon compounds related to glycerol was found to be incorporated non-randomly into ricinine, nicotine, anabasine, trigonelline and nicotinamide. These results are consistent with the view that the skeleton of the pyridine ring in these compounds arises from the union of a three carbon compound related to glycerol and a four carbon compound related to aspartic acid. The three carbon compound has been shown to be specifically incorporated into carbons 2, 3 and 4 of the pyridine ring. Nicotinic acid has been established as a precursor of these compounds, and, as expected, the carboxyl substituent of this molecule has been shown to arise from the γ-carboxyl group of aspartic acid. The α-carboxyl carbon is lost and does not provide any part of the nicotinic acid skeleton. These results, have been discussed in detail in Chapter II of this thesis. They are summarised in figure 11.

Radioactive dipicolinic acid and pyridomycin have also been isolated from experiments in which $[^{14}C]$ labelled three carbon compounds were administered to a biosynthesising system.

Proposed sequence leading from small molecules to Ricinine Trigonelline, Nicotine and Anabasine



The amino acid alanine provided the three remaining carbons of dipicolinic acid, when this compound was biosynthesised by cultures of Bacillus megaterium (Martin & Foster, 1958). In this species, dipicolinic acid is now known to arise from a union of a three carbon compound, probably pyruvic acid, and a four carbon compound, probably aspartic acid, by way of the diaminopimelic acid pathway of lysine biosynthesis (fig. 12).

Pyridomycin has two pyridine moieties. One of these, the 3-hydroxypicolinic acid moiety, became labelled when radioactive pyruvic acid was administered to growing cultures of Streptomyces pyridomyceticus The other, an unsubstituted pyridine ring, became labelled when either radioactive pyruvate or glycerol was administered to the same organism (Ogawara, Maeda & Umezawa, 1968). These results, together with the result in which radioactive aspartate was incorporated, suggested that both pyridine moieties arise via the union of a three and four carbon compound by a mechanism similar to that operating in the biosynthesis of nicotinic acid (fig. 5).

Fusaric acid, on the other hand, derives its remaining carbons from acetate. Three units were incorporated, together with aspartate (Dobson et al., 1967; Hill et al., 1966). One of these provides carbons 5 and 6 of the pyridine ring, the other two, the butyl sidechain (fig. 13).

This is not the only instance in which acetate has been shown to provide a portion of the pyridine ring of a naturally occurring substance.

Radioactivity from $[^{14}\text{-C}]$ labelled acetate was specifically incorporated into dipicolinic acid, when this substance was biosynthesised by the organism P. citreo-viride (Hodson & Foster, 1966); into piericidin

FIGURE 12

Scheme

Biosynthesis of dipicolinic acid in B. megaterium

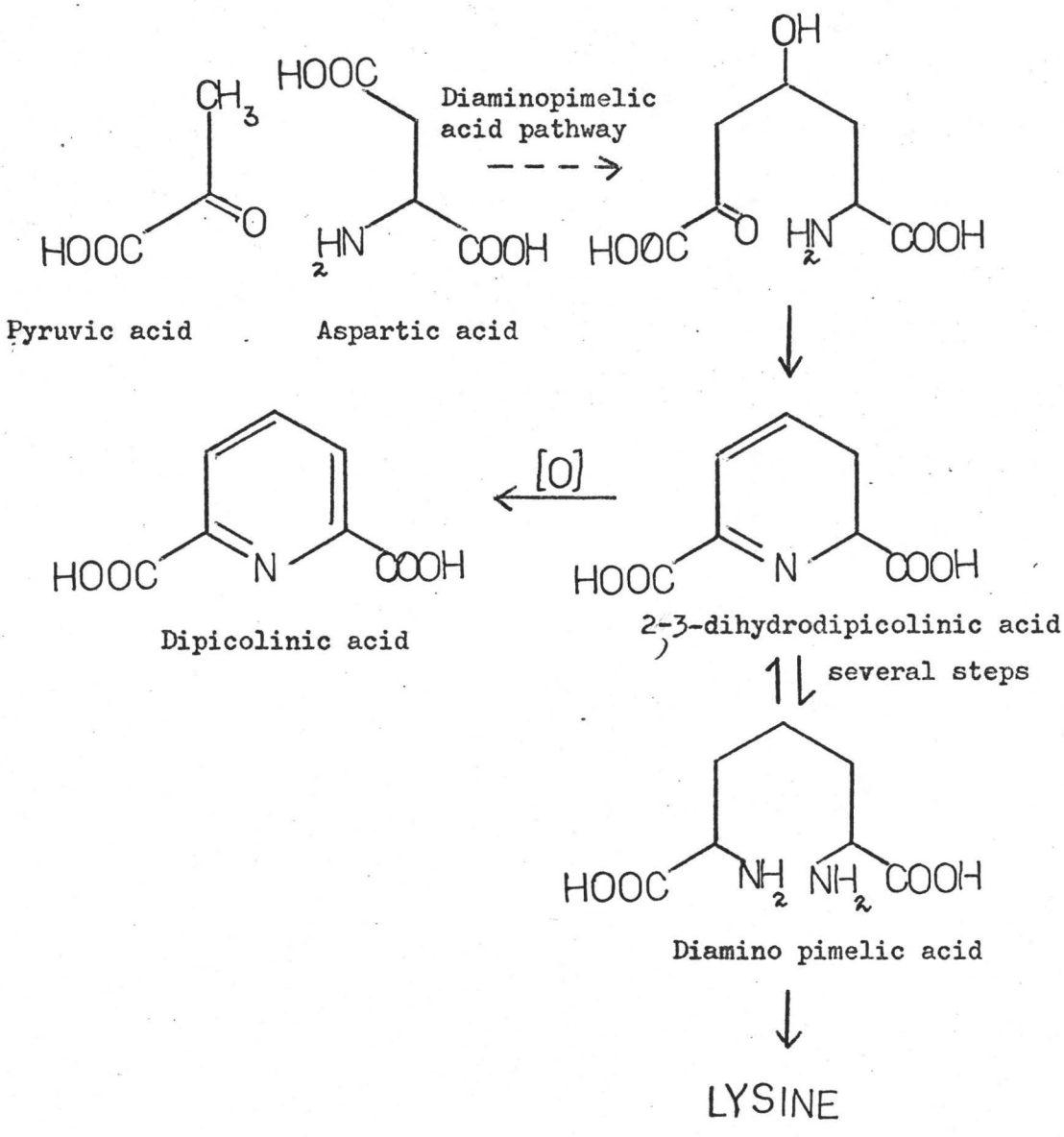
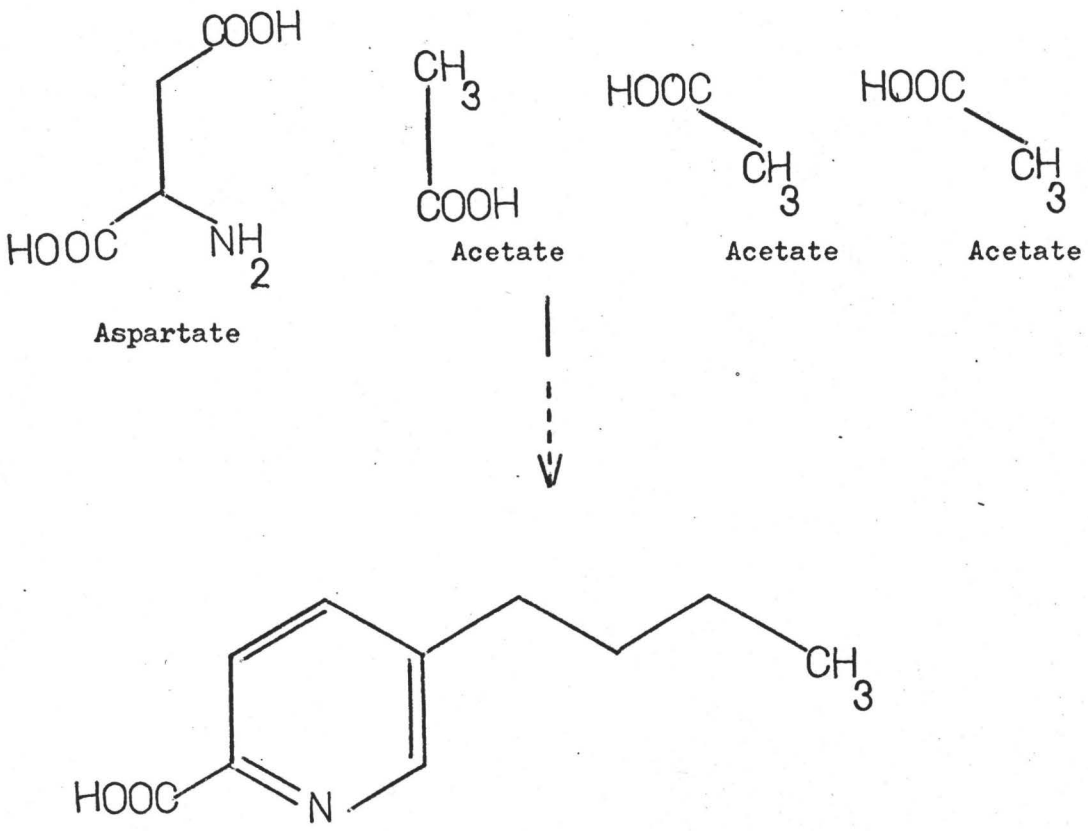


FIGURE 13

Aspartic acid and acetate as precursors of fusaric acid*



Aspartate

Acetate

Acetate

Acetate

Fusaric acid

* (Hill, Unrau & Canvin, 1966)

A and B (Kimura, Takahashi & Tamura, 1968) and into the alkaloid actinidine (Auda, Waller & Eisenbraun, 1967).

The result with dipicolinic acid shows that this compound is synthesised by different routes in different organisms. In this case the route is closely associated with the α-aminoadipic acid pathway to lysine. Consequently most of the remaining carbons of dipicolinic acid are derived from α-ketoglutarate, which combines with acetate, in the form of acetyl coenzyme A, to produce α-ketoadipic acid, an intermediate of the α-aminoadipic pathway. This compound is then converted to 2,6-diketopimelic acid by elongation of the carbon chain. The origin of this other carbon appears to be unknown. Finally the 2,6-diketo compound is converted to dipicolinic acid (fig. 14).

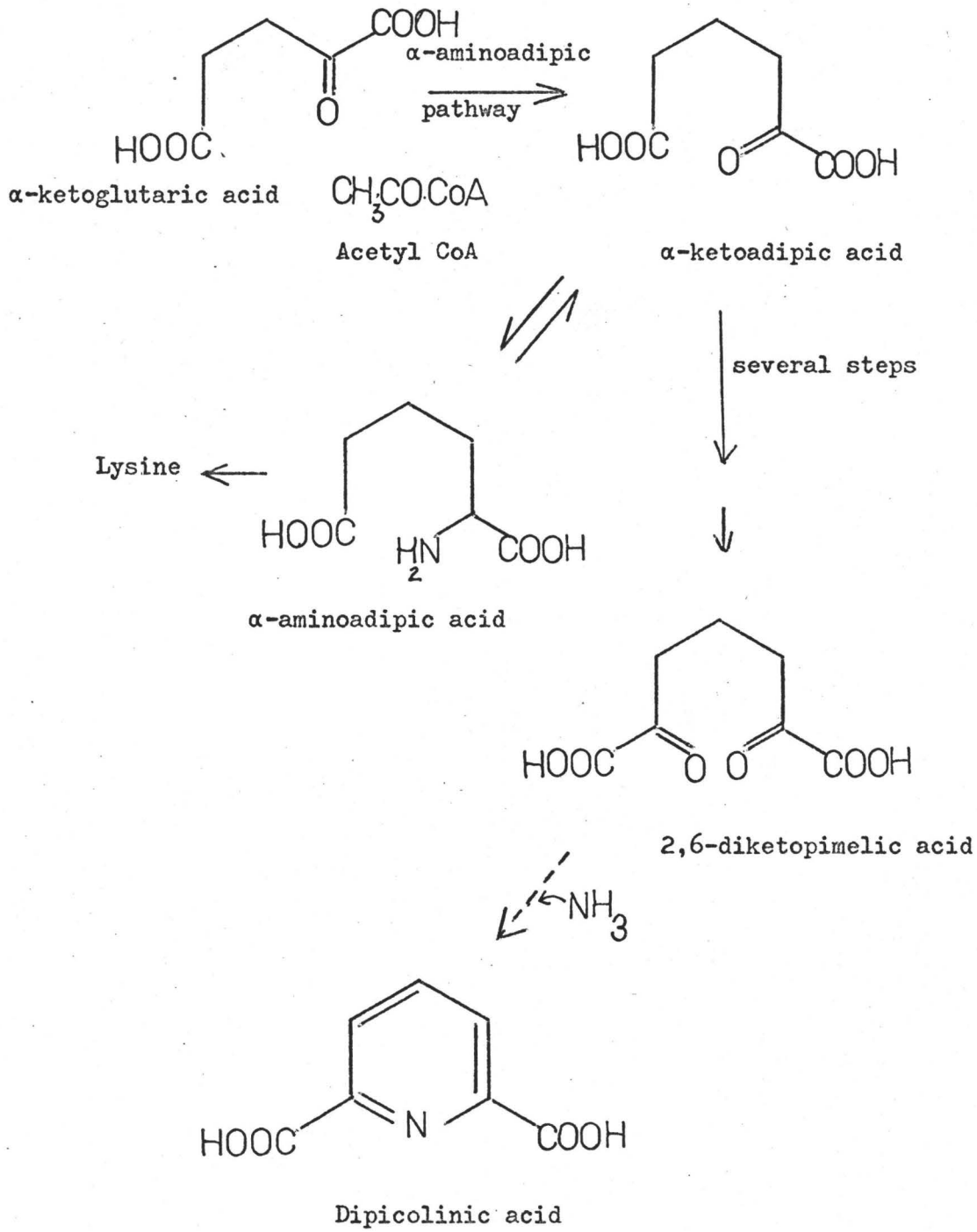
The structure of actinidine can be divided into two isoprene portions. On the basis of this structural hypothesis, [^{14}-C] labelled acetic acid and geranyl pyrophosphate were fed to intact Actinidia polygama (Auda et al., 1967). The distribution of activity was in accordance with this hypothesis of the origin of the actinidine skeleton (fig. 15).

Experiments designed to illustrate the origin of piericidin A and B in cultures of Streptomyces mobaraensis resulted in the non-random incorporation of [^{14}-C] labelled acetate and propionate into this substance. The evidence suggested that a long chain C_{23} moiety is first formed from four acetate and five propionate units. Formation of the pyridine ring follows and requires the inclusion of a nitrogen atom between C_{14} and C_{18} of the chain (fig. 16).

Three of the remaining four compounds from fig. 10 derive their

FIGURE 14

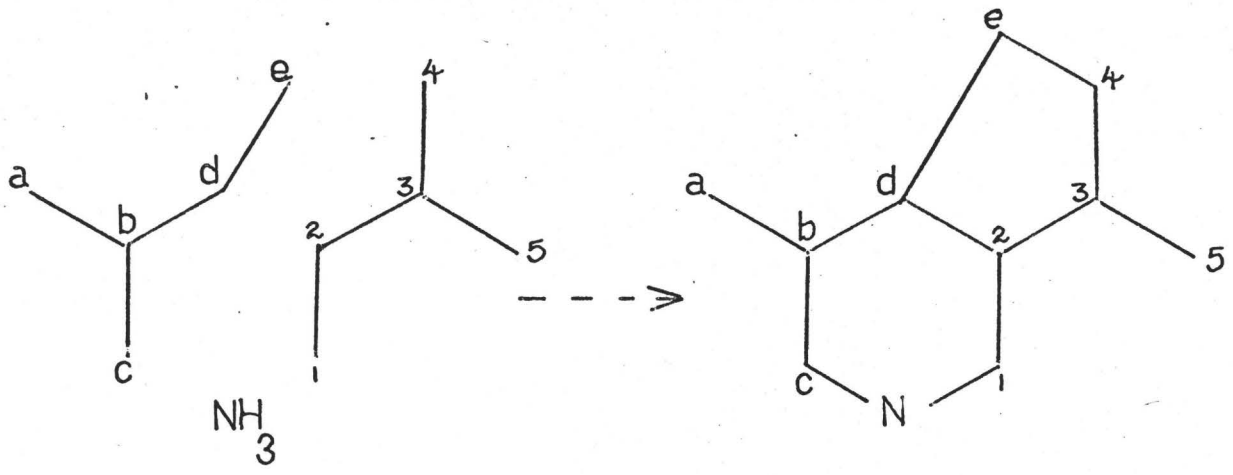
The route to dipicolinic acid in P. citreo-viride*



* (Hodson & Foster, 1966)

FIGURE 15

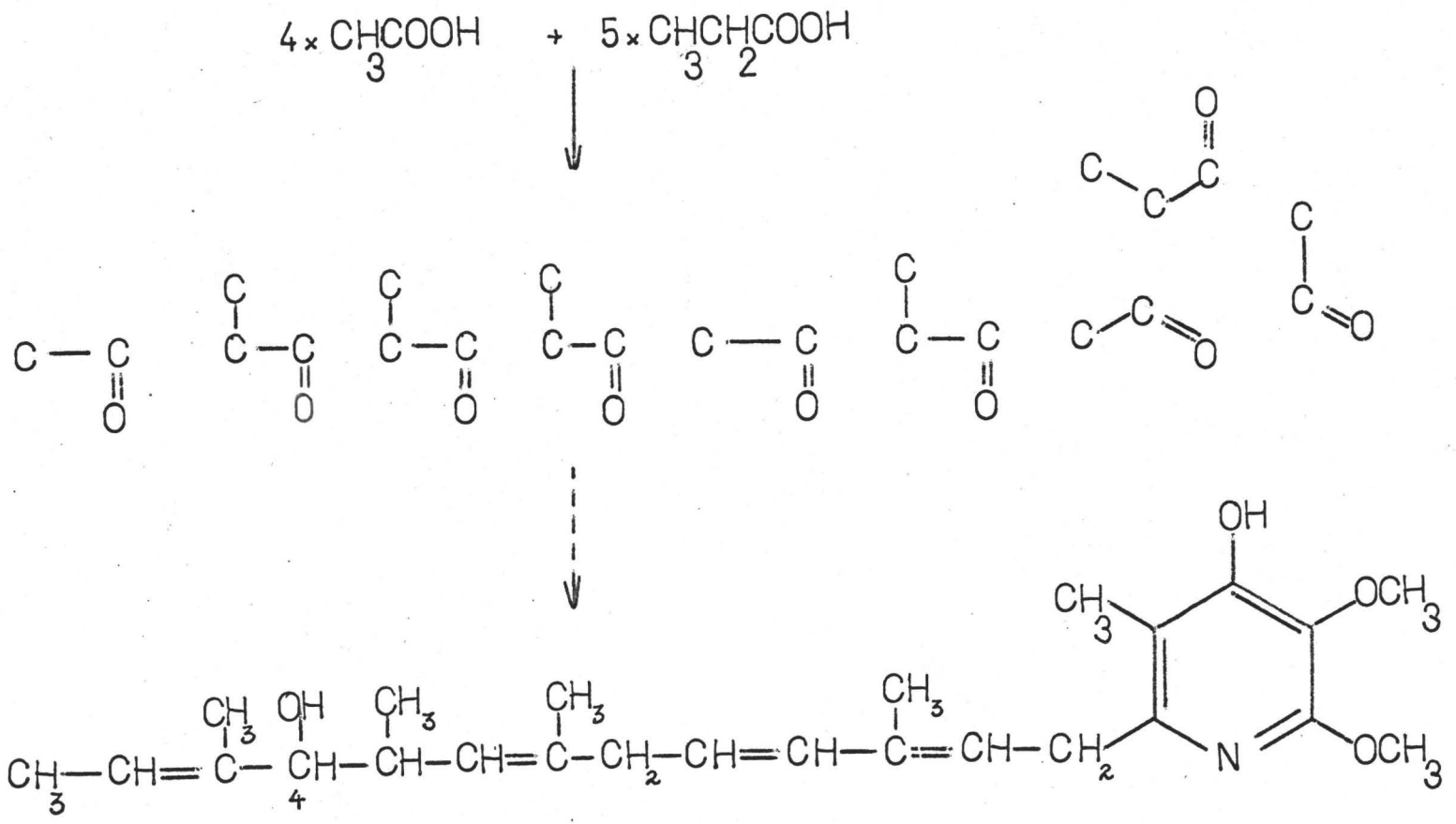
Isoprene units as precursors of actinidine*



* (Auda, Waller & Eisenbraun, 1967).

FIGURE 16

Fatty acids as precursors of piericidin A and B*



Piericidin A

Piericidin B

* (Kimura, Takahashi & Tamura, 1968).

pyridine moieties directly from the amino acid lysine. These are desmosine, isodesmosine and the derivative of 4-pyridone, mimosine.

Desmosine and isodesmosine have been isolated from a protein, elastin (Partridge, Elsden & Thomas, 1963). Structural hypotheses, which implicated lysine as a possible precursor stimulated tracer studies which subsequently showed that four lysine units account for the skeletons of desmosine and isodesmosine (Anwar & Oda, 1966) (fig. 17).

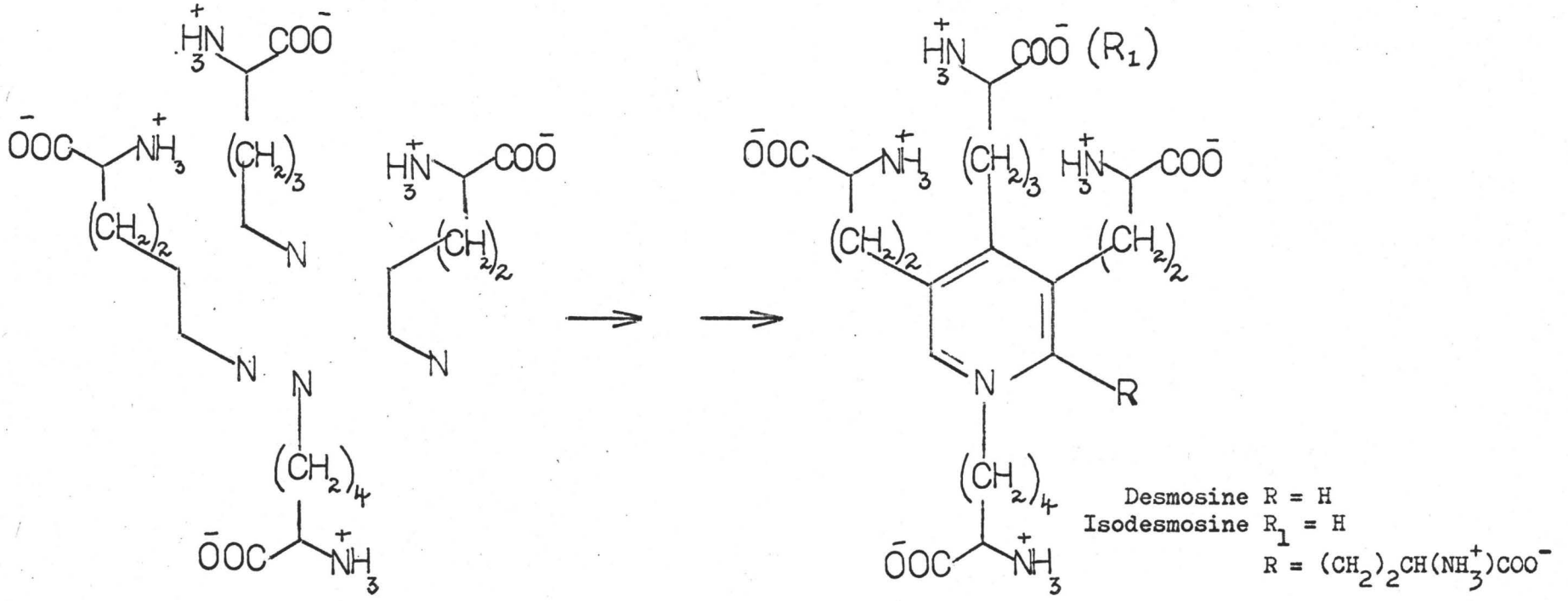
The substituted 4-pyridone nucleus of mimosine appears to be specifically derived from lysine. Administration of $[2\text{-}^{14}C]$ and $[6\text{-}^{14}C]$ DL-and L-lysine to Leucaena glauca and to Mimosa pudica (Hylin, 1964; Tiwari & Spenser, 1964) led to the isolation of radioactive mimosine. The label was shown to be incorporated non-randomly and this result supported the hypothesis that lysine serves as a specific precursor of mimosine (fig. 17).

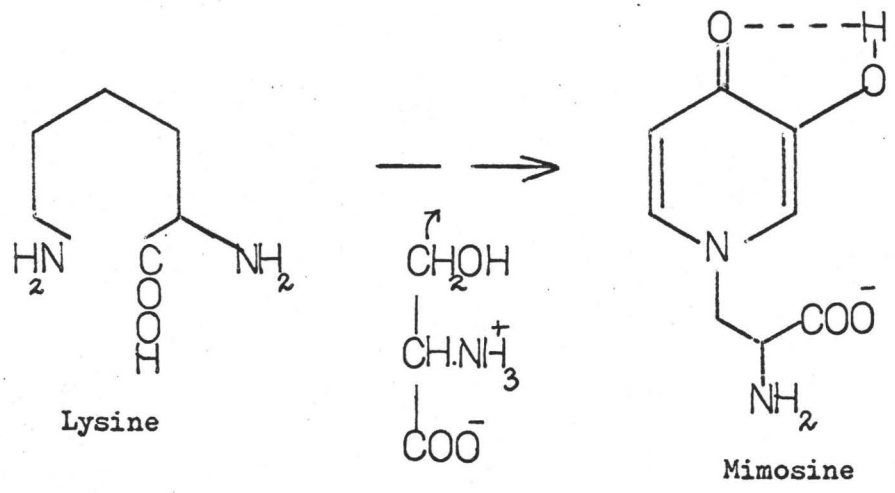
The alkaloid, indicaxanthin, a betaxanthin product of the Centrospermae family, contains a hydrogenated pyridine ring. The origin of this portion of the molecule was predicted on purely structural grounds, which suggested the amino acid derivative DOPA to be a precursor (Wyler, Mabry & Dreiding, 1963). Subsequent tracer studies supported this hypothesis (Minale, Piatelli & Nicolaus, 1965). The remaining portion of the molecule bears a close resemblance to, and was therefore thought to be derived from the amino acid proline. This hypothesis was also substantiated by tracer studies. The results of both studies are consistent with the scheme in figure 18.

Recently, a novel route to nicotinic acid has been demonstrated in the organism Clostridium butylicum (Scott & Mattey, 1968). The

FIGURE 17

Lysine units as precursors of desmosine, isodesmosine,[a] and mimosine[b]





[a] (Anwar & Oda, 1966)

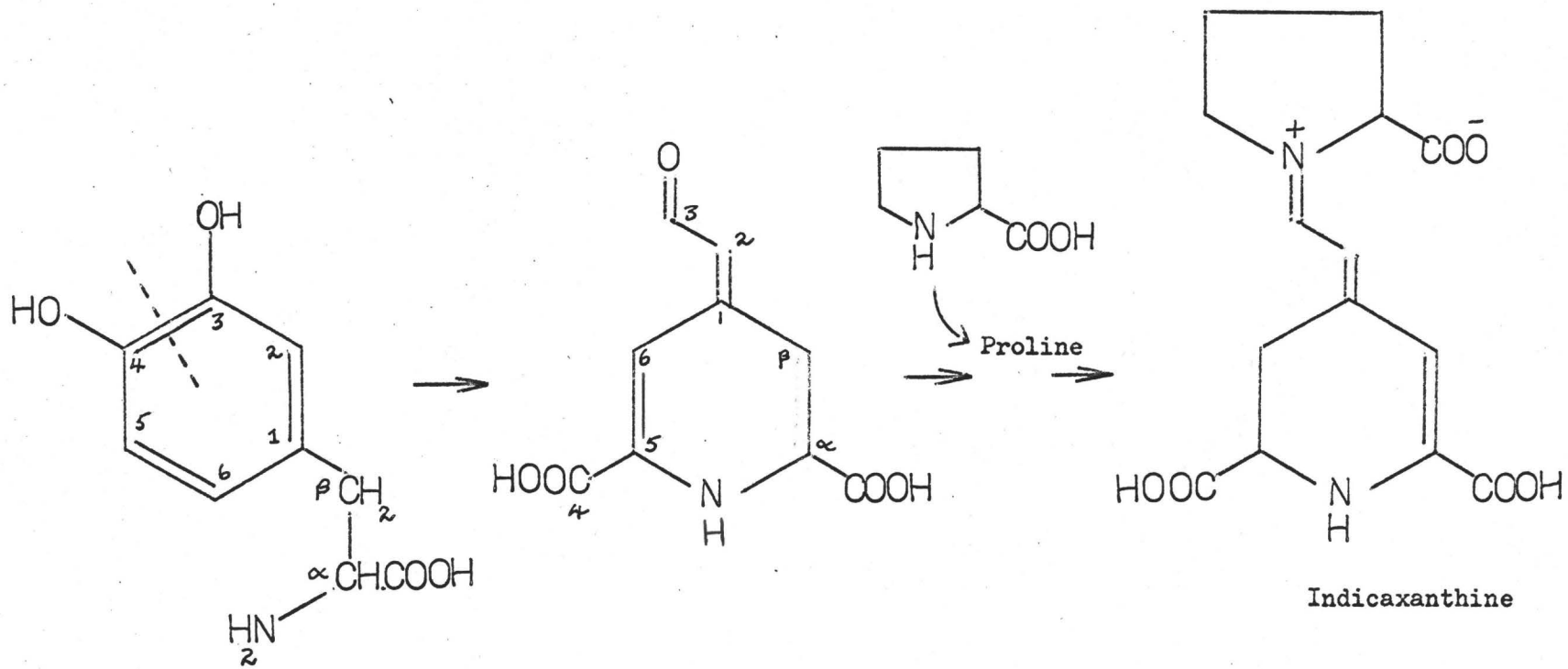
[b] (Hylin, 1964; Tiwari & Spenser, 1964)

[a] (Anwar & Oda, 1966)

[b] (Hylin, 1964; Tiwari & Spenser, 1964)

FIGURE 18

DOPA as a precursor of indicaxanthine*



Indicaxanthine

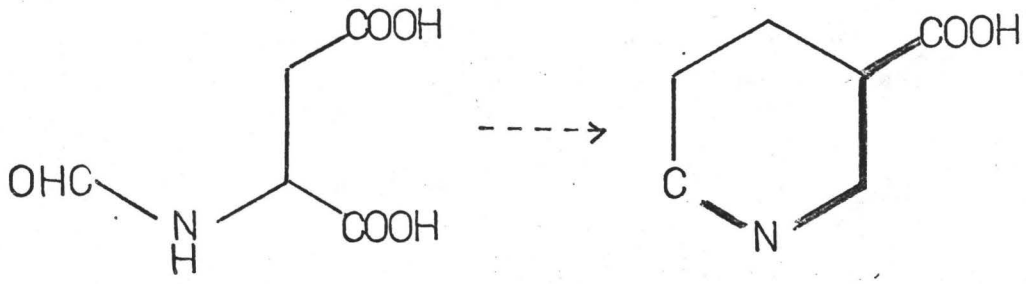
* (Minale, Piatelli & Nicolaus, 1965)

work provides the only evidence for a one carbon unit as a precursor of the pyridine ring in nature. In this system, as expected, aspartic acid provides carbons 5, 5' and 6 of the nicotinic acid skeleton. However label from $[^{14}C]$ formic acid was shown to be specifically incorporated into carbon 2 of the pyridine ring. This result precluded the possibility that a three carbon unit provides carbons 2, 3 and 4 and suggested N-formylaspartate to be the precursor of the ring nitrogen and carbons 2, 5, 5' and 6 of the nicotinic acid skeleton. Subsequent tracer studies with labelled N-formylaspartate showed this to be the case (fig. 19).

Finally, a pathway which leads to the pyridine ring, substantiated by tracer studies, but initially elucidated by the mutant organism approach, is the route from tryptophan to nicotinic acid in Neurospora and mammals. Although this route (fig. 2) has been discussed in detail in Chapter II, it is included here as it may be useful in the construction of working hypotheses of pyridoxine biosynthesis.

FIGURE 19

N-Formylaspartate as a precursor of nicotinic acid*

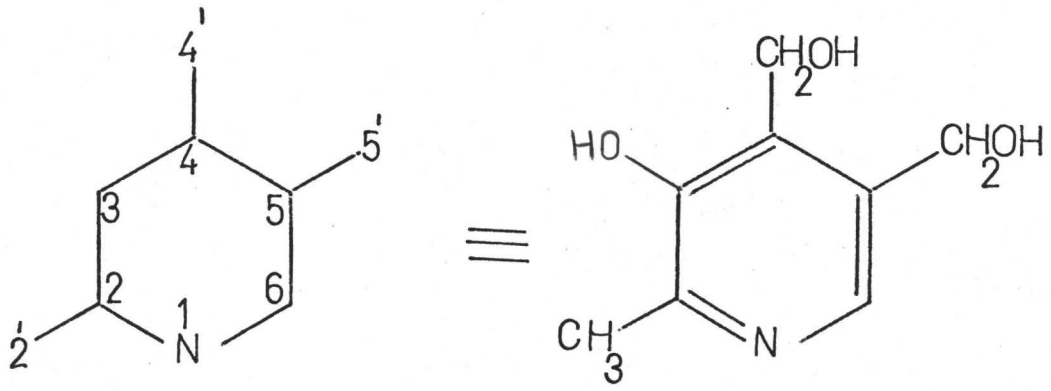


N-Formylaspartic acid Nicotinic acid skeleton

* (Scott & Mattey, 1968)

FIGURE 20

The carbon skeleton of pyridoxol



(3) Biogenetic hypotheses of the origin of vitamin B_6

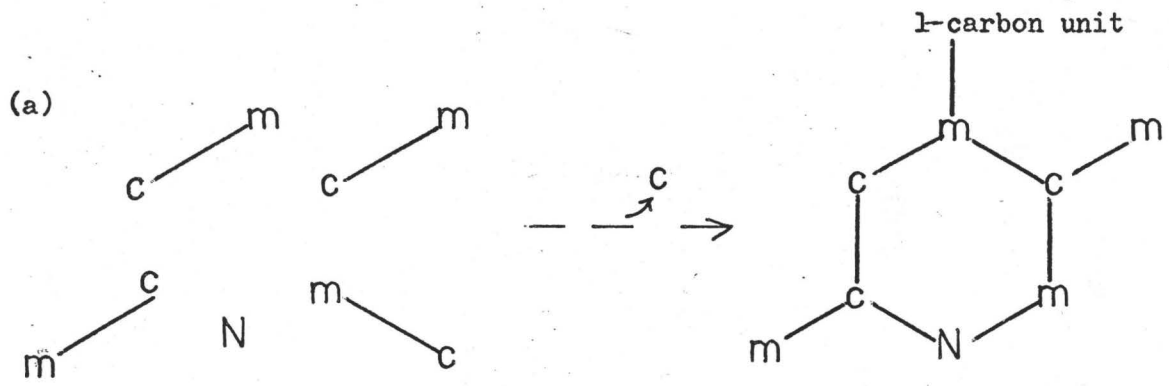
The carbon skeleton of vitamin B_6 is made up of a pyridine ring substituted with carbon at three positions. Each of the substituent groups contain one carbon atom (fig. 20). It is plausible therefore, that the biogenesis of vitamin B_6 might involve one-carbon metabolism.

The amino acid, methionine, is the biological donor of methyl groups (Greenberg, 1963). Hydroxymethyl, on the other hand, is more likely to arise from "active formaldehyde"; N^5, N^{10}-methylene tetrahydrofolate (Osborn, Talbert & Huennekens, 1960). There is ample precedent for the role of these two compounds as one carbon donors in biosynthesis. (Friedkin, 1963; Greenberg, 1963). However, the only known instance where a one carbon unit provides a portion of a naturally occurring pyridine ring is that in which formate supplies carbon 2 of nicotinic acid, when this compound is biosynthesised by cultures of Clostridium butylicuum. Moreover, this result shows a one carbon unit to provide a ring carbon and is thus somewhat removed from the present argument, which proposes one carbon units as a source of substituent carbons. Despite this lack of precedent several structural hypotheses can be constructed, based on the supposition that one carbon units provide substituent carbons. These are demonstrated in figures 21(a), 21(b) and 22(a).

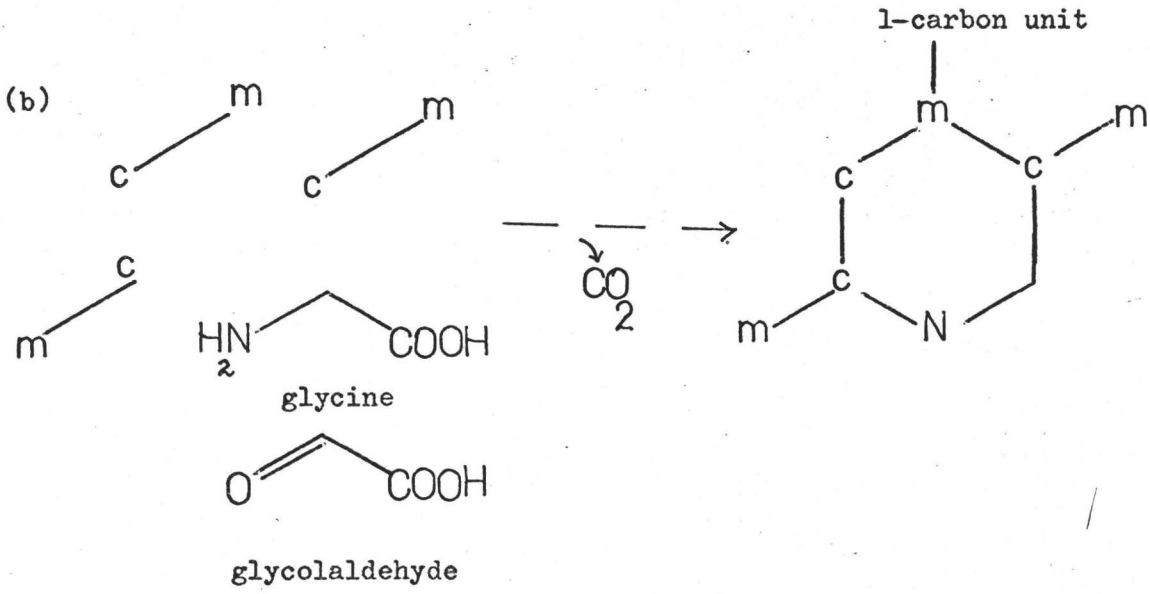
An arrangement leading to the carbon skeleton of the vitamin can be constructed from a polyketide (fig. 21(a)). This hypothesis is not without precedent. Polyketide units have been shown to provide

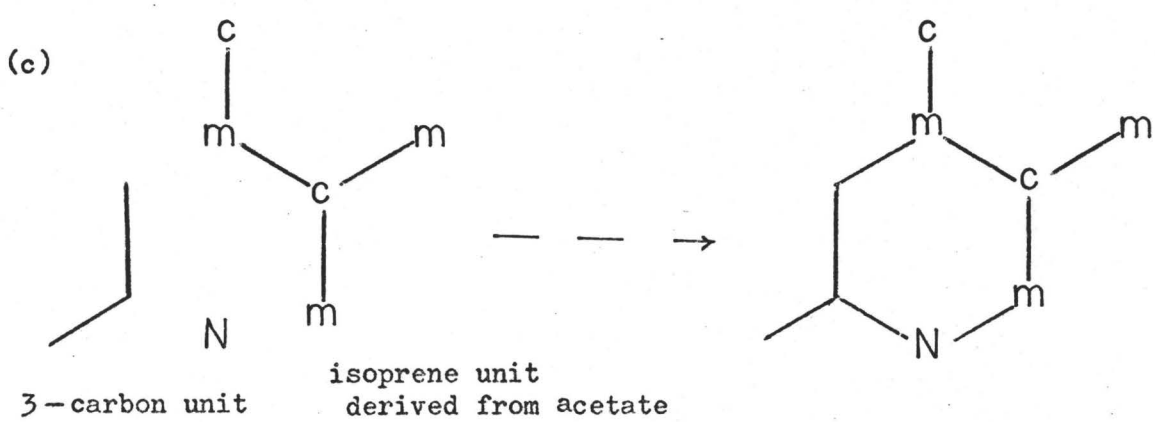
FIGURE 21

Acetate units as possible precursors of the skeleton of Vitamin B_6*



1. Carbon substitution at position 4'





* (m = methyl carbon of acetate, c = carboxyl carbon)

portions of the skeletons of other naturally occurring pyridine derivatives, namely fusaric acid (fig. 13) and piericidin A and B (fig. 16). In these systems, and in natural products of polyketide origin, in general, the polyketide chains arise by linear head to tail condensation of acetyl units. The present hypothesis, however, requires a carboxyl-carboxyl union. There is little precedent for such a union and it is therefore not very likely that the hypothesis represents the true nature of the origin of the pyridine ring of vitamin B_6.

A second acetate hypothesis is shown in figure 21(b). Here a triketide unit joins with the amino acid glycine or the corresponding keto acid, glycolaldehyde. The results of studies using pyridoxine auxotrophs of E. coli (Morris, 1959) suggested glycine and glycolaldehyde to be precursors of pyridoxine. Since the formation of the triketide unit again requires a carboxyl-carboxyl condensation, this variant is also somewhat unlikely.

The branched chain isoprene unit, mevalonic acid, is known to be derived from acetate. There is ample precedent for this unit as a biogenetic precursor (Richards & Hendrickson, 1964). A pertinent example is its role in the biosynthesis of the pyridine alkaloid, actinidine. The scheme in figure 21(c) illustrates how an isoprene unit could contribute to the skeleton of vitamin B_6.

Microorganisms and green plants are known to biosynthesise the pyridine ring of nicotinic acid from small molecules. A three carbon compound related to glycerol and a four carbon unit related to aspartate are implicated (fig. 5). The pyridine ring of dipicolinic

acid is derived from similar units when this compound is synthesised by the organism B. megaterium (Martin & Foster, 1958) (fig. 12). With these precedents in mind, and the knowledge that aspartic acid induces a high rate of synthesis of pyridoxine in the yeast Candida albicans (Sato, 1965b), it seems plausible that aspartic acid could contribute to the pyridine ring of vitamin B_6 (figs. 22(a) & (b)).

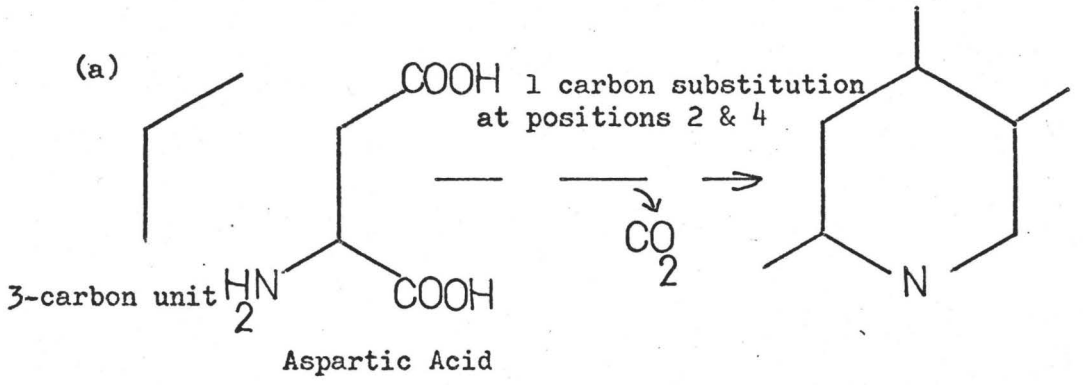
In the scheme in figure 22(b) three units, a three carbon moiety, related to pyruvate, a four carbon unit related to aspartate and a two carbon unit, presumably acetate, account for the carbon skeleton of pyridoxine. This hypothesis is somewhat analogous to the mode of biosynthesis of fusaric acid, and of dipicolinic acid, when this compound is synthesised by P. citreo-viride (fig. 14). Both of these pathways involve the condensation of an acetate unit with a large unit in order to form their pyridine nuclei.

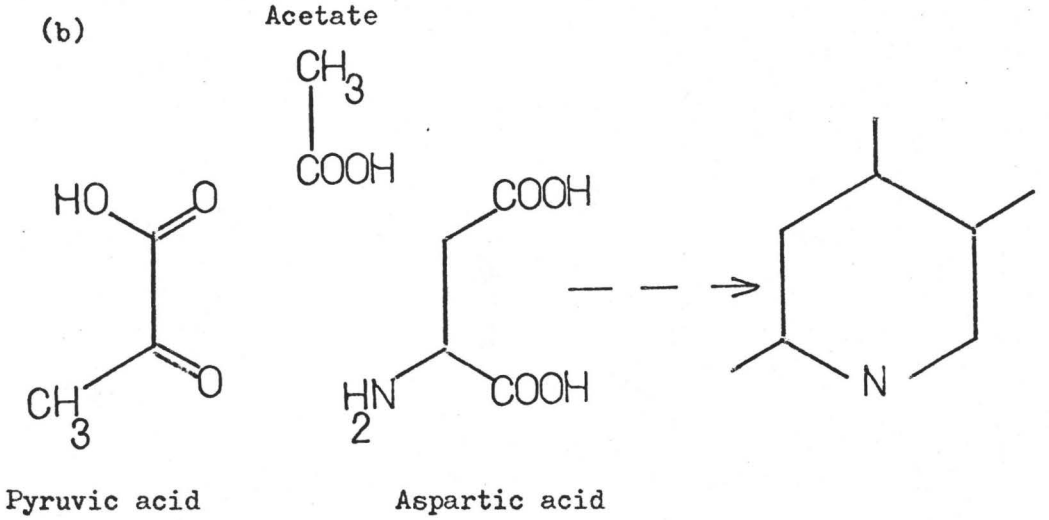
In Neurospora and mammals the pyridine ring of nicotinic acid arises by degradation of the amino acid tryptophan (fig. 2). Even though this route does not occur in microorganisms and in higher plants, the main sources of vitamin B_6, the possibility that the pyridine ring of pyridoxine might in fact arise in this way, cannot be completely rejected, a priori. Apart from this route, two other degradations of the tryptophan skeleton can be envisaged as leading to substantial fragments of the vitamin skeleton (fig. 23(a)&(b)).

In early work, Snell (1942) noticed the structural relationship between alanine and positions 2, 2' and 3 of pyridoxine (fig. 8). Even though early nutritional investigations did not substantiate such a relationship (Chapter III (i)), the hypothesis that a three

FIGURE 22

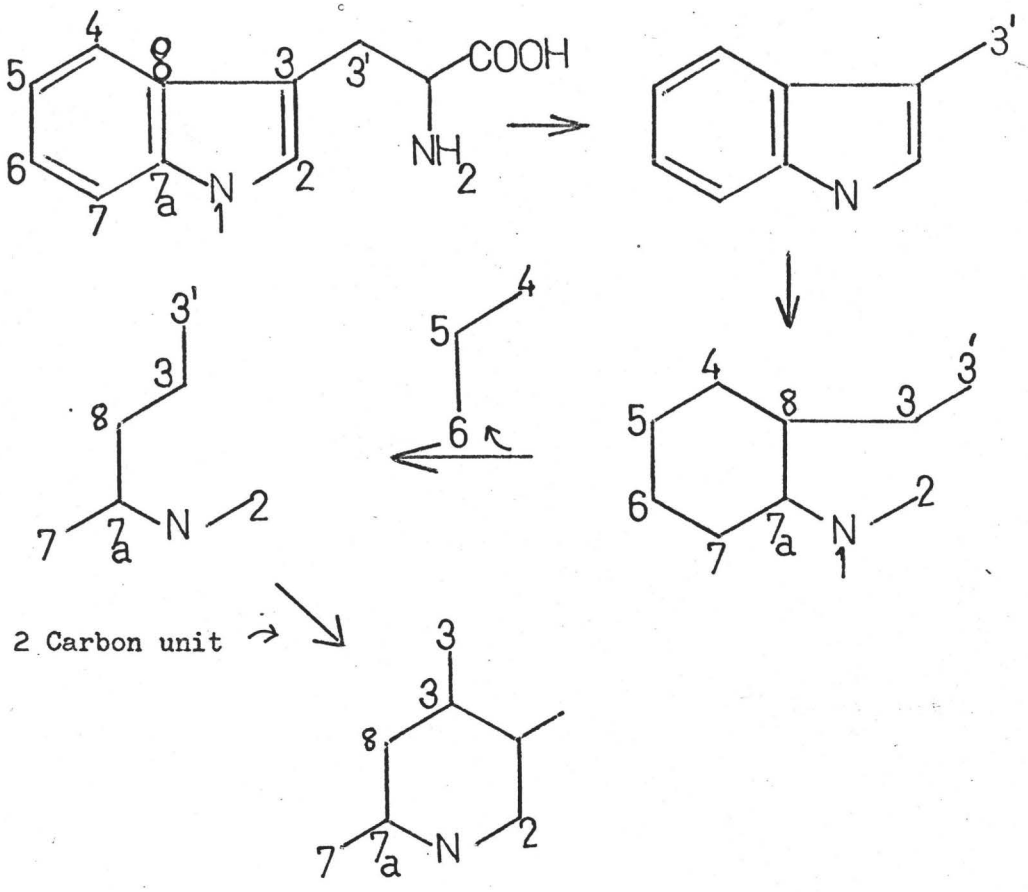
Aspartic acid as a possible precursor of Vitamin B_6





Novel degradations of Tryptophan leading to portions of the skeleton of Vitamin B_6

(a)



(b)

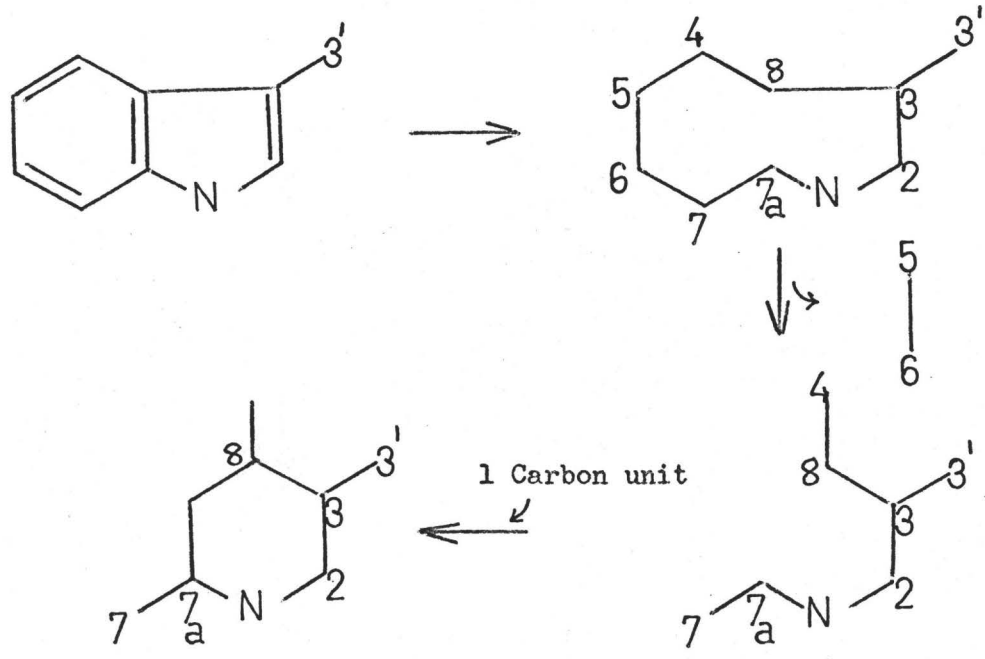
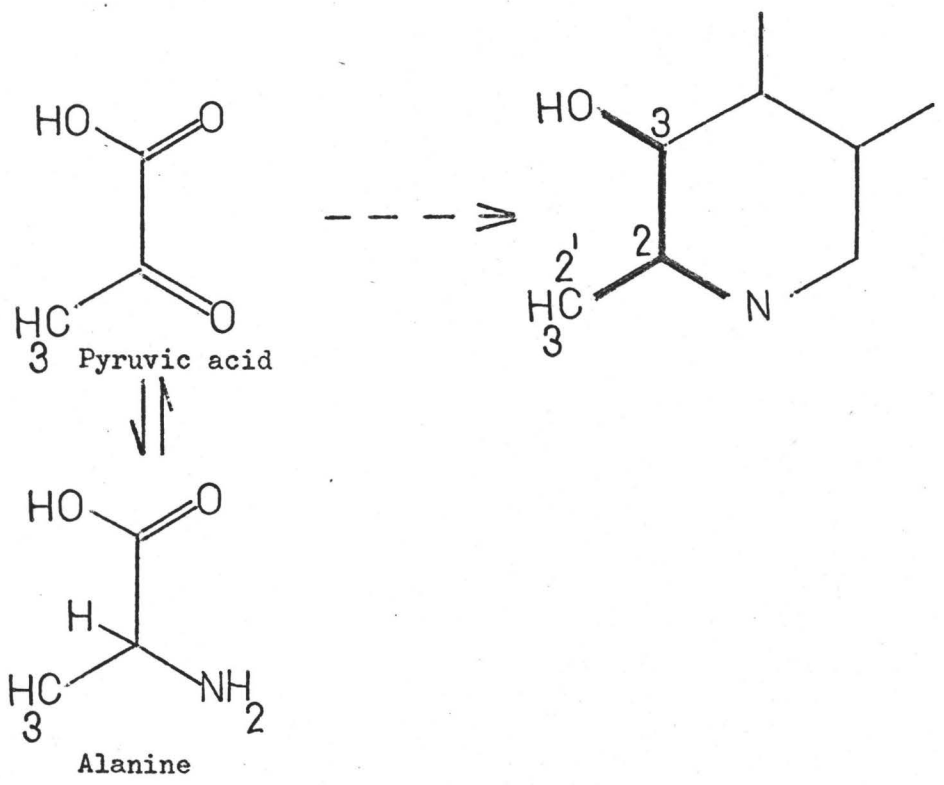


FIGURE 24

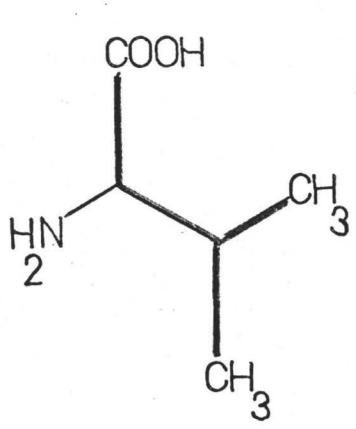
A three carbon unit, related to pyruvic acid as a precursor of carbons 2', 2 and 3 of Vitamin B_6*



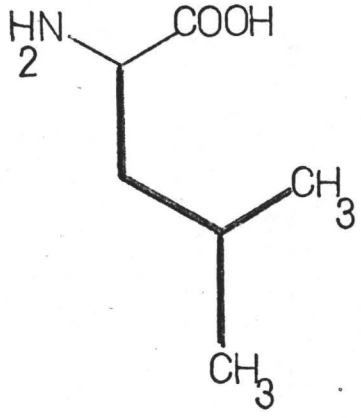
* (c.f. Snell, 1942) (fig. 8)

FIGURE 25

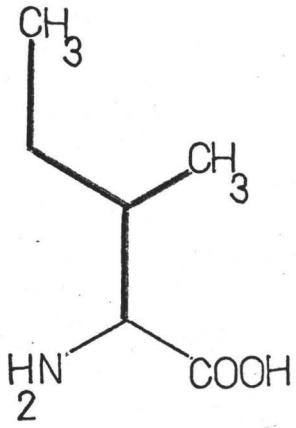
A five carbon branched chain structure, related to the branched chain amino acids, as the precursor of carbons 4',4,5,5' and 6 of Vitamin B_6.



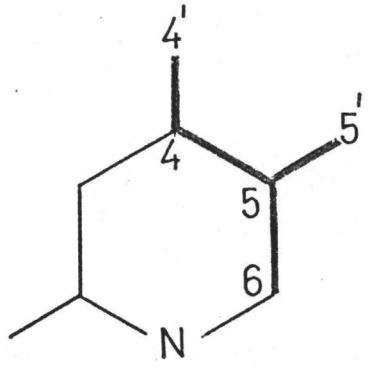
Valine



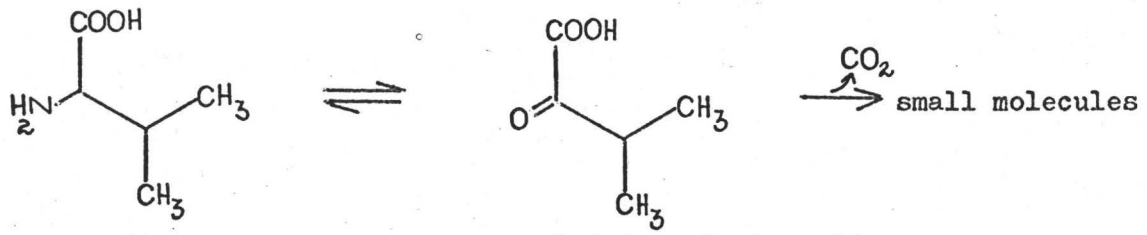
Leucine



Isoleucine

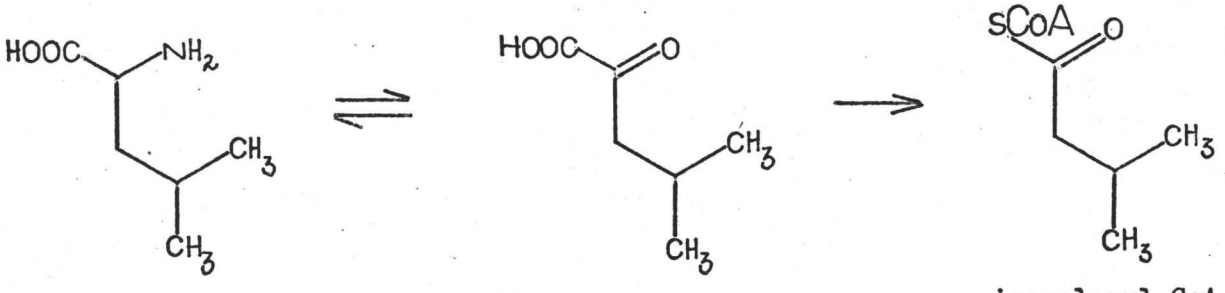


(a) The Degradative pathways of leucine, valine and isoleucine, showing branched units which may provide carbons 4,4',5,5' and 6 of Vitamin B_6



aline

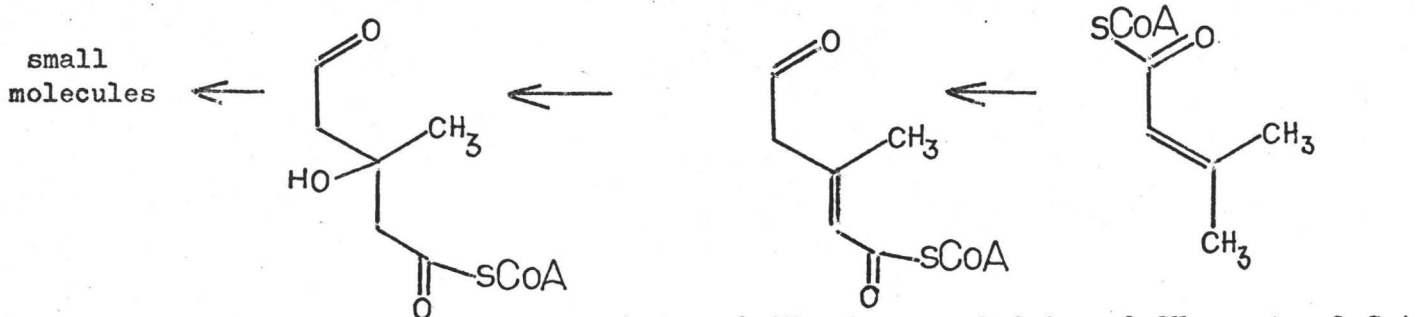
α-ketoisovaleric acid



leucine

α-ketoisocaproic acid

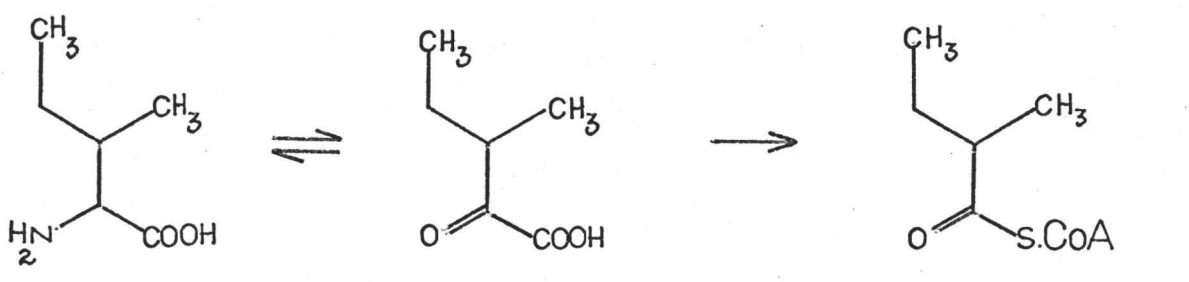
isovaleryl CoA



β-OH, β-CH_3-glutaryl CoA

β-CH_3-glutaconyl CoA

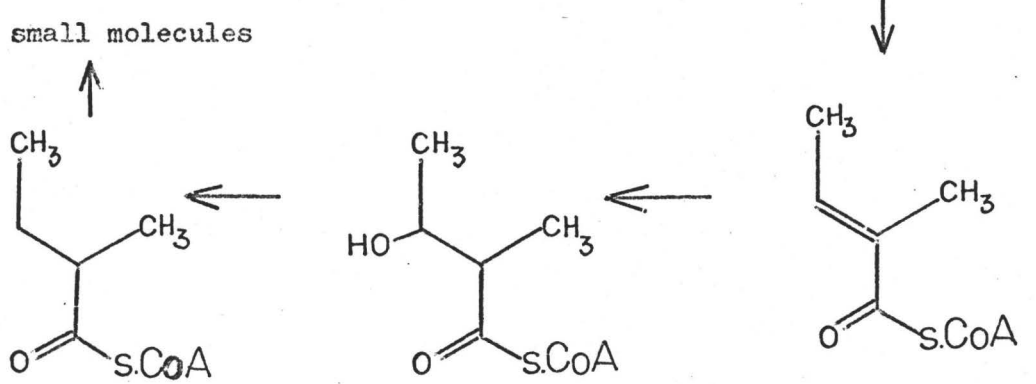
β-CH_3-crotonyl CoA



isoleucine

α-keto, β-CH_3-valeric acid

α-CH_3-butyryl-S.CoA

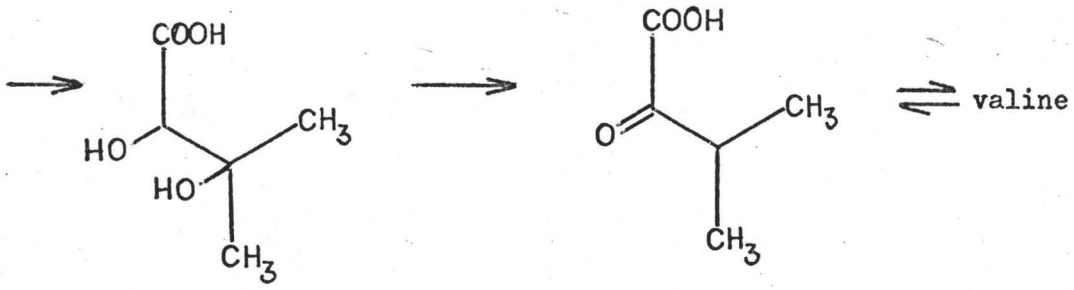


α-CH_3-acetoacetyl CoA

α-CH_3, β-OH-butyryl-S.CoA

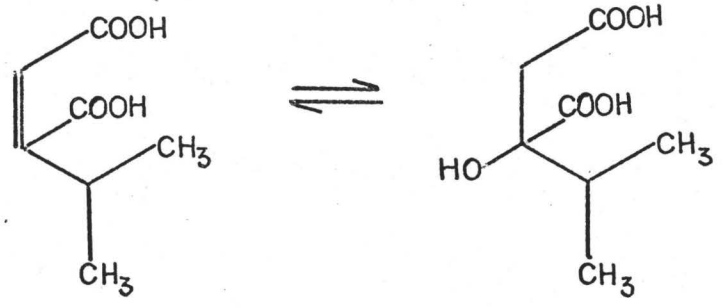
tigloyl CoA

(b) The biosynthetic schemes of valine, leucine and isoleucine, showing branched units which may contribute to carbons 4,4',5,5' and 6 of Vitamin B_6.



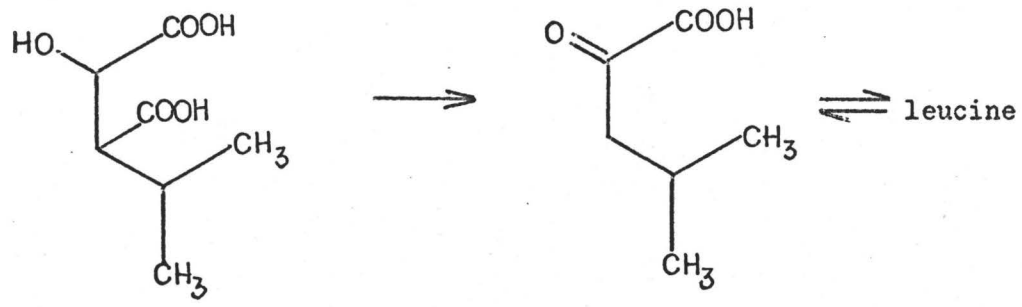
α,β-dihydroxyisovaleric acid

α-ketoisovalerate



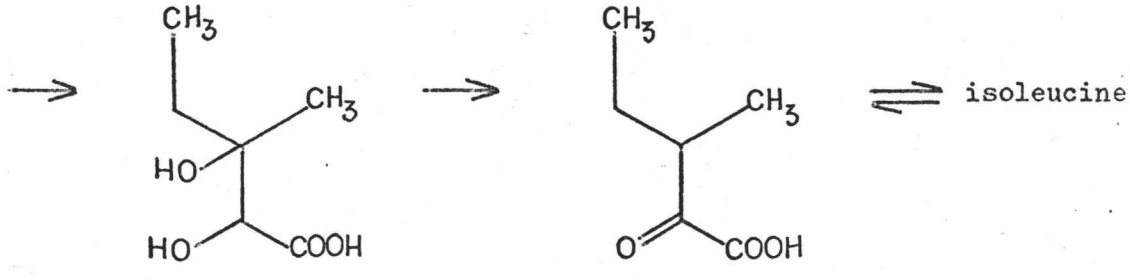
cis-dimethylcitraconic acid

β-OH,β-carboxyisocaproic acid



α-OH,β-carboxyisocaproic acid

α-ketoisocaproic acid

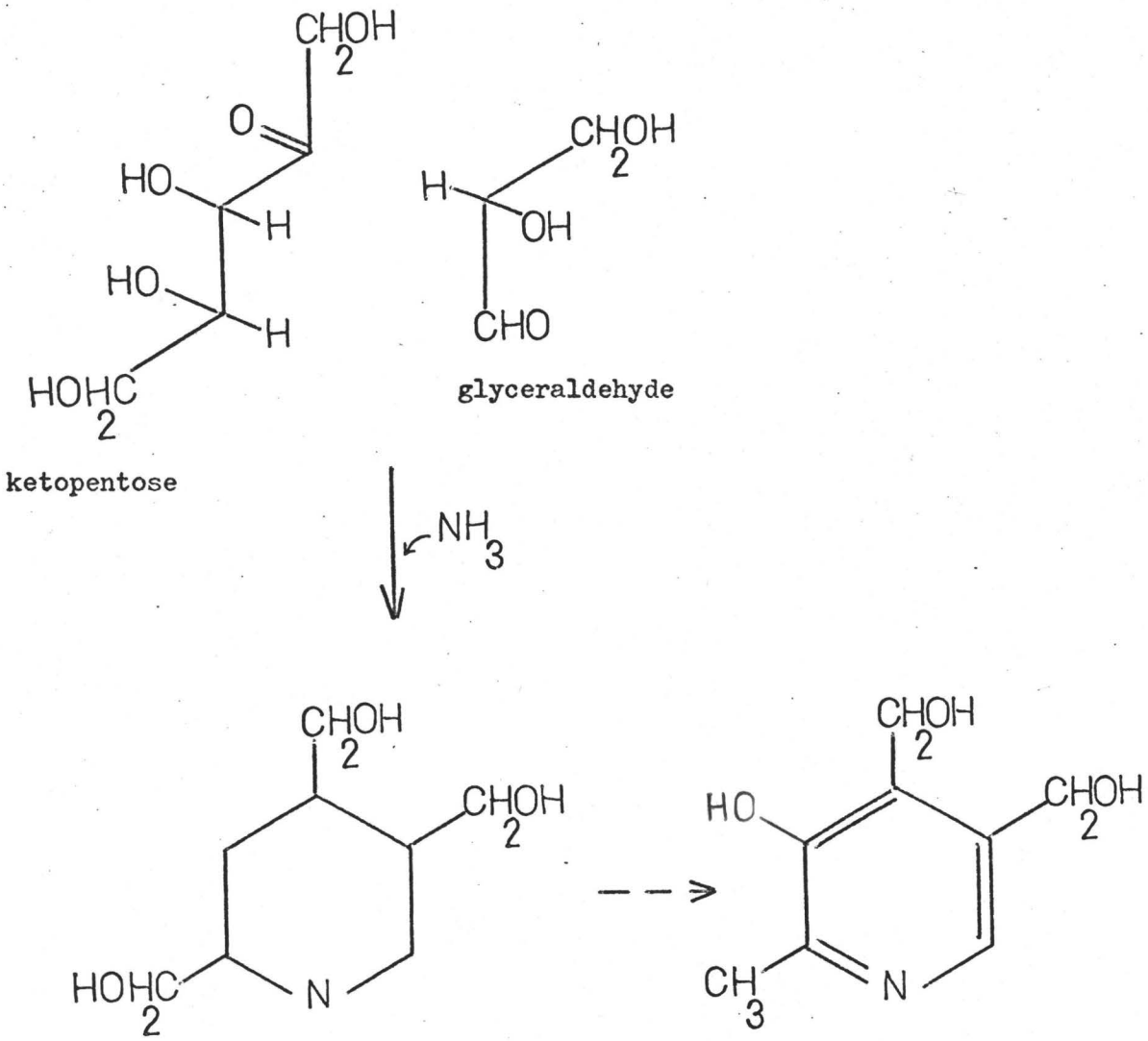


α,β-dihydroxy-β-methylvaleric acid

α-keto-β-methyl valeric acid

FIGURE 27

Carbohydrates as possible precursors of Vitamin B_6*



* (Leete, 1963)

carbon unit, related to alanine or pyruvic acid, might serve as a precursor of these carbons (fig. 24), is attractive on structural grounds.

This hypothesis leaves a branched chain five carbon unit, representing carbons -4, -4', -5, -5' and -6 of the pyridoxine skeleton, to be accounted for (fig. 25). The possible derivation of such a unit from a mevalonate moiety has been mentioned above. The only other widely distributed naturally occurring compounds from which a five-carbon branched chain structure might be derived are the amino acids, valine, leucine and isoleucine. The biosynthetic and degradative pathways of these three compounds (fig. 26) illustrate the units which could provide this fraction of the vitamin. One of these, β-methyl-β-hydroxyglutaryl CoA, was suggested to be a precursor on the basis of results of tracer studies (Suzue, 1964). Another, α,β-dihydroxy-β-methylvaleric acid, was used in a purely structural hypothesis, (Dalgliesh, 1958).

Finally, a novel hypothesis, suggested by Leete (1963), proposes that a ketopentose unites with glyceraldehyde to form the vitamin skeleton (fig. 27).

(4) Experimental approach to test the hypotheses

In principle the strategy capable of testing a biogenetic hypothesis is simple. Putative precursors, suitably labelled with ^{14}C, are administered to a system which biosynthesises vitamin B_6. After sufficient exposure to the labelled substrate the vitamin is isolated from the system, and, if radioactive, purified to constant specific activity. If the substrate has served as a direct precursor, the label should be present at a predictable site in the product. Selective degradation determines the position of the label. If non random incorporation is thus established and the results are interpretable, a direct product-precursor relationship may exist.

The solution of the tactical problems of translating this strategy into action determines the success or failure of the approach.

In all microbiological systems the amount of vitamin B_6 biosynthesised per mg dry weight of cells or per litre culture etc. is exceedingly small (Dempsey, 1967). Successful isolation and purification of these microgram quantities is a major stumbling block.

In previous tracer experiments isolation of the vitamin involved total hydrolysis of the harvested cells, followed by column chromatography. In such systems the chromatographic technique must distinguish between the heterocyclic ring of the vitamin and those of the nucleic acid bases. Not only is the concentration of the latter massive, compared to that of the vitamin, but due to the

similarity of their chromatographic properties, purification of the vitamin is tedious and difficult.

To overcome this problem it would be desirable to have access to a microbiological system which releases newly synthesised vitamin B_6 into its surrounding medium. In this way the contaminating presence of nucleic acid bases, as a consequence of cell hydrolysis, may be avoided.

Another prerequisite for success in this work, however, is the isolation of amounts of radioactive compound adequate for chemical degradation; the simplest reaction requires a minimum of 20 mg of pure crystalline pyridoxol hydrochloride.

A technique called "carrier dilution" overcomes this problem. This process rests on the premise that the biosynthesised product is indistinguishable chemically from unlabelled synthetic vitamin B_6. It is necessary to be sure of the identity of the vitamin congener that is biosynthesised by the system. However, this is a minor problem. Addition of known weights of identical unlabelled synthetic material to the crude system produces a pool of the vitamin which can be readily isolated and purified to constant specific activity. This specific activity must be high enough to permit counting and useful degradations. It is directly proportional to the extent of incorporation of the labelled substrate into the vitamin and inversely proportional to the dilution factor. This latter function is, in turn, directly proportional to the amount of unlabelled material added as carrier and inversely proportional to the amount of biosynthesised material. In this work, where the amount biosynthesised is small, carrier dilution

results in a large dilution factor. Thus isolation of a final product with a workable specific activity requires that the substrate be efficiently incorporated into the vitamin.

The isolation of any natural product relies heavily upon the techniques of chromatography. The isolation of vitamin B_6 is no exception, and ion exchange and thin layer chromatography can be used to this end. The carrier material will raise the total vitamin content of the system above the limit of detection by ultra-violet spectroscopy (the minimal concentration detectable is about M/60,000). The vitamin has a series of characteristic absorption spectra (Metzler & Snell, 1954), which permit fractions and spots containing it to be easily identified.

Finally, it is essential that the final crystalline product is pure, as trace amounts of highly labelled impurity will produce totally misleading results. With this in mind purification must be rigorous.

The degradation reactions used to determine the distribution of the label must proceed cleanly by known mechanisms. Since there are eight carbons in the pyridoxine molecule, 1/8 i.e. 12.5% of the total activity, in each carbon, represents random incorporation of the labelled substrate. Any other distribution is non-random and may reflect a precursor-product relationship. Thus although complete degradation is desirable, partial degradation can give useful results.

IV THE BIOSYNTHESIS OF PYRIDOXOL

(1) Introduction

A microbiological system that releases newly synthesised vitamin B_6 into its surrounding medium has been described by Dempsey (1966). The organism is a mutant of *E. coli* that contains a block at the oxidase step between pyridoxol phosphate and pyridoxal phosphate. The 5' phosphate ester of pyridoxal is the biologically active form of the vitamin. Therefore, this mutant, although it can synthesise pyridoxol, does not grow unless the medium is supplemented with pyridoxal. It follows that, if a growing culture is deprived of pyridoxal, growth will cease. However, under these conditions the organism, although not growing, continues to biosynthesise pyridoxol which it excretes into the surrounding medium. This synthesis continues for about three hours before all metabolism ceases due to the lack of pyridoxal. The rate of synthesis during this time is four to five times that of the wild type *E. coli* B.

All the tracer experiments described made use of this system. Addition of the labelled substrate coincided with the onset of pyridoxal starvation. Incubation was carried out for about five hours, with the aim of inducing incorporation during the phase of rapid pyridoxol synthesis.

Aside from satisfying the main requirements of the proposed experimental approach, this system has three desirable features. The pyridoxol which is released into the medium is not

subject to the degradative metabolism which can be assumed to be associated with the turnover of normally metabolising cells. The short incubation time is convenient and is likely to minimize the randomisation of the label, and finally, the absence of growth avoids the dissipation of the label into cellular macromolecules.

Both pyridoxol and its 5' phosphate ester are released into the medium (Dempsey, 1966). In order to isolate the total vitamin content in the form of pyridoxol, the 5'phosphate ester fraction was hydrolyzed. This was effected by the use of low molarity sulphuric acid (Morris, Hughes & Mulder, 1959). A known weight (generally 40 mg) of unlabelled pyridoxol hydrochloride was then added to the hydrolysate.

The mixture of newly biosynthesised pyridoxol and carrier pyridoxol was then re-isolated, using a combination of ion exchange and thin-layer chromatography techniques.

The final material was crystallised as the hydrochloride and its radioactivity assayed. The crystallization procedure was repeated until successive crystal batches showed the same specific activity. The final batch was further purified by high vacuum sublimation.

The purified pyridoxol hydrochloride was degraded by Kuhn-Roth degradation to yield acetic acid (isolated as the α-naphthylamide (Leete, Gregory & Gros , 1965)) from C-2 and the adjacent C-methyl group (C-2'). Degradation of the acetic acid by the Schmidt reaction in turn gave the C-methyl group as methylamine (isolated as the N-dinitrophenyl derivative).

(2) Materials & Methods

(i) Microbiological

The organism used in this investigation was a pyridoxine auxotroph of Escherichia coli, obtained from W. B. Dempsey. Its original designation, E. coli $B.B_{6-2}$ (Dempsey, 1965) was recently changed and the mutant has now been reclassified as E. coli B. W.G.2. (Dempsey, 1968). It grows well on a medium supplemented with pyridoxal but cannot utilize pyridoxol.

Stock cultures were maintained on monthly slants of minimal medium containing glycerol (0.5%) as a carbon source, supplemented with pyridoxal hydrochloride ($6x10^{-7}$ M). These slants were incubated for about 48 h. at 37°C and then stored at 6°C.

Media

All bacterial stocks were maintained on a minimal medium containing glycerol (0.5%) in the salts medium of Rickenberg, Yanofsky and Bonner (1953), (KH_2PO_4 7g, K_2HPO_4 3g, $(NH_4)_2SO_4$ 1g, $MgSO_4$ 0.1g, $CaCl_2$ 0.01g; made up to 1000 ml with glass distilled water). This medium was supplemented with pyridoxal hydrochloride (final concentration $6x10^{-7}$ M) and will be referred to as Supplemented glycerol (0.5%) medium; when solid medium was required Bacto agar (20 g) was added.

In all tracer experiments the same salts medium was used, but the carbon source was reduced to glycerol (0.2%) and pyridoxal supplementation was omitted; this medium will be called Unsupplemented glycerol (0.2%) medium. All pyridoxal solutions were sterilized by filtration.

In one experiment glycerol was replaced by glucose (0.2%). This medium will be referred to as Unsupplemented glucose (0.2%) medium.

Reisolation Procedure

Mutant stocks were reisolated before each tracer experiment. Stock cells were suspended in sterile distilled water (10 ml) and the absolute count of this suspension estimated using a haemocytometer. Successive dilutions in sterile distilled water yielded a final suspension containing approximately 200 cells/ml. Samples of this suspension ($\frac{1}{2}$ml) were spread on plates of supplemented glycerol (0.5%) medium. The plates were incubated at 37°C to yield single colonies of approximately 2mm diameter.

Two series of plates, one of supplemented glycerol (0.5%) medium, the other of unsupplemented glycerol (0.5%) medium were prepared. Each plate was allotted twenty inoculation sites, eighty sites were used on each medium. Both media were inoculated at a given site with cells from a single colony; the plates were incubated (37°C) and inspected for growth after 36h. Cells from supplemented sites, which had no growth at the corresponding unsupplemented site, were subcultured onto slants of supplemented glycerol (0.5%) medium. These slants were incubated (37°C, 24h) before use in a tracer experiment.

Growth Experiments

Supplemented glycerol (0.5%) medium (250 ml) was placed in a one litre Erlenmeyer flask. The medium was inoculated with freshly isolated cells and the culture incubated on a rotary shaker (37°C, 400 rev/min.).

Growth curve of E. coli WG2 ($B\text{-}B_{6\text{-}2}$) on supplemented glycerol (0.5%) medium.
(for incubation conditions see text p. 80).

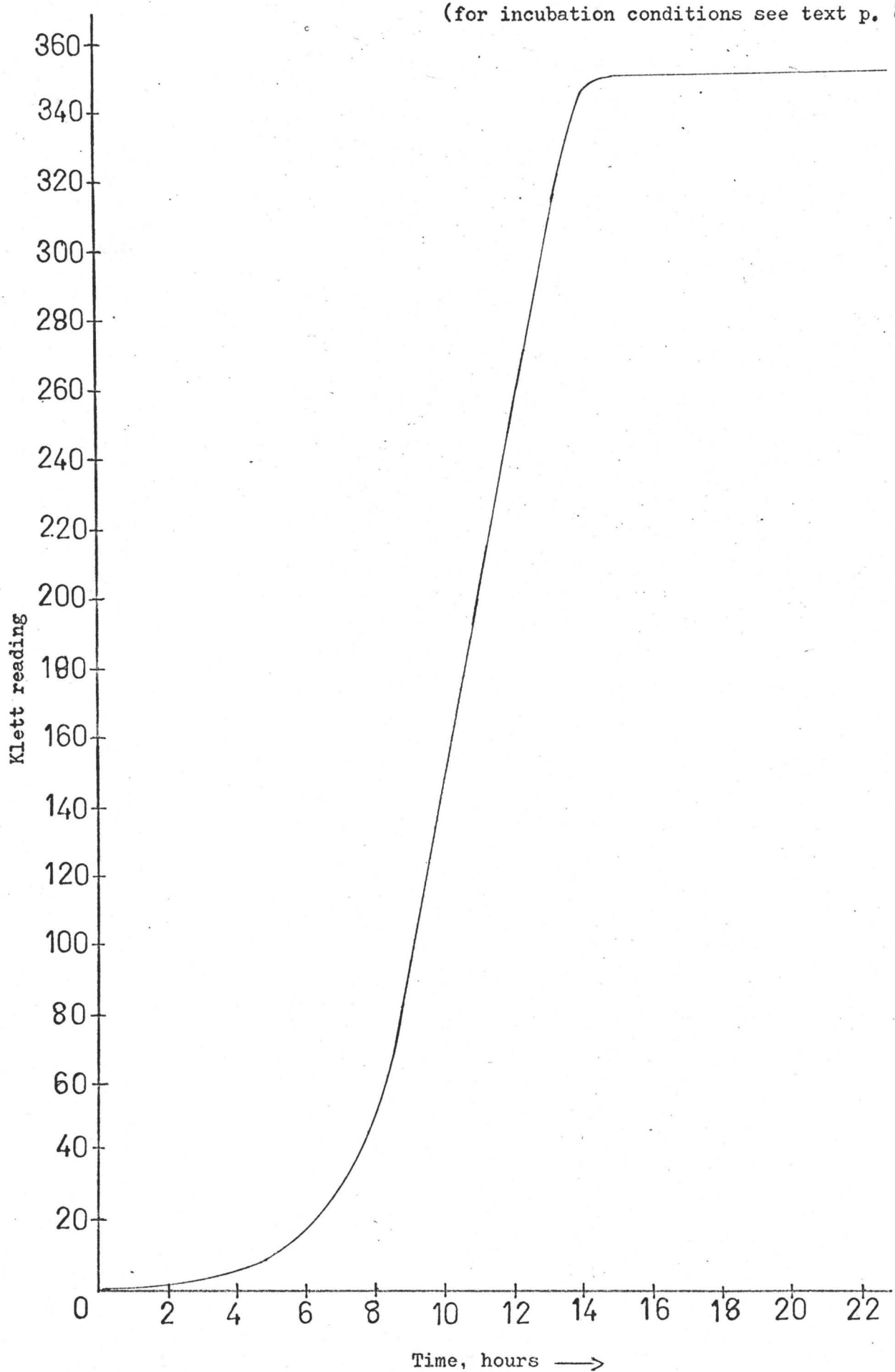


TABLE V

Radioactive Compounds

Expt. No.	Compound	Nominal Specific Activity (mCi/m mol)	Nominal Total Activity (μCi)	Source
1	[1-^{14}C] Glycerol	10	500	(a)
2	[1-^{14}C] Glycerol	15.4	100	(b)
3	Sodium [3-^{14}C] pyruvate	35.6	100	(b)
4	Sodium [3-^{14}C] pyruvate	35.6	100	(b)
5	Sodium [2-^{14}C] pyruvate	31.7	100	(b)
6	Sodium [1,3-^{14}C] pyruvate			
	Sodium [1-^{14}C] pyruvate	27.2	150	(b)
	Sodium [3-^{14}C] pyruvate	35.6	150	(b)
7	Sodium [2-^{14}C] acetate	54.7	100	(b)
8	Sodium [1,3-^{14}C] pyruvate			
	Sodium [1-^{14}C] pyruvate	27.2	100	(b)
	Sodium [3-^{14}C] pyruvate	35.6	100	(b)
9	Sodium [^{14}C] formate	36	100	(b)
10	[2-^{14}C]-DL-Leucine	48	100	(a)
11	[3-^{14}C]-DL-Aspartic acid	1.9	100	(d)
12	[7a-^{14}C]-DL-Tryptophan	3.7	100	(c)
13	[3,3',4,5-^{14}C$_4$]-DL-Isoleucine	160	100	(a)
	[3,3',4,5-^{14}C$_4$]-DL-Alloisoleucine	160	100	(a)
14	[Methyl-^{14}C] Methionine	53.6	200	(b)

(a) Commissariat a l'Energie Atomique, France

(b) Amersham/Searle

(c) Tracerlab

(d) Cal Biochem

The optical density of the culture was measured on a Klettometer (Klett Summerson photoelectric colorimetor, model 800-3) at zero time and every hour until a constant value was observed and the stationary phase was thus evident. Distilled water was used as a blank in every measurement. The resultant growth curve (fig. 28) was used to determine the period of logarithmic growth.

(ii) Tracer Experiments

Labelled Compounds

The labelled compounds used in individual tracer experiments are listed in Table (V). Fourteen tracer experiments were carried out. In eleven of these (Expts. 1-5, 7, 9-12, 14) the substrate was dissolved in sterile distilled water (10 ml), and this solution was added to the culture fluid by means of a disposable syringe. Mixtures of trace labelled compounds were used in the three remaining experiments. In Experiment 13 a mixture of DL-isoleucine and DL-allisoleucine was used. In Experiments 6 and 8 intermolecularly doubly labelled 1,3-$^{14}C_2$-pyruvate was used which was prepared as follows.

Sodium 1,3-$^{14}C_2$-Pyruvate

Samples of sodium 1-^{14}C-pyruvate (100 μCi) and sodium 3-^{14}C-pyruvate (100 μCi) were each dissolved in sterile distilled water (10 ml). Samples (6 x 10 μl) were withdrawn from each solution for liquid scintillation counting; the solutions were then mixed and made up to a volume of 25 ml with sterile water. This solution of intermolecularly labelled 1,3-$^{14}C_2$-pyruvate was added to the culture fluid in Experiment 8. The same procedure was adopted in Experiment 6

but in this case the amount of each radiomer was 150 μCi.

Two samples (2x100 μl) were withdrawn from the solution of intermolecularly labelled 1,3-$^{14}C_2$-pyruvate. Each 100 μl was added to a solution containing sodium pyruvate (110 mg) in distilled water (100 ml). To each of these solutions was added 20 ml of a solution of phenylhydrazine hydrochloride, which had been prepared from 0.25 ml phenylhydrazine, made up to 50 ml with 0.1 M HCl. The reaction mixture was allowed to stand overnight, when crystals of 1,3-$^{14}C_2$-pyruvic acid phenylhydrazone were filtered and dried. For Kuhn-Roth oxidation this doubly labelled material was diluted with unlabelled carrier as follows.

1,3-$^{14}C_2$-Pyruvic acid phenylhydrazone (11.3 mg) was dissolved in 0.1 N NaOH (1 ml) and this solution was added to a solution of unlabelled pyruvic acid phenylhydrazone (120 mg dissolved in 8 ml 0.1 N NaOH). Water (25 ml) was added, the solution was thoroughly mixed and filtered and water (33 ml) was added to the filtrate. Hydrochloric acid(0.1 N, 18 ml) was added and the mixture was allowed to stand overnight. The diluted 1,3-$^{14}C_2$-pyruvic acid phenylhydrazone was filtered and dried. This sample was counted and then subjected to Kuhn-Roth oxidation in order to determine the ratio of the activities at the two labelled centres.

Feeding Incubations

Freshly isolated cells from a 24h slope were used to inoculate two 250 ml samples of supplemented glycerol (0.5%) medium in 1 litre Erlenmeyer flasks. The cultures were incubated on a rotary shaker (36°C, 400 rev/min) until the optical density indicated that growth

was well into the exponential phase. The cells were harvested by centrifugation for 10 min at 7000 g and washed with sterile distilled water (3x100 ml).

The cells were resuspended in four 250 ml samples of unsupplemented glycerol (0.2%) medium in 1 litre Erlenmeyer flasks. The solution of radioactive tracer was equally divided among these cultures which were then incubated for 6h on a rotary shaker (36°C, 400 rev/min). Harvesting was by centrifugation for 10 min at 7000 g, the culture fluid was decanted and stored at 4°C.

This procedure was used in all the experiments except 2 and 6. In Experiment 2 the unsupplemented glycerol (0.2%) medium was replaced by unsupplemented glucose (0.2%) medium. In Experiment 6 four 250 ml samples of supplemented glycerol (0.5%) medium was inoculated and incubated, the harvested cells were then resuspended in six 330 ml samples of unsupplemented glycerol (0.2%) medium; incubation conditions remained the same.

Isolation of Pyridoxine

Remaining cells in the decanted fluid were removed by filtration, (0.2 μ membrane filter, Nagle Co.). The filtrate was reduced in volume to 200 ml by vacuum distillation by means of a rotary evaporator. The residual solution was then subjected to acid hydrolysis. Sulphuric acid (1M) (~80 ml) was added to the solution until the final pH was 1.5. This solution was autoclaved (121°C, 3h) to complete the hydrolysis. The hydrolysate was lyophilised and stored at 4°C.

Chromatography

Dowex 50x8 (200-400 mesh) (Dow Chemical Co.) was subjected to a washing cycle (water, 3N HCl, water, 6N KOH) until free of fines. A column (15 cm by 1.5 cm) was prepared and treated with 6N KOH followed by water until the pH of the eluate was approximately 9.

The lyophilised material, obtained from a feeding incubation above, was dissolved in 0.2M potassium acetate-acetic acid buffer, pH 4.5, (200 ml), and the solution was filtered through a fine sintered glass filter. Pyridoxine hydrochloride (5 mg) (Eastman Organic Chemicals) was added to the clear filtrate and this solution was loaded onto the prepared ion exchange column.

Elution was effected by stepwise increase in pH using the following buffer series:

0.2M potassium acetate-acetic acid, pH 5.00 (100 ml),

0.2M potassium acetate-acetic acid, pH 5.50 (100 ml),

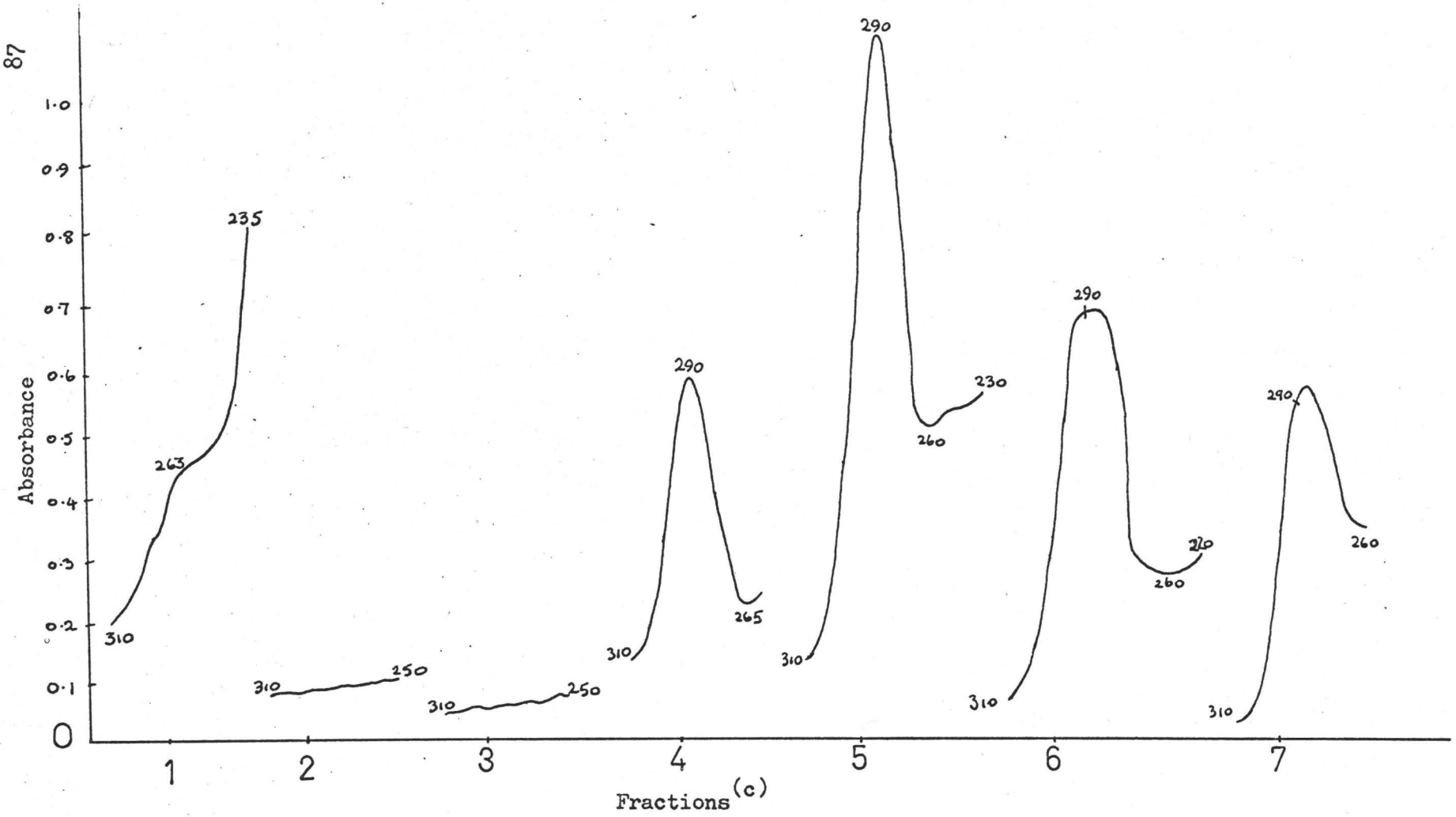
0.2M potassium acetate-acetic acid, pH 6.00 (50 ml),

and 0.2M sodium borate, 0.2M potassium chloride, 0.2M sodium hydroxide, pH 6.60 (200 ml).

Fractions (10 ml) were collected and assayed by ultra-violet spectroscopy in order to determine the position of pyridoxine in the elution sequence. Pyridoxine was eluted in the fractions following the addition of the final buffer (pH 6.6) (fig. 29).

A second Dowex 50x8 (200-400 mesh) column (6 cm by 1 cm) was prepared and washed with 0.1M hydrochloric acid until the eluate was acidic. The pyridoxine fractions from the first column were pooled, evaporated under reduced pressure, and redissolved in 0.1M hydrochloric

Typical UV profile[a] (acid conditions[b]) of fractions[c] containing pyridoxol. FIGURE 29



(a) The wavelengths (mμ) are given, (pyridoxol absorbs at 290 mμ in acid conditions).

(b) A few drops of 0.1M HCl was added to the cuvette before each scan.

(c) Fraction 1 is the first fraction eluted after addition of the KCl, borate, NaOH buffer (pH 6.6).

acid (50 ml). Pyridoxine hydrochloride (15 mg) was added to this solution which was then applied to the second column. The column was eluted with distilled water until the eluate was neutral, and then with dilute ammonia (3%).

The ammoniacal eluate (10 ml) was taken to dryness under reduced pressure and the solid residue dissolved in a small volume of anhydrous methanol (2 ml). This solution was applied to a preparative plate for thin layer chromatography, (thickness 2mm, Silica Gel G according to Stahl). Development was for 15 cm in the solvent system tert. butyl alcohol, methyl ethyl ketone, ammonia (.880), water; (4:3:2:1). The plate was dried and the pyridoxine band was identified by its characteristic blue fluorescence under ultra-violet light. The band was removed from the plate and the pyridoxine extracted from the silica gel by stirring at 40°C, overnight, in anhydrous methanol (40 ml). The resultant slurry was filtered through a fine sintered glass filter and remaining traces of silica were removed from the filtrate by further filtration through a P.V.C. membrane (0.1 μm, Sartorium Co.). The filtrate was reduced to a small volume (1-2 ml) and pyridoxine hydrochloride (20 mg) was added, together with a few drops of 0.1M hydrochloric acid. On addition of anhydrous ether pyridoxine hydrochloride crystallized in good yield.

The product was repeatedly recrystallized from the same solvent system until successive crystal batches did not change in specific activity. Final purification was effected by high vacuum sublimation (2×10^{-3} mm, 150°C). The sublimed pyridoxine monohydrochloride (m.p. 206°C) (~30 mg, 75% yield) was used for degradation.

The above procedure was used in all the experiments except 1 and 6. In Experiment 1 the incorporation efficiency was high enough to permit dilution with 100 mg unlabelled pyridoxine hydrochloride. In this case the yield of sublimed material was about 85 mg.

Experiment 6 was carried out using twice the usual amount of tracer and culture fluid. This permitted dilution with 80 mg of unlabelled pyridoxine hydrochloride, and yielded ~70mg resublimed product.

DEGRADATIONS

Kuhn-Roth oxidation (Leete, Gregory & Gros, 1965).

Pyridoxine hydrochloride (20 mg) was added to a chromic acid mixture consisting of chromium trioxide (2 g) in dilute sulphuric acid (2N, 10 ml), and the reaction flask was connected to a condenser. Steam was passed through the reaction mixture until the volume of the distillate was approximately 80 ml. The volume of the reaction mixture was maintained by addition of distilled water (5 ml at a time) and a continuous stream of nitrogen gas was passed through the system during the distillation. The distillate containing acetic acid was neutralized by titration with 0.1M sodium hydroxide. The resulting sodium acetate solution was placed in an oven at 100°C and allowed to evaporate to dryness.

Sodium acetate from this reaction was converted to the α-naphthylamide derivative or subjected to further degradation by the Schmidt reaction.

Acetyl-α-naphthylamide (Leete, 1963)

Sodium acetate obtained from pyridoxine hydrochloride (20 mg) by Kuhn-Roth oxidation was dissolved in water (½ ml) and a solution (½ ml) containing a slight excess of α-naphthylamine (usually about 18 mg), was added. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (approximately 50 mg) was added to the mixture as a condensing agent. The resulting oily precipitate was stirred until a solid precipitated. The crude material was filtered, dried and subjected to high vacuum sublimation (2×10^{-3} mm, 100°C). The sublimate, acetyl-α-naphthylamide (m.p. 160°C), was recrystallized from hot benzene until the specific activity of successive crystal batches did not change. The final material was stored in a vacuum desicator before counting.

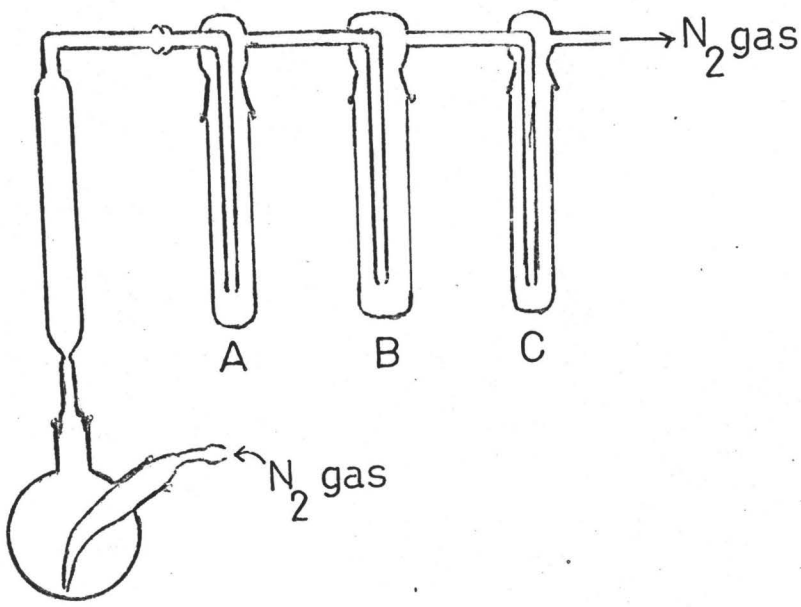
Schmidt reaction (Vogel, 1956)

Pyridoxine hydrochloride (50 mg) was oxidised by the Kuhn-Roth method. The solid sodium acetate so obtained was transferred into a suitable reaction flask (fig. 30) and dissolved in concentrated sulphuric acid (1 ml). The flask was kept cool in an ice bath whilst sodium azide (50 mg) was added to the reaction mixture. The flask was then attached to a gas trap assembly (fig. 30) and heated to 70°C. The carbon dioxide which evolved was carried into the traps, containing dilute potassium hydroxide (10%), by a current of nitrogen gas and there collected.

The reaction flask was disconnected and cooled in a mixture of dry ice and acetone. Distilled water (4 ml) was added, followed by dilute sodium hydroxide (10%), until the conditions were strongly alkaline (pH 14.0).

FIGURE 30

Apparatus for Schmidt reaction



Collection of CO_2:

Trap A - water

Trap B - 10% KOH, w/v

Trap C - empty

Collection of CH_3NH_2:

Trap A - empty

Trap B - anhydrous methanol (15 ml),
2,4-dinitrofluorobenzene (~25 µl),
sodium bicarbonate (0.5g)

Trap C - anhydrous methanol.

The flask was connected to a second gas trap assembly (fig. 30) and heated to 70°C. A continuous stream of nitrogen gas carried the evolved methylamine into a trap containing a solution of 2,4-dinitrofluorobenzene (~25 μl) in anhydrous methanol (15 ml) containing sodium bicarbonate (0.5 g). The methylamine was collected in this trap as its 2,4-dinitrophenyl derivitive. The trap was disconnected and the solution containing the derivitive was taken to dryness under reduced pressure and the remaining yellow solid was dissolved in anhydrous ether (10 ml). The ether solution was washed with distilled water (3x$\frac{1}{2}$ ml), dried and then reduced in volume (2 ml). Crystals of N-methyl-2,4-dinitroaniline (m.p.. 170°C) appeared on standing. The product was further purified by high vacuum sublimation (1.5×10^{-4} mm, 140°C) and stored in a vacuum desicator before counting.

Kuhn-Roth oxidation of pyruvic acid phenylhydrazone

Diluted 1,3-$^{14}C_2$ pyruvic acid phenylhydrazone (50 mg) was subjected to Kuhn-Roth oxidation in order to determine the ratio of activities at the two labelled centres. The reaction conditions and the procedure used to collect the sodium acetate were exactly the same as those used in the oxidation of pyridoxine hydrochloride.

The sodium acetate was converted to acetyl α-naphthylamide as described above and this product was subjected to the same purification procedures before counting.

Radioactivity Measurements

Radioactivity was assayed on samples of finite thickness on aluminium planchettes with a low background gas flow Geiger counting

system (Nuclear Chicago Corporation Model 4342). Corrections for background and self absorption were applied. Samples for counting were prepared as follows: Solid materials (0.5 - 3 mg, weighed to the nearest microgram on a Micro Gram-atic balance (Mettler Instrument Corp.) were weighed on aluminium planchettes (diameter 1.5 cm), and dissolved in suitable solvent systems. Aqueous sucrose solution (10% w/v) was used for pyridoxol hydrochloride samples, dimethyl-formamide containing collodion (1%) was found to be suitable for all other substances. Even distribution of the samples was ensured by the lens tissue technique and the planchettes were thoroughly dried before counting.

Liquid scintillation counting was carried out on a Mark I Liquid Scintillation Computer (model 6860, Nuclear Chicago Corporation). Aqueous samples were dispersed with the aid of "Aquasol" (10 ml) (New England Nuclear Corporation). The efficiency of counting for ^{14}C was determined by external standardisation counting with ^{133}Ba.

(3) Results

Pyridoxol, isolated from the experiments with [methyl-^{14}C] methionine and [3,3',4,5-$^{14}C_4$]-DL-isoleucine and DL-alloisoleucine was essentially inactive. Activity of pyridoxol, derived from [^{14}C] formate and [2-^{14}C]leucine was low and apparently randomly distributed. Incorporation of activity from [2-^{14}C]acetate was also low; however, incorporation was non-random as 60% of the total activity of the pyridoxol was localised at the C_2 unit C-2',-2. Activity from [3-^{14}C]aspartic acid was adequately incorporated into pyridoxol; 60% of the total activity was localised at the C_2 unit C-2',-2. Label from [7α-^{14}C]tryptophan entered pyridoxol in good radiochemical yield; the Kuhn-Roth acetate was shown to be inactive, reflecting non-random incorporation.

Pyridoxol, obtained from the cultures incubated with [1-^{14}C]-glycerol was labelled in good yield and contained approximately one-fifth of its label in the C_2 unit, C-2',-2 (acetic acid), irrespective of whether glycerol or glucose had served as the general carbon source. Since virtually all the activity of this acetic acid was located at the C-methyl group (C-2') (methylamine), distribution of label from [1-^{14}C] glycerol was non-random.

The radiomers of pyruvic acid labelled pyridoxol in good yield. All activity of pyridoxol from [2-^{14}C] pyruvate and from [3-^{14}C] pyruvate was found in the Kuhn-Roth acetate (C-2',-2), whose C-methyl carbon (C-2') was supplied entirely by the C-methyl group of pyruvate.

The Kuhn-Roth acetate from experiments in which $\left[1,3\text{-}^{14}C_2\right]$ pyruvate was administered, contained nearly all the activity of the pyridoxol. All this activity was shown to be located in the C-2' of pyridoxol.

These data, along with all other relevant results are summarised in Tables VI, VII and VIII.

TABLE VI

Incorporation of labelled substrates into pyridoxol

Expt. No.[a,b]	Substrate	Nominal Total Activity (mCi)	Nominal Specific Activity (mCi/mmole)	Carrier added (g)	Pyridoxol Hydrochloride Specific activity x 10^{-4} (counts min^{-1} $mmole^{-1}$)
1	[1-^{14}C] Glycerol	0.1	15.4	0.04	1.78 ± 0.05
2	[1-^{14}C] Glycerol	0.5	0.023[c]	0.12	2.38 ± 0.06
3	[3-^{14}C] Pyruvic Acid	0.1	35.6	0.04	1.20 ± 0.03
4	[3-^{14}C] Pyruvic Acid	0.1	35.6	0.04	
5	[2-^{14}C] Pyruvic Acid	0.1	31.7	0.04	0.72 ± 0.02
6	[1,3-$^{14}C_2$] Pyruvic Acid[d]	0.3	30.8	0.08	1.05 ± 0.02
7	[2-^{14}C] Acetic Acid	0.1	54.7	0.04	0.36 ± 0.01

(a) The general carbon source in Expt. 1 was glucose (2% w/v). In all other experiments the general carbon source was glycerol.

(b) The culture volume in Expt. 6 was 2 l. In all other experiments it was 1 l.

(c) Obtained by mixing 1-^{14}C glycerol, nominal specific activity 10 mCi/mmole (4.6 mg) with inactive glycerol (2 g).

(d) Obtained by mixing 1-^{14}C pyruvic acid (0.15 mCi, 27.2 mCi/mmole) and 3-^{14}C pyruvic acid (0.15 mCi, 35.6 mCi/mmole). For determination of activity, see Table VII, Footnote f.

TABLE VI (continued)

Expt. No.	Substrate	Nominal Total Activity mCi	Nominal Specific Activity mCi/mmole	Carrier Added (g)	Pyridoxol Hydrochloride Specific Activity ($\times 10^{-4}$)
8	[1,3-$^{14}C_2$] Pyruvic Acid	0.2	30.8	0.07	0.60 ± 0.01
	[1-^{14}C] Pyruvic Acid	0.1	27.2		
	[3-^{14}C] Pyruvic Acid	0.1	35.6		
9	[^{14}C] Formic Acid	0.1	36	0.04	0.16 ± 0.01
10	[2-^{14}C]-DL-Leucine	0.1	48	0.04	0.31 ± 0.02
11	[3-^{14}C]-DL-Aspartic Acid	0.1	1.9	0.04	0.25 ± 0.01
12	[7a-^{14}C]-DL-Tryptophan	0.1	3.7	0.04	0.75 ± 0.02
13	[3,3',4,5-$^{14}C_4$]-DL-Isoleucine	0.1	160	0.05	Inactive
	[3,3',4,5-$^{14}C_4$]-DL-Alloisoleucine	0.1	160		
14	[Methyl-^{14}C]-L-Methionine	0.2	53.6	0.03	Inactive

TABLE VII

Specific activity of pyridoxol and its degradation products

Expt. No.	Substrate	Pyridoxol SA[c]	Acetic Acid[a] (C-2',2) SA[c]	RSA[d]	Methylamine[b] (C-2') SA[c]	RSA[d]
1	[^{14}C]Glycerol	1.78 ± 0.05	0.39 ± 0.01	22 ± 1	-	-
2	[1-^{14}C]Glycerol	2.38 ± 0.06	0.51 ± 0.01	21 ± 1	-	-
		1.12 ± 0.03[e]	-	-	0.20 ± 0.01	18 ± 1
3	[3-^{14}C]Pyruvic Acid	1.20 ± 0.03	1.15 ± 0.03	96 ± 3	-	-
4	[3-^{14}C]Pyruvic Acid	0.54 ± 0.01	-	-	0.47 ± 0.01	88 ± 2
5	[2-^{14}C]Pyruvic Acid	0.72 ± 0.02	0.68 ± 0.02	94 ± 3	-	-
6	[1,3-$^{14}C_2$]Pyruvic Acid[f]	1.05 ± 0.02	0.94 ± 0.02	90 ± 2	0.87 ± 0.02	84 ± 2
7	[2-^{14}C]Acetic Acid	0.36 ± 0.01	0.21 ± 0.01	58 ± 2	-	-

(a) Isolated as acetyl α-naphthylamide

(b) Isolated as N-methyl-2,4-dinitroaniline

(c) Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

(d) Relative specific activity (percent) (intact pyridoxol = 100)

(e) Obtained from the pyridoxol, specific activity (2.38 ± 0.06) x 10^4, by further dilution with inactive pyridoxol.

(f) To confirm the distribution of label in this substrate, a small sample (~2 μCi) was diluted with inactive pyruvic acid and converted to the phenylhydrazone (SA = (3.47 ± 0.02) x 10^4 counts min^{-1} $mmole^{-1}$) and degraded by Kuhn-Roth oxidation to yield acetic acid[(a)] (SA = (1.65 ± 0.01 x 10^4 counts min^{-1} $mmole^{-1}$) (RSA = 48 ± 1).

TABLE VII (continued)

Expt. No.	Substrate	Pyridoxol SA[c]	Acetic Acid[a] SA[c]	(C-2,2') RSA[d]	Methylamine[b] SA[c]	(C-2') RSA[d]
8	[1,3-$^{14}C_2$]Pyruvate[f]	0.60 ± 0.01	0.48 ± 0.01	79 ± 2	-	-
9	[^{14}C]Formate	0.16 ± 0.01	0.04 ± 0.002	24 ± 2	-	-
10	[2-^{14}C]-DL-Leucine	0.31 ± 0.02	0.08 ± 0.004	24 ± 2	-	-
11	[3-^{14}C]-DL-Aspartic Acid	0.25 ± 0.01	0.15 ± 0.01	59 ± 2	-	-
12	[7a-^{14}C]-DL-Tryptophan	0.75 ± 0.02	0.02 ± 0.002	2 ± 0.3	-	-
13	[3,3',4,5-$^{14}C_4$]Isoleucine and Alloisoleucine	Inactive	----	--	-	-
14	[Methyl-^{14}C]Methionine	Inactive	----	--	-	-

TABLE VIII

Efficiency of Incorporation of Labelled Substrates into Pyridoxol

Expt. No.	Substrate	Nominal Specific Activity (mCi/mmol)=A	Specific Activity x 10^{-5} of isolated pyridoxol (mCi/mmol)[a]	Dilution Factor[b]	Specific Activity x 10^{-3} of biosynthesised pyridoxol = B B = (a)(b)	Efficiency of Incorporation of the label[c]
1	[1-^{14}C] Glycerol	15.4	4.01	400	16.04	0.104
2	[1-^{14}C] Glycerol	0.023[d]	5.36	1200	64.32	279.6
3	[3-^{14}C] Pyruvic acid	35.6	2.70	400	10.80	0.031
4	[3-^{14}C] Pyruvic acid	35.6	1.22	900	10.95	0.031
5	[2-^{14}C] Pyruvic acid	31.7	1.80	400	6.50	0.021
6	[1,3-$^{14}C_2$] Pyruvic acid	30.8	2.36	400[e]	9.46	0.031
7	[2-^{14}C] Acetic acid	54.7	0.81	400	3.24	0.006
8	[1,3-$^{14}C_2$] Pyruvic acid	30.8	1.35	700	9.45	0.031
9	[^{14}C] Formic acid	36	0.26	400	1.44	0.004
10	[2-^{14}C]-DL-Leucine	48	0.69	400	2.79	.006
11	[3-^{14}C] Aspartic acid	1.9	0.56	400	2.25	0.120

TABLE VIII (continued)

Expt. No.	Substrate	Nominal Specific Activity (mCi/mmol)=A	Specific Activity x 10^{-5} of isolated pyridoxol (mCi/mmol)(a)	Dilution Factor(b)	Specific Activity x 10^{-3} of biosynthesised pyridoxol = B B = (a)(b)	Efficiency of Incorporation of the label(c)
12	[7a-^{14}C]-DL-Tryptophan	3.7	1.69	400	6.76	0.180
13	[3,3',4,5]-DL-Isoleucine [3,3',4,5]-DL-Alloisoleucine	160	Inactive	500	Inactive	0
14	[Methyl-^{14}C]-L-Methionine	53.6	Inactive	300	Inactive	0

(a) This value is calculated from the specific activity (cts./min/mmol), given that 1 Ci = 3.7×10^{10} d.p.s. and assuming the efficiency of counting to be 20%.

(b) Dilution factor = carrier added (g) (Table VI)/amount of pyridoxol biosynthesised (assumed to be 100 μgm (1×10^{-4}g)/1 litre of culture).

(c) The values given equal the ratio B/A x 100% for each substrate.

(d) Obtained by mixing 1-^{14}C glycerol, nominal specific activity 10 mCi/mmol (4.6 mg) with inactive glycerol (2 g).

(e) Although 0.08 g carrier was added (Table VI), the dilution factor remains 400 as 2 l of culture was used and therefore 200 μgm of pyridoxol is assumed to be biosynthesised (see footnote (b)).

(4) A critical Examination of the Biogenetic Hypotheses

(i) One carbon hypotheses:

The hypotheses in figures 21(a), 21(b) and 22(a) are based on the premise that one or more of the carbon atoms of the pyridoxol skeleton are derived from one carbon units. Biological one carbon units arise at three levels of oxidation, methyl, hydroxymethyl and aldehyde. The scheme in figure 31 illustrates the metabolic interconversions of these oxidation states. Two separate experiments (Table V, Expts. 9,14) one with methionine, the other with $[^{14}C]$ formic acid were designed to test the role of these one carbon moieties in pyridoxol biosynthesis.

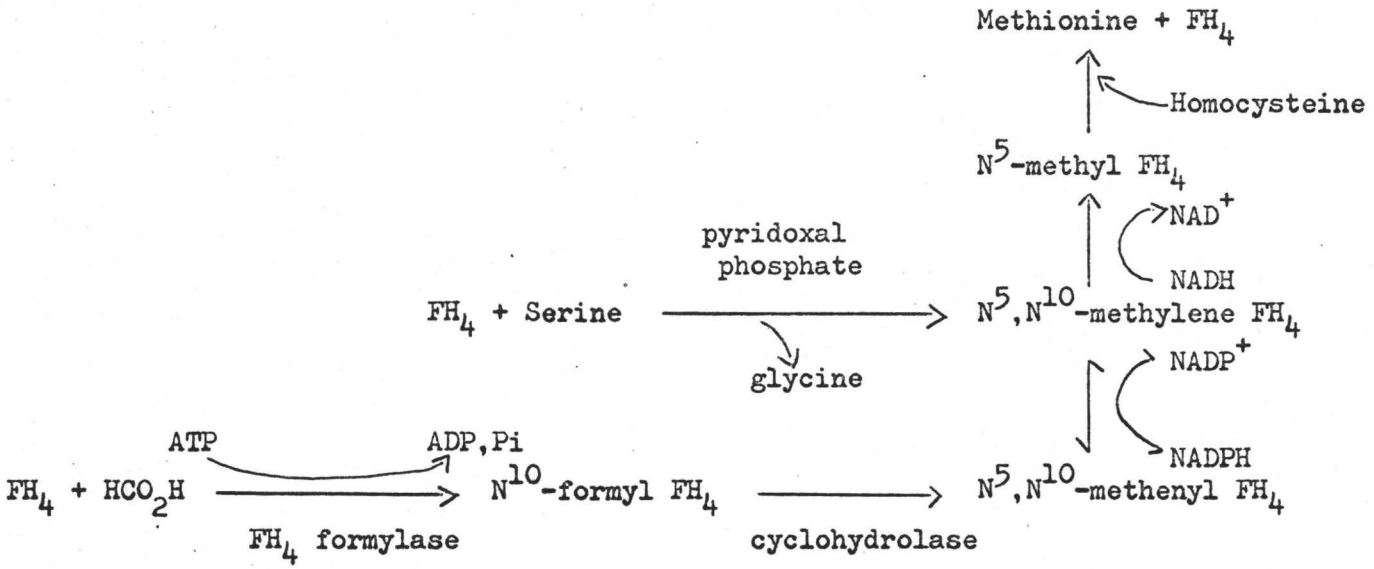
The results of these experiments (Table VI, Expts. 9,14) show that activity from the labelled methionine was not incorporated into pyridoxol and that activity from labelled formic acid was incorporated only in very poor radiochemical yield. Furthermore upon degradation, 25% of the activity from the formic acid was found to be in the Kuhn-Roth acetate (C-2',2 of pyridoxol) (Table VII, Expt. 9). This suggests that the label was distributed randomly amongst the pyridoxol carbons. A direct precursor-product relationship between formate and pyridoxol is thus unlikely and it would seem that one carbon units do not provide any of the carbons of pyridoxol. Thus the structural hypotheses in figures 21(a), 21(b) and 22(a) can be dismissed.

(ii) Polyketide hypotheses:

Even though both polyketide hypotheses (figs. 21(a) (b)) require the intervention of one carbon units, and can therefore be regarded as

FIGURE 31

Metabolic interconversions of biological "one carbon" units



unlikely on the basis of the results discussed above, they were tested independently. One of these hypotheses envisages the incorporation of four acetate units, the other, of three units. If either were operative, activity from $[2\text{-}^{14}C]$ acetate would be expected to be incorporated into pyridoxol in good radiochemical yield, and with a characteristic distribution.

Acetic acid from a Kuhn-Roth degradation should contain 25% of the total activity if four acetate units were incorporated; and 33% if the triacetyl structure served as a precursor. However, the results of experiment 7, in which 2-^{14}C acetic acid was administered to the mutant, are totally inconsistent with these predicted values. The radiochemical yield (Table VIII, Expt. 7) was relatively poor and the Kuhn-Roth acetate contained 60% of the total activity of the isolated pyridoxol (Table VII, Expt. 7). The conclusion, already suggested by the results of the "one-carbon" experiments, is that neither polyketide hypothesis operates in the biosynthesis of pyridoxol.

The mevalonate hypothesis (fig. 21(c)) is also disproved by these results. Again, if this route operated, the radiochemical yield would be good. In this case however, activity from $[2\text{-}^{14}C]$ acetate would give rise to labelled pyridoxol, which, on Kuhn-Roth degradation, would yield unlabelled acetic acid.

Despite the fact that all the polyketide hypotheses are disproved, 60% of the label from $[2\text{-}^{14}C]$ acetate was found in carbons C-2',2 of pyridoxol. This indicates that the label was incorporated non-randomly. It would appear therefore, that acetate

may act as a precursor of one of the true precursors of pyridoxol. This suggestion will be expanded in a later section, where the acetate result is rationalised within the framework of the conclusions of this thesis.

(iii) Aspartic acid as a precursor (figs. 22(a), (b)).

Although activity from [3-^{14}C] aspartic acid was incorporated into pyridoxol in relatively high radiochemical yield (Table VIII, Expt. 11), Kuhn-Roth degradation showed that 60% of this activity was situated in carbons 2',2 of the molecule. This result is at variance with the distribution predicted by the hypotheses in figures 22(a) & (b). In both cases aspartic acid is envisaged as providing carbons 5,5' and 6 of the pyridoxol skeleton, and label from 3-^{14}C aspartic acid is expected to be situated almost entirely in carbon 5. It appears therefore that neither of these hypothesis can account for the biosynthesis of the vitamin.

On the other hand, the incorporation of the label was non-random and may reflect a precursor-product relationship between aspartic acid and pyridoxol. Since a Schmidt reaction was not carried out, the distribution of the activity between carbons 2 and 2' of pyridoxol remains unknown. It is therefore difficult to rationalise the result in terms of the direct incorporation of an aspartate moiety. However, the distribution of the label can be rationalised in terms of the conclusions of this thesis. This will be discussed in a later section (Section 5 (ii)).

(iv) Five carbon branched structures as precursors:

$[2\text{-}^{14}C]$-DL-leucine and a mixture of $[3,3',4,5\text{-}^{14}C_4]$-DL-isoleucine and DL-alloisoleucine were administered to the mutant in separate experiments (Table V, Expts. 10, 13). Both experiments were designed to test the hypothesis that carbons 4',4,5,5' and 6 of the pyridoxol skeleton are derived from a five carbon branched structure related to the branched chain amino acids (fig. 25). Neither experiment succeeded in establishing such a relationship.

Activity from $[3,3',4,5\text{-}^{14}C_4]$-DL-isoleucine and DL-alloisoleucine was not incorporated into the isolated pyridoxol. On the other hand, administration of $[2\text{-}^{14}C]$-DL-leucine did lead to radioactive pyridoxol but, the radiochemical yield (Table VIII, Expt. 10) was extremely poor and the results of the Kuhn-Roth degradation (Table VII, Expt. 10) suggest that the label was distributed randomly throughout the pyridoxol molecule.

(v) Tryptophan as a precursor:

If tryptophan provides a portion of the pyridoxol skeleton via the degradative routes shown in figure 23, activity from $[7a\text{-}^{14}C]$ tryptophan would give rise to pyridoxol labelled entirely at the C2 position. In this event the Kuhn-Roth acetate (C2',2 of pyridoxol) would contain 100% of the activity of the isolated pyridoxol. However, despite adequate incorporation of activity from $[7a\text{-}^{14}C]$ tryptophan (Table VIII, Expt. 12), the acetic acid from the Kuhn-Roth degradation was found to be inactive (Table VII, Expt. 12). Whilst this result disproves the hypotheses of figure 23, it does indicate that the incorporation was non-random and may be indicative of a precursor-product relationship between tryptophan and pyridoxol.

In fact such a result can be rationalised if tryptophan provides the pyridine ring of pyridoxol by way of quinolinic acid (c.f. fig. 5). In this event all the activity from [7a-^{14}C] tryptophan would be situated at carbon 6 of pyridoxol and the Kuhn-Roth acetate (C2',2) would be inactive as shown.

However, on the basis of the results from a single experiment, it is premature to suggest that this route to pyridoxol operates in E. coli, especially since the degradation evidence from this experiment failed to establish the site of the label and was therefore indirect and may have been fortuitous. Further experiments with other radiomers of tryptophan and some of quinolinic acid would support or undermine the hypothesis. Until such time, and probably not until more comprehensive degradative sequences are available, the result will remain controversial.

Firstly, it cannot be rationalised within the framework of the final hypothesis of this thesis. Secondly, it may be indicative of a route leading to the pyridine ring from tryptophan, a sequence thought to be inoperative in bacteria. If this were the case, one-carbon units are envisaged as providing the substituent carbons at positions 2' and 4' of the pyridoxol skeleton. However, the results of the experiments with labelled methionine and formic acid (Tables VI and VII, Expts. 9,14) preclude one carbon substitution at these positions. Furthermore, the results discussed in section (5) lead to the conclusion that this organism does in fact synthesise pyridoxol from small molecules.

This paradoxical situation can be rationalised only if it is assumed that pyridoxol can arise via two routes, one starting from small molecules, the other from tryptophan. The tryptophan pathway may be an inducible system which operates only when tryptophan is available from the culture medium. Such a hypothesis, which is pure conjecture, could account for the lack of incorporation of methionine and formate in experiments 9 and 14 where exogenous tryptophan was not available.

(5) The biosynthesis of pyridoxol

(i) Incorporation of three carbon units:

The structural hypothesis in figure 24 shows the close resemblance between pyruvic acid and the corresponding amino acid alanine, and carbons 2', 2 and 3 of the pyridoxol skeleton.

In an attempt to define the relationship of these compounds with pyridoxol incorporation of label from pyruvic acid was investigated. Activity from $[2\text{-}^{14}C]$ pyruvate (Expt. 5) and from $[3\text{-}^{14}C]$ pyruvate (Expts. 3,4) was incorporated into pyridoxol in adequate radiochemical yield (Table VIII, Expts. 3,4 & 5). Furthermore all activity of pyridoxol from these two radiomers of pyruvic acid was found in the Kuhn-Roth acetate (C2',2) (Table VII, Expts. 3,4 & 5). It seems likely therefore that the C2 unit, C2',2 of pyridoxol, is derived specifically from the methyl and the carbonyl carbons of pyruvate. This theory is confirmed by the results of experiment 4 (Table VII), in which Schmidt degradation of the Kuhn-Roth acetate showed the C-methyl carbon (C2') to be supplied entirely by the C-methyl group of pyruvate.

To test whether the carboxyl carbon of pyruvate serves as the source of C-3 of pyridoxol, as the hypothesis suggests, experiments with $[1,3\text{-}^{14}C_2]$ pyruvate (Table V, Expts. 6 & 8) were carried out. Intact incorporation of $[1,3\text{-}^{14}C_2]$ pyruvate (52 ± 1% of total activity of this pyruvate was located at the carboxyl carbon, 48 ± 1% of total activity at the methyl carbon, c.f. Table VII, footnote f) would have led to pyridoxol which on degradation should have yielded acetate

(C2',2) and methylamine (C2') containing 48% of the activity of the isolated pyridoxol.

In fact in two experiments the acetate contained 80% (Table VII, Expt. 8) and 90% (Table VII, Expt. 6) of the activity, most of which was located at C2' of pyridoxol (Table VII, Expt. 6) and was therefore derived (c.f., Expts. 3 & 4) from the C-methyl group of pyruvate. It follows that the carboxyl group of pyruvate had not been incorporated into pyridoxol. Thus it is a C_2 unit, corresponding to the methyl and carboxyl groups of pyruvate, which supplies the C_2 unit, C-2',-2 of pyridoxol. Since the incorporation of acetate into this C_2 unit is not quantitative (Table VII, Expt. 7) and takes place in low radiochemical yield (Table VIII, Expt. 7) this compound does not appear to be a direct precursor and it is likely that the pyruvate-derived 2-carbon unit enters at the oxidation level of acetaldehyde.

Evidence from experiments 1 & 2 in which $\left[1\text{-}^{14}C\right]$ glycerol was administered to the mutant supports this role for an acetaldehyde moiety in the biosynthesis of pyridoxol. What is more important, the mode of this incorporation is consistent with the view that the remaining carbons of pyridoxol are derived from two 3-carbon units related to glycerol.

Pyridoxol obtained from the cultures incubated with $\left[1\text{-}^{14}C\right]$ glycerol (Table VI, Expts. 1 & 2) contained one fifth of its label in the C_2 unit, C-2'-2 (acetic acid), irrespective of whether glycerol or glucose had served as the general carbon source (Table VII, Expt. 1 & 2). Since virtually all the activity of this acetic acid

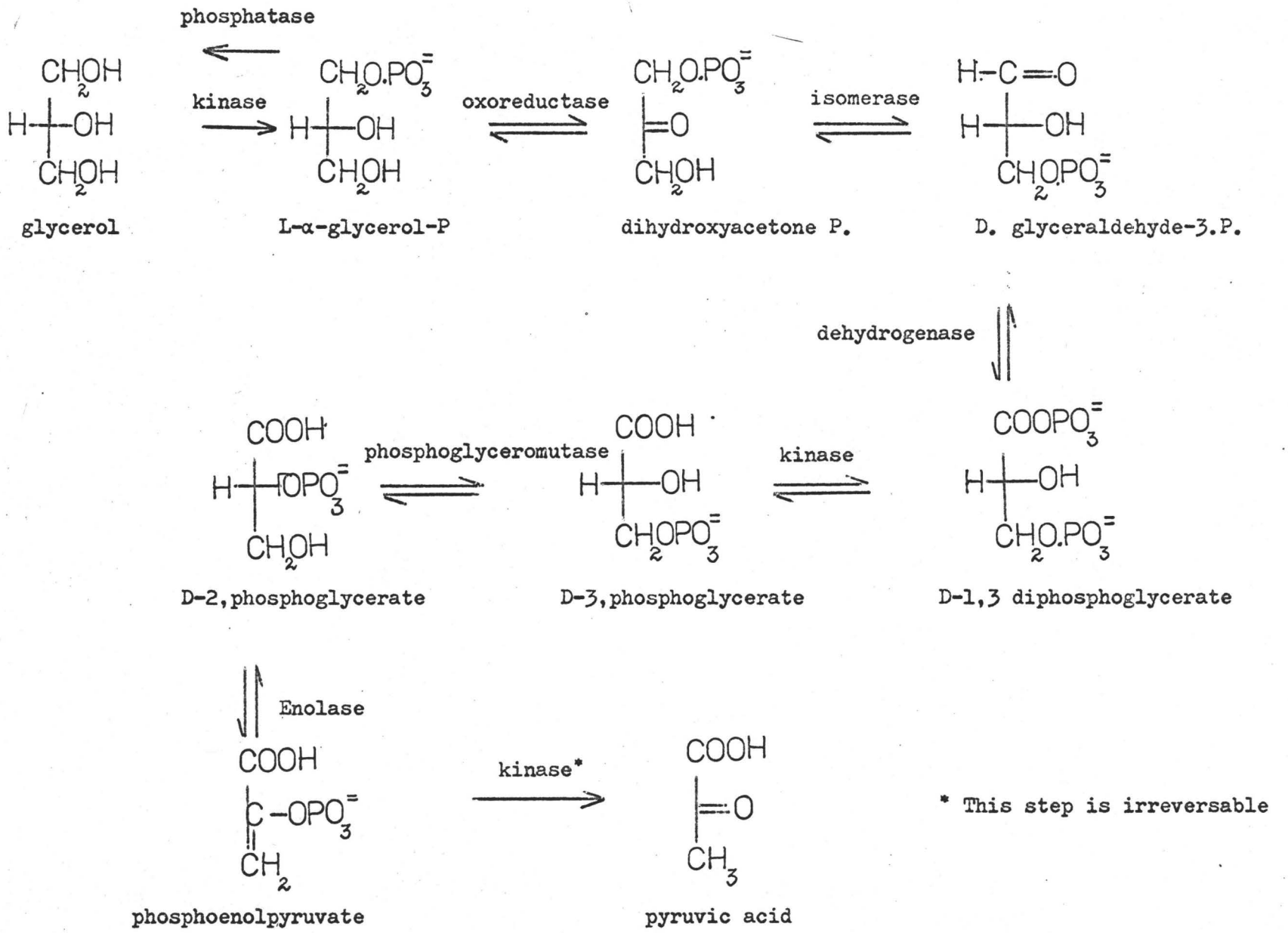
was located at the C-methyl group (C-2') (Table VII, Expt. 2), distribution of label from [1-^{14}C] glycerol was non-random. It may be inferred from this result that five of the eight carbon atoms of pyridoxol might be ultimately derived from the terminal carbon atoms of glycerol.

This hypothesis is consistent with the results of the experiments with pyruvic acid. In these experiments it was shown that the entire activity from [3-^{14}C] pyruvate was located at C-2' of pyridoxol whereas the experiments with [1-^{14}C] glycerol demonstrated that only 20% was incorporated into this carbon from [1-^{14}C] glycerol. This observation can be explained in terms of the conversion of glycerol, via glycerol phosphate and phosphoglyceraldehyde, to pyruvic acid (fig. 32). The final step of this sequence, the conversion of phosphoenolpyruvate to pyruvic acid, catalysed by the enzyme pyruvate kinase, is an irreversible reaction. It follows that activity introduced as pyruvic acid does not enter any of the intermediates which occur prior to the irreversible step. The activity is therefore incorporated only into positions C2' and C2 of pyridoxol.

Activity from [1-^{14}C] glycerol, on the other hand, will label pyruvic acid and all the intermediates equally at positions C_1 and C_3 of their carbon chains (fig. 32). If two of these labelled intermediates then unite, and join with the pyruvate-derived 2-carbon unit, five centres of the pyridoxol skeleton would be equally labelled. Kuhn-Roth degradation of this pyridoxol would yield acetic acid (C-2', -2) that contained 20% of the total activity. Further degradation by the Schmidt reaction should then show this activity to be located entirely at

FIGURE 32

The conversion of glycerol to pyruvate via the glycolysis pathway.



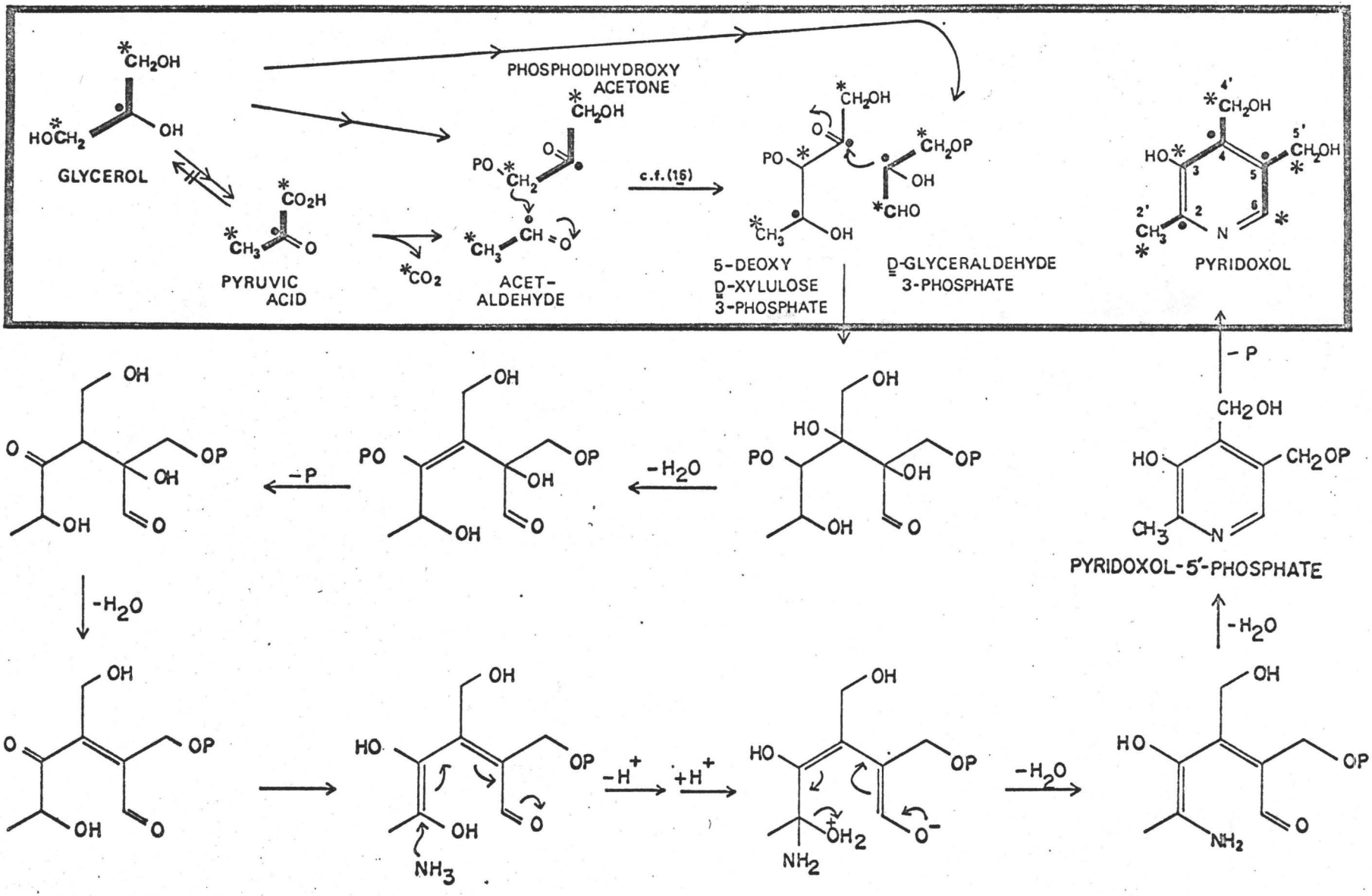
the C-methyl of the acetic acid (C-2'). The results of experiments 1 and 2 are consistent with this prediction. It is therefore suggested that the carbon atoms of pyridoxol arise from two 3-carbon units related to glycerol and a 2-carbon fragment, at the oxidation level of acetaldehyde, derived from pyruvic acid. In the light of the degradation data, it is interesting to note that the radiochemical yield of the experiments, in which $[1\text{-}^{14}C]$ glycerol was administered in the presence of inactive glucose as the general carbon source, was approximately three times that of the experiments in which radiomers of pyruvic acid were administered (Table VIII, Expts. 2,3,4,5,6 & 8). Furthermore, when $[1\text{-}^{14}C]$ glycerol was administered in the presence of glycerol as the general carbon source (Expt. 2), the radiochemical yield was of the order of 300% (Table VIII, Expt. 2). Both these results are consistent with the hypothesis.

The structural correspondence of glycerol, pyruvate and pyridoxol (fig. 33, box) and the steps of a plausible reaction sequence leading from the precursors to the product are shown in figure 33.

The first step of this scheme envisages an aldol type condensation between acetaldehyde and dihydroxyacetone-3-phosphate to produce the intermediate 5-desoxy-D-xylulose-3-phosphate. This reaction bears a close resemblance to the production of 5-desoxy-D-xylulose-1-phosphate, from dihydroxyacetone phosphate and acetaldehyde, catalysed by the enzyme aldolase (Gorin, Hough & Jones, 1953; Rutter, 1961). This enzyme is known to be present in microorganisms which utilise carbohydrates, and therefore could account for the first reaction of the scheme. Furthermore, ferrous ion, which has been shown to affect

FIGURE 33

Scheme for the biosynthesis of pyridoxol



the production of vitamin B_6 in some microorganisms (Lewis, 1944), stimulates this enzyme in microbiological systems (Rutter, 1961). On the other hand, aldolase appears to be specific for D-dihydroxyacetone-1-phosphate (Rutter, 1961) whereas the scheme uses the 3-phosphate ester. With this in mind it must be emphasised that the scheme is hypothetical and that 5-desoxy-D-xylulose-1-phosphate does contain the keto group at position 2 essential for the condensation step with glyceraldehyde-3-phosphate and the production of the vitamin skeleton.

Finally, it is noteworthy that a speculative hypothesis, embodying the major features of the scheme, was put forward on purely structural grounds (figure 27) (Leete, 1963).

(ii) The incorporation of acetate and aspartate (Expts. 7 & 11).

Rationalization within the framework of the scheme in figure 33 of the results obtained with acetate and aspartate rests on the postulated reconversion of these substrates into phosphoenolpyruvate, by action of the enzyme phosphoenolpyruvate carboxykinase. This enzyme catalyses the conversion of oxaloacetic acid to phosphoenolpyruvate (figure 34).

Activity from $\left[2\text{-}^{14}C\right]$ acetic acid gives rise to labelled oxaloacetic acid via the Krebs' cycle. The action of phosphoenolpyruvate carboxykinase thence gives rise to phosphoenolpyruvate labelled in all three carbons (figure 34). The hydrolysis of this phosphoenolpyruvate, catalysed by the enzyme pyruvate kinase produces labelled pyruvic acid, which is incorporated into positions C-2',2 of pyridoxol.

In this experiment, these carbons were, in fact, shown to contain 60% of the total activity of the isolated pyridoxol.

It is plausible that label from phosphoenolpyruvate is also incorporated into 3-carbon structures, related to glycerol, by a reversal of the glycolytic pathway. This incorporation is suggested to account for the remaining 40% of the activity distributed among the remaining carbons of the vitamin. The fact that 60% resided in the two carbons derived directly from pyruvate whereas only 40% was found in the remaining six carbons, which according to the hypothesis should have all been labelled, indicates that most of the labelled phosphoenolpyruvate was converted to pyruvate. This high conversion rate is attributable to three major factors.

Firstly, phosphoenolpyruvate has such a large negative free energy of hydrolysis (~12 k cals/mole) that its conversion to pyruvic acid proceeds very nearly to completion.

Secondly, the continuous uptake of glycerol from the culture medium ensures an adequate supply of the substrates required for gluconeogenesis.

Finally, as growth is absent, and as normal metabolism becomes impaired due to lack of pyridoxal in the culture medium, it is unlikely that gluconeogenesis is a major factor in the organism's metabolism.

Incorporation of activity from $[3\text{-}^{14}C]$ aspartate can be rationalised in a similar manner.

A transamination reaction converts $[3\text{-}^{14}C]$ aspartate to $[3\text{-}^{14}C]$ oxaloacetate, which, in turn, undergoes decarboxylation to $[3\text{-}^{14}C]$ phosphoenolpyruvate (figure 35). Some of the $[3\text{-}^{14}C]$

oxaloacetate enters the Krebs' cycle and thereby produces doubly labelled oxaloacetate (figure 35). This, in turn undergoes decarboxylation to $[1\text{-}^{14}C]$ labelled phosphoenolpyruvate. However, despite this the oxaloacetate available for decarboxylation is still heavily labelled at the 3 position. The phosphoenolpyruvate produced by this reaction should thus be labelled predominantly in the methyl carbon (figure 35).

On the one hand, this leads to pyruvic acid labelled predominantly at the C-3 position, and thereby, to pyridoxol similarily labelled at the C-2' carbon. On the other hand, the label is incorporated into the 3-carbon intermediates of the glycolysis pathway and thence into the remaining six carbons of pyridoxol (figure 35).

As this situation is analogous to that arising from the administration of $[2\text{-}^{14}C]$ acetate, a similar distribution of label in the isolated pyridoxol would be expected.

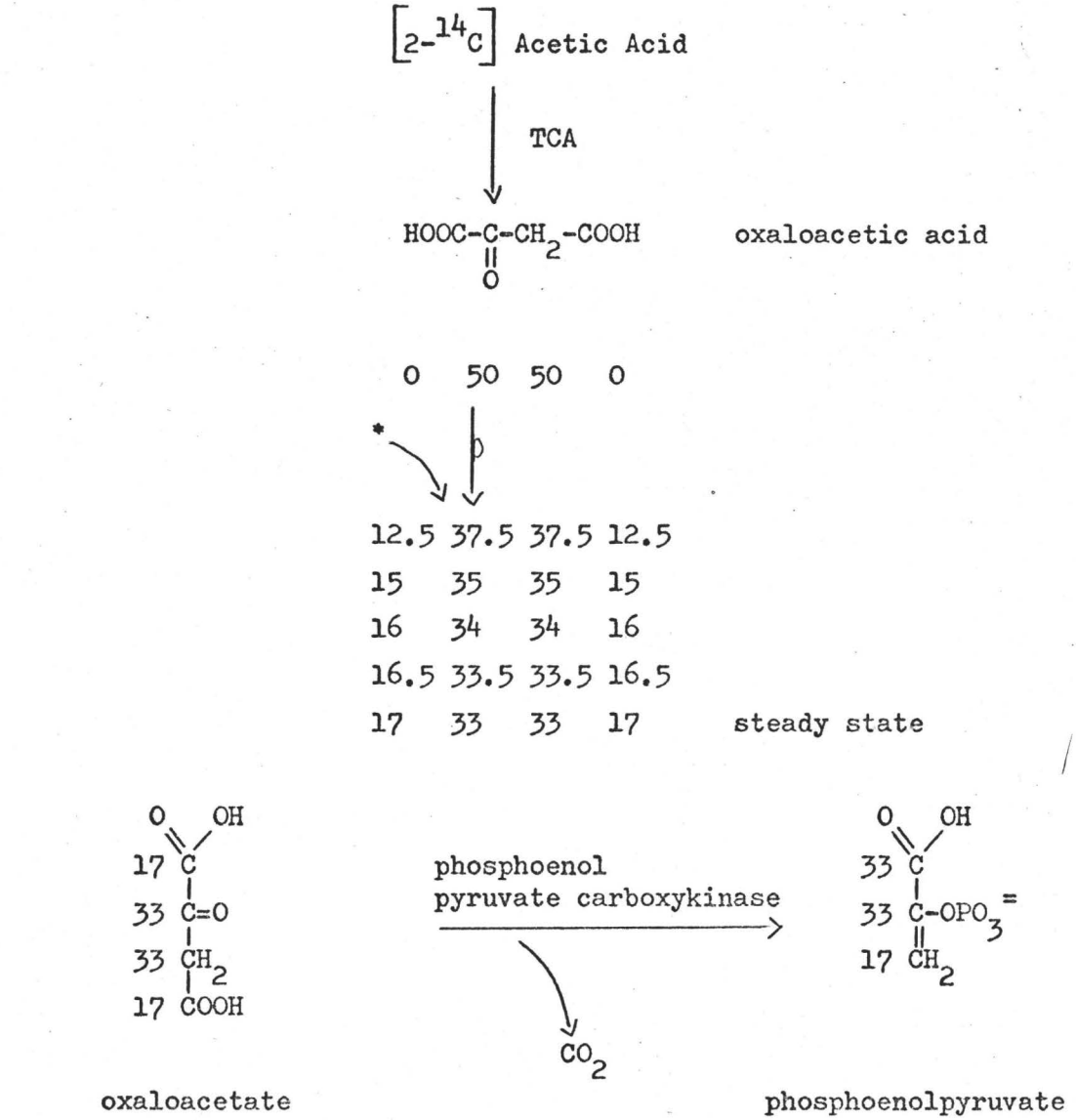
The distributions were, in fact, found to be identical (Table VII, Expts. 7 & 11), 60% residing in the Kuhn-Roth acetate (C2'-2) and 40% in the remaining six carbons.

However, despite the identical nature of these degradation data and despite the fact that they can be rationalised by identical arguments, the radiochemical yields of the "aspartate" experiment exceeded that of the "acetate" experiment by a factor of 20 (Table VIII, Expts. 7 & 11). This result can be explained as follows.

Label from $[2\text{-}^{14}C]$ acetate enters oxaloacetate via eight intermediates of the Krebs' cycle (figure 34). As each of these intermediates is present at substrate level concentration, this route

FIGURE 34

Distribution of ^{14}C in phosphoenolpyruvate from $[2\text{-}^{14}C]$ Acetic Acid



Denotes recycling

* Denotes entry of new label

dilutes the specific activity of the original label. Furthermore, as the production of labelled oxaloacetate is controlled by the action of the Krebs' cycle, the amount available for decarboxylation never rises above normal endogenous levels. Consequently the production of labelled phosphoenolpyruvate is unexceptional and may, in fact, be somewhat less than normal, due to the adverse gluconeogenetic conditions of the culture. The incorporation of label into the isolated pyridoxol is therefore relatively poor.

In contrast, $[3\text{-}^{14}C]$ aspartic acid is converted directly to $[3\text{-}^{14}C]$ oxaloacetate (fig. 35). Endogenous oxaloacetate produces only a relatively small decrease in the specific activity of the label. Furthermore, the concentration of oxaloacetate becomes appreciably higher than normal and, despite the adverse gluconeogenetic conditions, should lead to an abnormally large production of labelled phosphoenol-pyruvate with a specific activity close to that of the $[3\text{-}^{14}C]$ aspartate. Under these conditions, incorporation of label from this substrate into pyridoxol takes place in relatively good radiochemical yield.

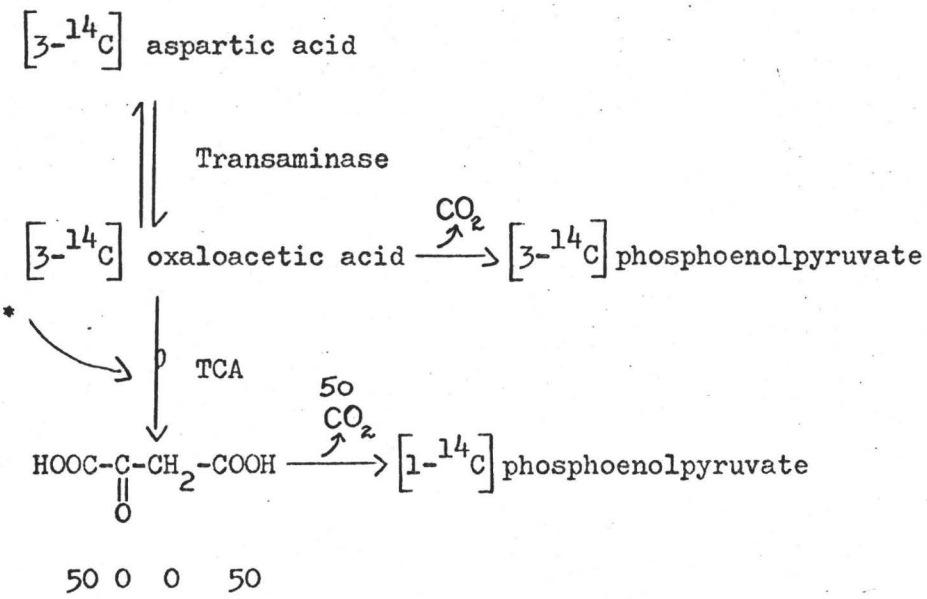
(iii) Alternative routes from pyruvate to the glycolytic intermediates:

Label from pyruvic acid can be returned into phosphoenol-pyruvate and thence into glycerol, by routes other than a reversal of glycolysis. If this occurs, the rationalisations of the results from the experiments with labelled glycerol and labelled pyruvate, which lead to the conclusions of this thesis, would be of doubtful validity.

There are two routes by which this reconversion might occur. One of these, already alluded to, is via acetyl-CoA and the Krebs'

FIGURE 35

Distribution of ^{14}C in phosphoenolpyruvate from [3-^{14}C]aspartic acid



* Denotes entry of new label

Denotes recycling

FIGURE 36

Pyruvate Synthase reaction*

$$CH_3\text{-}CO\text{-}CO_2H + ATP \longrightarrow CH_2{=}\underset{\underset{PO_3^{=}}{|}}{\overset{\overset{O}{|}}{C}}\text{-}COOH + AMP + Pi$$

* Cooper & Kornberg, 1965

cycle. The other is the direct conversion of pyruvate to phosphoenol-pyruvate, catalysed by "phosphoenolpyruvate synthase" (Cooper & Kornberg, 1965), (fig. 36).

The low radiochemical yield and the degradation evidence from the experiment with $\left[2\text{-}^{14}C\right]$ acetate (Tables VII & VIII, Expt. 7) demonstrates that the "Krebs' cycle route" via acetyl CoA, terminating with the decarboxylation of oxaloacetate (figure 34), cannot account for any appreciable redistribution of label from pyruvic acid into the 3-carbon intermediates of glycolysis.

There is no direct evidence against the operation of the phosphoenolpyruvate synthase catalysed reaction. However, as in the reaction catalysed by phosphoenolpyruvate carboxykinase, the product is phosphoenolpyruvate and the step is essentially gluconeogenetic. As previously discussed, it is likely that the culture conditions preclude gluconeogenesis. Therefore, the majority of phosphoenol-pyruvate produced by this reaction is reconverted to pyruvic acid and only insignificant amounts of activity from any radiomer of pyruvate enters the 3-carbon units which supply carbons 3, 4, 4', 5, 5' and 6 of pyridoxol.

(6) Future Investigations

If the scheme in figure 33 is correct activity from [1-^{14}C] glycerol should enter C-2',-3,-4',-5' and -6 of pyridoxol equally. Degradation schemes are now available for the isolation of C-4' and C-5' (Rowell & Gupta, 1970). Each of these carbon atoms, along with C-2', isolated by the Schmidt reaction (Expt. 2, Table VIII), should contain 20% of the total activity of the isolated pyridoxol.

A complementary experiment using [2-^{14}C] glycerol should, in turn, yield pyridoxol with C-2, -4 and -5 equally labelled (33% of the total activity) and C-2', -3, -4', -5' and -6 unlabelled. Degradations leading to the isolation of C-2', -2, -4' and -5' will confirm or refute this predicted distribution.

Due to the symmetry of the glycerol molecule, the label in [1-^{14}C] glycerol is located equally at C-1 and C-3. On the other hand, [1-^{14}C] glyceric acid and [3-^{14}C] glyceric acid are labelled exclusively at C-1 and C-3 respectively. Administration of either of these compounds to the mutant should lead to labelled pyridoxol. Three carbons of pyridoxol should be labelled when [3-^{14}C] glyceric acid serves as the precursor, whereas only two centres of pyridoxol should be labelled when [1-^{14}C] glyceric acid is administered, since C-1 of pyruvic acid is not incorporated (Expts. 6 & 8, this thesis).

The actual distribution of the label from these substrates should also give additional insight into the initial steps of the biosynthetic scheme outlined in figure 33.

The two carbons of pyridoxol that derive activity from [1-^{14}C] glyceric acid will be C-6, since it is suggested to arise from the carbonyl carbon of 3-phosphoglyceraldehyde, which, in turn, is derived directly from the carboxyl carbon of glyceric acid; and either C-3 or C-4'. The distribution between C-3 and C-4' is decided by the orientation of the phosphate ester of dihydroxyacetone which is proposed as providing C-3, -4 and -4' of pyridoxol.

Label from [3-^{14}C]glyceric acid, on the other hand, should label C-2', via pyruvic acid and C-5', via 3-phosphoglyceraldehyde together with C-3 or -4' again depending upon the orientation of the dihydroxyacetone phosphate ester.

The above experiments, together with those already carried out and described in this thesis are aimed at the identification of primary precursors; the arbitrary first phase of any biosynthetic investigation.

Identification of intermediates and the isolation of enzymes followed by successful <u>in vitro</u> experiments is necessary in order to establish a sequence with finality. Incorporation of any of the radiomers of 5-desoxy-D-xylulose would strengthen the view that this compound or one of its phosphate esters are intermediates in the biosynthesis of pyridoxol (fig. 33). Unfortunately labelled 5-desoxy-D-xylulose is unavailable, commercially, but a synthesis should not be an insurmountable problem.

It has already been suggested (Chapter IV, Section 5) that the enzyme aldolase catalyses the first step of the sequence in figure 33. The procedure for its isolation is well documented (Rutter, 1961).

Work with this system could provide the starting point for *in vitro* investigations.

V SUMMARY

The biosynthesis of vitamin B_6 has been studied by administering radioactive putative precursors to *E. coli* WG2 ($B\text{-}B_{6\text{-}2}$), an organism known to biosynthesise and excrete the vitamin congener, pyridoxol (1.2). The isolation of the biosynthesised material was facilitated by a "carrier dilution" technique and effected by using a combination of ion exchange and thin layer chromatography. The radiochemical purity of the final material was ensured by repeated recrystallisations and high vacuum sublimation.

The radioactive samples of pyridoxol hydrochloride so obtained were partially degraded to determine the distribution of activity. Kuhn-Roth oxidation yielded the 2-carbon unit, C-2',-2 of pyridoxol, as acetic acid. Further degradation of the acetic acid by the Schmidt reaction yielded C-2' as methylamine. Both of these products were converted into solid derivatives for radioactive assay.

Non-random incorporation of activity into pyridoxol was observed when radiomers of glycerol, pyruvate, aspartate and acetate were administered to the mutant. These data were consistent with the view that the eight carbons of pyridoxol arise from the union of three pieces. One is a two carbon unit, at the oxidation level of acetaldehyde, supplied by the methyl and the carbonyl carbon of pyruvic acid; the other two are three carbon units closely related to glycerol. On the basis of these results a biosynthetic scheme leading to pyridoxol is proposed (fig. 33).

Activity from some substrates was not incorporated into pyridoxol. From others incorporation was poor and the label was found to be distributed randomly between the pyridoxol carbons. These results precluded several biogenetic schemes which had been constructed on purely structural grounds and from analogy with other pyridine systems.

The non-random incorporation of activity from labelled tryptophan could not be rationalised within the framework of the hypothesis shown in figure 33. It may, however, reflect a second, inducible route to pyridoxol in this organism.

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