Biosynthesis of Vitamin B_6. Incorporation of Glycolaldehyde into Pyridoxal

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Radioactivity from [^{14}C]glycolaldehyde enters pyridoxal phosphate specifically, and is confined to the two-carbon unit C(5)–C(5′). Glycolaldehyde enters as an intact two-carbon unit, the aldehyde carbon atom supplying C-5, and the carbinol carbon atom C-5′ of the vitamin. These observations are interpreted in terms of the early stages of the biosynthesis of vitamin B_6.

We have demonstrated [1-3] that radioactivity from specifically labelled D-glucose and glycerol is incorporated non-randomly into pyridoxol, biosynthesized by *Escherichia coli* B mutant WG2. The data are consistent with the hypothesis that the eight-carbon skeleton of pyridoxol is generated from three precursor units. Two of these, giving rise to the C_3 fragments of pyridoxol, C(4′)–C(4)–C(3) and C(5′)–C(5)–C(6), are three-carbon compounds closely related to triose phosphate, whereas the third, which yields the C_2 fragment of pyridoxol, C(2′)–C(2), is a two-carbon compound derivable from glycolytic intermediates or from pyruvate. We inferred [1,3] on mechanistic grounds that this two-carbon compound was an aldehyde, and suggested that it might be at the oxidation level of acetaldehyde [3] or of glycolaldehyde.[4]

The notion that glycolaldehyde might be implicated in the biosynthesis of vitamin B_6 was first mooted [5] on the basis of the finding [6] that this compound replaced vitamin B_6 in promoting the growth of two B_6-requiring strains of *E. coli*. Recently Dempsey characterized an *E. coli* B mutant, WG3, with a nutritional requirement for pyridoxol which was satisfied by glycolaldehyde,[7] and isolated radioactive samples of the vitamin from cultures of the mutant which had been incubated with [1,2-$^{14}C_2$]- and with [2-^{14}C]-glycolaldehyde.[8] Glycolaldehyde thus apparently served as a precursor of vitamin B_6. Degradation of the labelled samples of pyridoxol obtained from these experiments showed that, contrary to prediction, little, if any, radioactivity was present at the C_2 fragment, C(2′)–C(2), of these samples.[4] This indicated non-random distribution of label, but the location of labelling was not determined. We now provide evidence that glycolaldehyde is incorporated as an intact unit into the C_2 fragment, C(5′)–C(5), of pyridoxol and that C-5′ is specifically derived from the carbinol carbon atom and C-5 from the carbonyl carbon atom of glycolaldehyde.

RESULTS AND DISCUSSION

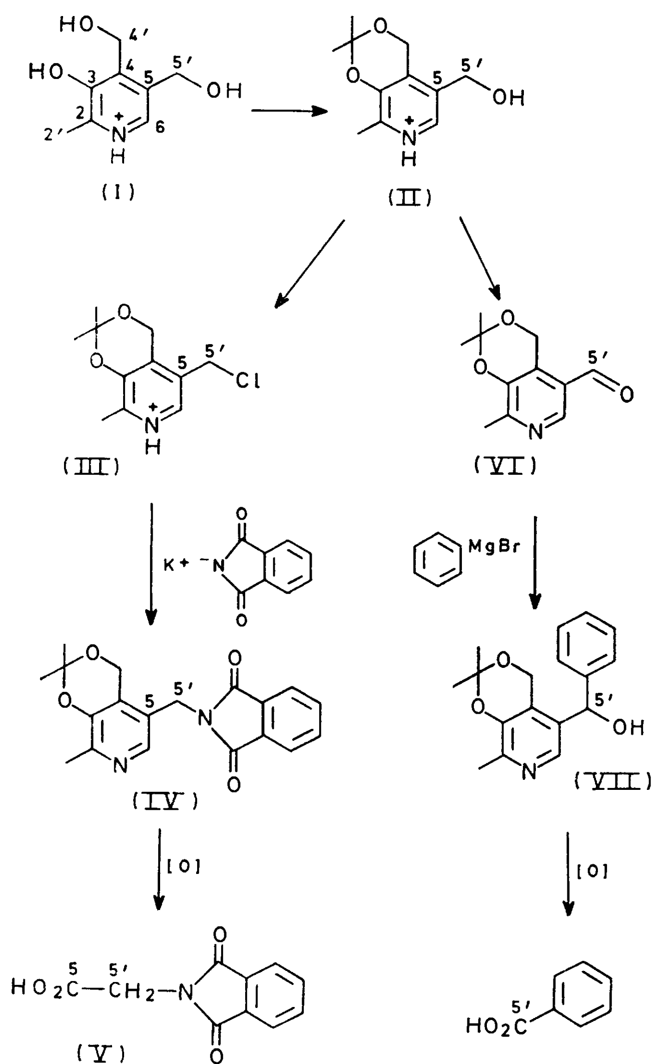
Radioactive samples of pyridoxol hydrochloride, obtained by Tani and Dempsey from pyridoxal phosphate which they had isolated from cultures of *E. coli* B strain WG3 after incubation with [1,2-$^{14}C_2$]glycolaldehyde (ref. 8, expts. III-6, III-9) or with [2-^{14}C]glycolaldehyde (ref. 8, expts. III-5, III-8), were diluted with inactive carrier and degraded, by the reactions shown in Scheme 1, to locate the sites of labelling. The specific activities of the degradation products are listed in Table 1.

Within experimental error, all activity of the pyridoxol hydrochloride derived from [2-^{14}C]glycolaldehyde † was recovered in benzoic acid, representing C-5′ of pyridoxol.

† This sample, prepared by oxidative decarboxylation of [3-^{14}C]serine, was assumed to be labelled solely at the carbinol carbon atom on the basis of its mode of preparation, but rigorous evidence proving the position of ^{14}C was not secured.[8]

1 R. E. Hill and I. D. Spenser, *Science*, 1970, **169**, 773.
2 R. E. Hill, R. N. Gupta, F. J. Rowell, and I. D. Spenser, *J. Amer. Chem. Soc.*, 1971, **93**, 518.
3 R. E. Hill, F. J. Rowell, R. N. Gupta, and I. D. Spenser, *J. Biol. Chem.*, 1972, **247**, 1869.
4 R. E. Hill and I. D. Spenser, *Canad. J. Biochem.*, 1973, **51**, 1412.
5 J. G. Morris, *J. Gen. Microbiol.*, 1959, **20**, 597.
6 J. G. Morris and D. D. Woods, *J. Gen. Microbiol.*, 1959, **20**, 576.
7 W. B. Dempsey, *J. Bacteriol.*, 1971, **108**, 1001.
8 Y. Tani and W. B. Dempsey, *J. Bacteriol.*, 1973, **116**, 341.

The benzoic acid, *i.e.* C-5′, from the pyridoxol derived from [1,2-$^{14}C_2$]glycolaldehyde,* on the other hand,

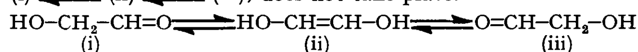


SCHEME 1 Chemical degradation of pyridoxol, permitting isolation of C-5′ and C-5

contained only half the label of the intact vitamin. The other half must be located at C-5, since the phthaloylglycine (*i.e.* C-5 plus C-5′) obtained from this sample of pyridoxol accounted for all its activity. It follows that glycolaldehyde enters the two-carbon unit, C(5′)–C(5), of pyridoxol as an intact unit, the aldehyde carbon atom (*i.e.* C-1) supplying C-5, and the carbinol carbon atom (*i.e.* C-2) C-5′ of pyridoxol.†

* This was prepared by oxidative decarboxylation of [U-^{14}C] serine, and was assumed to have activity equally distributed between the carbinol and the carbonyl carbon atoms. Rigorous proof for equal distribution of label was not obtained, however.[8]

† These results also show that the two carbon atoms of glycolaldehyde maintain their chemical individuality in the course of metabolism, *i.e.* that equilibration of label, by way of enolization (i) ⇌ (ii) ⇌ (iii), does not take place.



$$\underset{(i)}{HO{-}CH_2{-}CH{=}O} \rightleftharpoons \underset{(ii)}{HO{-}CH{=}CH{-}OH} \rightleftharpoons \underset{(iii)}{O{=}CH{-}CH_2{-}OH}$$

Growth of *E. coli* B WG3, a pyridoxol-requiring mutant, can be maintained only if minimal medium, containing either 0.2% glucose [7,9] or 0.2% glycerol [8] as general carbon source, is supplemented by pyridoxol, or by glycolaldehyde.[7] It follows that this mutant cannot biosynthesize glycolaldehyde from glucose or from glycerol. Since specific incorporation of glycolaldehyde into pyridoxal phosphate is now demonstrated, and it is shown that a single glycolaldehyde unit enters the pyridoxal molecule, it follows that the samples of pyridoxal phosphate, produced by *E. coli* B WG3 cultures on incubation with labelled glycolaldehyde, must show a molar specific radioactivity (mCi mmol^{-1}) identical with that of the samples of [^{14}C]glycolaldehyde from which they are derived. That is, the specific radiochemical yield (= 100 × molar specific radioactivity of product/molar specific radioactivity of precursor) must be 100%. Published data [8] tend to bear out this prediction.‡

Failure to recognize that in these experiments maintenance within the product of the molar specific activity of the precursor was a consequence of the experimental conditions prompted the conclusion in a recent review [10] that whereas '[^{14}C]glycolaldehyde served as a highly efficient precursor of labelled pyridoxol,' the importance of glycerol and glucose in pyridoxol biosynthesis was questionable, since in experiments with ^{14}C-labelled samples of these substrates 'the total incorporation § of radioactivity into pyridoxol was slight.' [10]

Before attempting an interpretation of the mode of incorporation of glycolaldehyde into vitamin B_6, it is necessary to correct the impression conveyed by this misleading comparison.

Non-random incorporation of radioactivity from specifically labelled radiomers of glycerol into pyridoxol was demonstrated by chemical degradation.[1-3] It was shown that approximately one third of the molar specific activity of pyridoxol derived from [2-^{14}C]-glycerol was located at each of C-2 and C-4. The remaining third was predicted to reside at C-5 of pyridoxol. This prediction is now confirmed. Whereas benzoic acid (*i.e.* C-5′) obtained from [2-^{14}C]glycerol-derived pyridoxol was devoid of radioactivity, phthaloylglycine (*i.e.* C-5′ plus C-5) accounts for one-third of its

‡ The apparent discrepancies in the molar specific activities of glycolaldehyde and of pyridoxal phosphate observed in several experiments (Table 2 of ref. 8) (specific radiochemical yields reported for four experiments with [2-^{14}C]glycolaldehyde 80, 100, 90, 50% and for three experiments with [1,2-$^{14}C_2$]glycolaldehyde 114, 86, 66%) are a measure of the accuracy of the radioactivity determinations on which these data are based.

The two low values (50 and 66%) were obtained in cultures of WG3 which had undergone partial reversion to wild type.[8] The reduction in the specific radiochemical yields in these experiments indicates that, in wild type cultures, [^{14}C]glycolaldehyde or one of its metabolites encounters a corresponding unlabelled pool prior to incorporation into the product.

§ *i.e.* the percentage incorporation (= 100 × total activity recovered in product/total activity administered in precursor).

[9] W. B. Dempsey, *J. Bacteriol.*, 1969, **100**, 295.
[10] G. W. E. Plaut, C. M. Smith, and W. L. Alworth, *Ann. Rev. Biochem.*, 1974, **43**, 899.

label, which is therefore located at C-5, as predicted (Table 2).

Similarly, it is now shown that C-5 of the pyridoxol derived from [1-^{14}C]glycerol is devoid of activity (Table 2). This is also according to prediction, which placed one-fifth of the molar specific activity of pyridoxol derived from [1-^{14}C]glycerol at each of C-2′, -3, -4′, -5′, and -6. It is thus established [1-3] that one-fifth of the molar specific activity does indeed reside at each of C-2′, -4′, and -5′. It is also established that C-2, -4, and -5 are free of activity. This leaves two carbon atoms, C-3 and C-6, to accommodate two-fifths of the molar specific activity. It is a plausible assumption that each of these two carbon atoms, like the other three labelled centres, contains one-fifth of the total label. These conclusions are summarized in Scheme 2.

The eight carbon atoms of pyridoxol are thus accounted for by three glycerol units which are incorporated non-randomly into the vitamin. Two of these glycerol units, giving rise to the C_3 units C(4′)–C(4)–C(3) and C(5′)–C(5)–C(6) are incorporated intact. The third, giving rise to the C_2 unit, C(2′)–C(2), suffers loss of a terminal carbon atom.

Since specific incorporation of glycerol into pyridoxol is demonstrated and it is shown that three glycerol units (less one terminal carbon atom) enter the pyridoxol molecule, and account for all its carbon atoms, it follows that a sample of pyridoxol synthesized by *E. coli* B WG2

TABLE 1

Incorporation of glycolaldehyde into pyridoxol

Products	C Atoms of Pyridoxol	Substrate: [2-^{14}C]Glycolaldehyde SA [a]	RSA [b]	[1,2-$^{14}C_2$]Glycolaldehyde SA [a]	RSA [b]
Pyridoxol hydrochloride (I)	All	1.18 ± 0.04 [c]	100 ± 3	1.11 ± 0.03 [d]	100 ± 2
Isopropylidenepyridoxol (II)	All	1.20 ± 0.04	102 ± 4		
Isopropylideneisopyridoxal (VI)	All	1.23 ± 0.03	105 ± 4	1.07 ± 0.03	97 ± 4
Isopropylidene-5′-phenylpyridoxol (VII)	All	1.16 ± 0.04	99 ± 4	1.11 ± 0.04	100 ± 4
Benzoic acid	C-5′	1.11 ± 0.02	94 ± 3	0.58 ± 0.01	52 ± 2
Isopyropylidenepyridoxol (II)	All			0.95 ± 0.03 [e]	100 ± 3
Isopropylidenephthaloylisopyridoxamine (IV)	All			0.91 ± 0.05	96 ± 6
Phthaloylglycine (V)	C-5′, -5			0.91 ± 0.03	97 ± 4
Phthaloylglycine minus benzoic acid	C-5 (by difference)				45 ± 5
Pyridoxol hydrochloride	All	0.69 ± 0.02 [f,h]	100 ± 3	0.76 ± 0.03 [g,h]	100 ± 3
Isopropylidenepyridoxol	All	0.70 ± 0.02	101 ± 4		
K/R acetate from pyridoxol	C-2′, 2	0.01 ± 0.003	2 ± 0.4	0.02 ± 0.02	3 ± 3
4′-Deoxypyridoxol	All	0.66 ± 0.03	95 ± 5		
K/R acetate from 4′-deoxypyridoxol	C-2′, -2, -4′, -4	0.001 ± 0.01	0.1 ± 2		

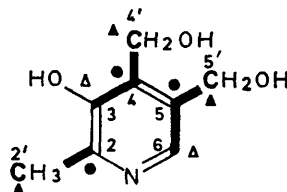
[a] Specific activity (disint. min^{-1} mmol^{-1}) × 10^{-4}. [b] Relative specific activity (%) (pyridoxol hydrochloride = 100). [c] Obtained from pyridoxal phosphate (expt. III-8, ref. 8) by conversion into pyridoxol hydrochloride [15] and dilution with inactive carrier. [d] As *c*, but expt. III-9, ref. 8. [e] As *c*, but expt. III-9, ref. 8, and followed by conversion to the isopropylidene derivative. [f] As *c*, but expt. III-5, ref. 8. [g] As *c*, but expt. III-6, ref. 8. [h] *Cf.* ref. 4.

TABLE 2

Degradation to locate label at C-5 of pyridoxol derived from [1-^{14}C]glycerol and from [2-^{14}C]glycerol

Products	C Atoms of Pyridoxol	Substrate: [1-^{14}C]Glycerol SA [a]	RSA [b]	[2-^{14}C]Glycerol SA [a]	RSA [b]
Pyridoxol hydrochloride (I)	All	5.15 ± 0.04 [c]	100 ± 1	3.15 ± 0.02 [d]	100 ± 1
Isopropylidenepyridoxol (II)	All	5.03 ± 0.04	98 ± 1	3.21 ± 0.03	102 ± 1
Isopropylidenephthaloylisopyridoxamine (IV)	All	5.00 ± 0.04	97 ± 1	3.04 ± 0.02	96 ± 1
Phthaloylglycine (V)	C-5′, -5	1.02 ± 0.01	20 ± 0.3	1.07 ± 0.01	34 ± 0.3
Benzoic acid	C-5′		22 ± 1 [e]		0.5 ± 0.2 [e]
Phthaloylglycine minus benzoic acid	C-5 (by difference)		Inactive		33 ± 0.4

[a,b] As Table 1. [c] Portion of the sample of pyridoxol hydrocyloride isolated in expt. 15, ref. 3. [d] As *c*, but expt. 16, ref. 3. [e] Quoted from Table IV of ref. 3.



SCHEME 2 Mode of incorporation of glycerol into pyridoxol; sites of activity derived from [1-^{14}C]glycerol (▲,△) (relative specific activity *ca.* 20%) and from [2-^{14}C]glycerol (●) (relative specific activity *ca.* 33%) shown by degradation (▲ ●) or inferred (△)

cultures on incubation with [2-^{14}C]glycerol, in the presence of glycerol as the sole general carbon source, must show a molar specific radioactivity (mCi mmol^{-1}) three times that of the labelled precursor, *i.e.* the specific radiochemical yield must be 300%. It follows also that a sample of pyridoxol produced similarly on incubation with chemically labelled [1-^{14}C]glycerol (*i.e.* an equimolar mixture of *sn*-[1-^{14}C]- and *sn*-[3-^{14}C]-glycerol) must show a molar specific radioactivity 2.5 times that of the labelled precursor, *i.e.* a specific radiochemical yield of 250%. Since the distribution pattern of label within pyridoxol derived from [^{14}C]glucose [3,4] is not as simple as that within glycerol-derived pyridoxol, the specific radiochemical yield cannot be predicted on the basis of existing evidence. Nor is there a simple relationship between molar specific activity of precursor and product when labelled precursor (*e.g.* [^{14}C]glycerol) has to compete for incorporation into product with another, unlabelled substrate (*e.g.* glucose) which serves as the general carbon source and is present in large excess.

Our experiments, in which [1-^{14}C]- and [2-^{14}C]-glycerol were tested as a precursor of pyridoxol in cultures of *E. coli* B WG2, with glycerol as the sole carbon source (ref. 3, expts. 2, 15, 16), were designed solely with a view to establishing the distribution of radioactivity within the newly synthesized pyridoxol. To achieve this purpose it was not essential to know the amount of pyridoxol formed or its specific radioactivity, and these values were not determined. Precise data to confirm the above predictions that glycerol-derived pyridoxol should have a molar specific activity 3 times or 2.5 times that of the precursor, are therefore lacking.

However, from data which are available it can be calculated,* that the predicted specific radiochemical yield of 300% in the experiment with [2-^{14}C]glycerol (expt. 16) and of 250% in those with [1-^{14}C]glycerol (expts. 2 and 15) would obtain if 60, 75, and 72 μg, respectively, of pyridoxol had been synthesized *de novo* in the course of these experiments. These values are indeed within the expected order of magnitude, 60—180 μg l^{-1} of bacterial culture.[11,12]

It is evident from the foregoing discussion that a comparison of specific radiochemical yields obtained in experiments with glycolaldehyde and with glycerol does not serve as a fruitful basis on which conclusions regarding their position in the biosynthetic pathway leading to pyridoxol can be drawn.

It remains to examine whether a comparison of percent incorporation of radioactivity in the various experiments leads to useful conclusions. For nine experiments with labelled glycerol,[3] glucose,[3,4] and pyruvate [3] the percent incorporation (100 × total activity recovered within pyridoxol †/total activity in precursor added to the medium) ranged from 2×10^{-3} to 8×10^{-3}.

For six experiments with labelled glycolaldehyde [8] the percent incorporation ranged from 3.5×10^{-3} to 9.5×10^{-3}. One experiment gave an exceptionally high yield of product and thus an exceptionally high value of 1.6×10^{-2} for the percent incorporation.

The recovery of radioactivity within the products of the two series of experiments is thus of the same low order of magnitude. This is hardly surprising. In each case the molar amount of product formed is several orders of magnitude lower than the molar amount of labelled substrate which is available. In the experiments with *E. coli* B WG2 [3] *ca.* 0.3 μmol of pyridoxol per litre was formed whereas *ca.* 5 mmol of substrate per litre (glucose, expt. 14), *ca.* 11 mmol of substrate per litre (glycerol, expts. 15, 16), and *ca.* 22 mmol of substrate per litre (glycerol, expt. 2), were available. In the experiments with *E. coli* B WG3,[8] *ca.* 0.03 μmol of pyridoxal per litre was formed whereas *ca.* 0.4 mmol of glycolaldehyde per litre was available.

Neither percent incorporation nor specific radiochemical yield thus serves as a guide to precursor status in this instance. The only reliable guide is distribution of label within the product.

We have reported the pattern of labelling within pyridoxol generated from a number of ^{14}C-labelled substrates.[1-4] This established the distribution and accounted for all the radioactivity within samples of pyridoxol derived from [2-^{14}C]- and [3-^{14}C]-pyruvate and from [1-^{14}C]- and [6-^{14}C]-glucose. Our published work, together with results reported here, similarly clarify the quantitative distribution of label within pyridoxol derived from [2-^{14}C]glycerol and, with a plausible assumption (see above), from [1-^{14}C]glycerol.

These results are entirely consistent with the hypothesis which is referred to in the Introduction. The non-random and specific incorporation of glycolaldehyde into pyridoxal, now demonstrated, must be reconcilable with this hypothesis. Any attempt to interpret the role of glycolaldehyde in pyridoxol biosynthesis must be tentative, because of the dearth of knowledge concerning the metabolism of this simple two-carbon compound.

In attempting to account for the position of glycolaldehyde in pyridoxal biosynthesis (Scheme 3), relevant available evidence must be reviewed.

In *E. coli* mutant WG2 the eight carbon atoms of pyridoxol are derived from two C_3 precursors and one C_2 precursor. All three are generated from glycolytic intermediates, presumably trioses, related to glucose

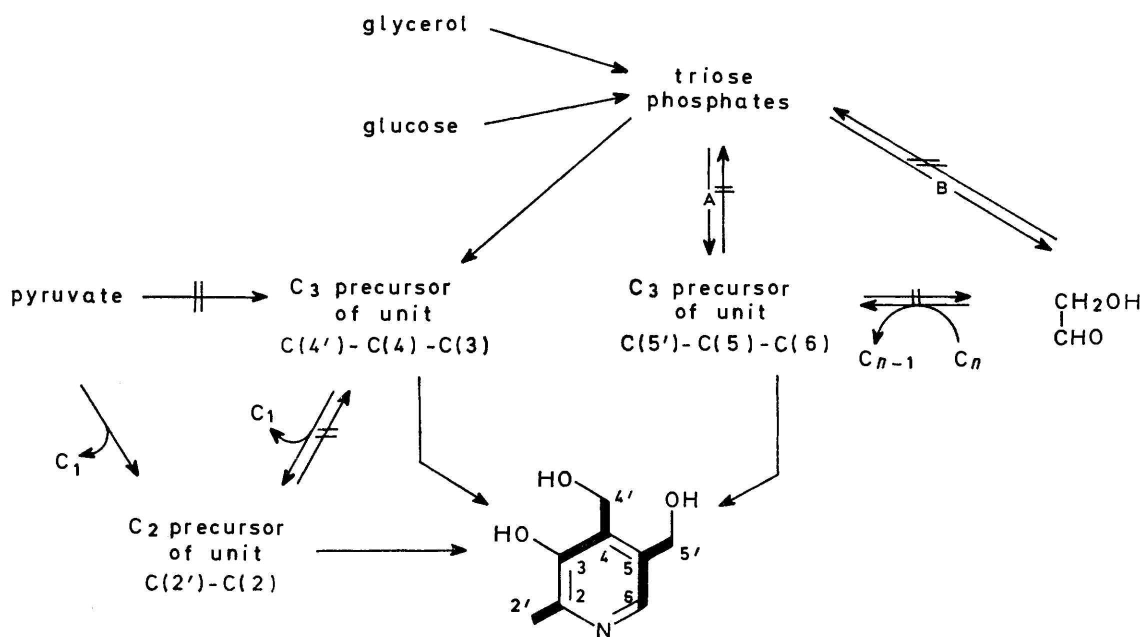
* *E.g.* for expt. 16, specific activity (nominal) of [2-^{14}C]-glycerol: 0.091 mCi mmol^{-1}; weight of pyridoxol added as carrier 1 000 mg; specific activity of pyridoxol isolated after carrier dilution 1.09×10^4 counts min^{-1} mmol^{-1}; efficiency of counting system *ca.* 30%; 1 mCi = 2.22×10^9 disint. min^{-1}; weight (mg) of newly synthesized pyridoxol, of specific activity 3×0.091 mCi mmol^{-1}:

$$\frac{1.09 \times 10^4}{3 \times 0.091} \times 1\,000 \times \frac{100}{30} \times \frac{1}{2.22 \times 10^9} = 0.06.$$

† *Cf.* footnote † and ref. 3, Table 1: total activity recovered within pyridoxol = total activity (counts min^{-1}) recovered per 0.1 mCi of substrate $\times \frac{100}{30} \times \frac{1}{2.22 \times 10^9}$.

[11] W. B. Dempsey, *J. Bacteriol.*, 1966, **92**, 333.
[12] W. B. Dempsey, *J. Bacteriol.*, 1967, **93**, 1179.

and glycerol. That the three precursors are closely related to each other is shown by the observation that, when derived from [2-^{14}C]glycerol, each of the fragments within pyridoxol, corresponding to the three precursors, contained the same molar specific activity. That the three precursors are all different is shown by the facts (i) that pyruvate supplies the C_2 unit, C(2′)–C(2) of pyridoxol, but neither of the C_3 units, C(4′)–C(4)–C(3) and C(5′)–C(5)–C(6), and (ii) that these two C_3 units show different molar specific activities when [^{14}C]glucose is the labelled substrate. Finally, the C_2 precursor is -5, then leads to the inference that, even though glycolaldehyde metabolism excludes its conversion into glycolytic intermediates, it leads to a C_3 unit which serves as the precursor of the pyridoxal unit C(5′)–C(5)–C(6). This chain extension might involve a one-carbon unit, but this is unlikely since one-carbon donors do not participate in pyridoxol biosynthesis in *E. coli* B WG2.[3] A possible pathway for such a chain elongation, represented in general terms as glycolaldehyde + $C_n \longrightarrow C_3 + C_{n-1}$, might proceed by a three-step sequence, involving a ketolase, a kinase, and an aldolase reaction.



SCHEME 3 Route of precursor fragments into pyridoxol; A and B denote possible sites of the genetic glock in *E. coli* B mutant WG3 [the arrows showing the paths from the C_2 and C_3 precursors are intended to indicate the sites of entry of these units; it is not implied that these units are the immediate precursors of pyridoxol (*cf.* ref. 3)]

more closely related to the precursor of the C_3 unit C(4′)–C(4)–C(3), than to that of the C_3 unit C(5′)–C(5)–C(6) since, in two samples of glucose-derived pyridoxol the molar specific activity of the C_2 unit was identical with that of the C_3 unit C(4′)–C(4)–C(3) but different from that of C(5′)–C(5)–C(6).

It is two carbon atoms of the precursor of this C_3 unit, C(5′)–C(5)–C(6), which in *E. coli* B mutant WG3 are generated from glycolaldehyde. Since glycerol served as the general carbon source in these experiments it must have supplied the other six carbon atoms of pyridoxal. The mode of entry of glycerol carbon into these sites has not yet been investigated. For purposes of the present discussion we will assume, in the absence of evidence to the contrary, that the origin of the C_2 unit C(2′)–C(2) and the C_3-unit C(4′)–C(4)–C(3) of the pyridoxal generated in mutant WG3 follows the pattern demonstrated for the origin of pyridoxol in mutant WG2.

The mode of incorporation of glycolaldehyde into pyridoxal, exclusively into two carbon atoms, C-5′ and Mulder [13] has reported the production of erythrulose in cultures of *E. coli* mutant B 166 growing on a glycolaldehyde-enriched medium.

The entry of glycolaldehyde into the precursor of the C_3 unit C(5′)–C(5)–C(6) may be dictated by the nature of the genetic block in mutant WG3. If the direct route to this precursor from glycerol and the triose intermediates of glycolysis were blocked in WG3 (Scheme 3, block A), an irreversible route *via* glycolaldehyde may be induced, and in the absence of adequate *de novo* biosynthesis of glycolaldehyde, this compound must be supplied to support pyridoxal biosynthesis and growth of the mutant.

Alternatively, glycolaldehyde may be a mandatory intermediate on the route to the precursor of the C_3 unit C(5′)–C(5)–C(6) whose formation from glycolytic intermediates, in an irreversible step, has been blocked in the mutant (Scheme 3, block B).

[13] C. Mulder, D.Phil. Thesis, University of Oxford, 1959; personal communication.

It may be significant in this context that reduction of glycolic acid to glycolaldehyde, a process which occurs in wild type *E. coli*, is blocked in mutant WG3.[14]

These possibilities are open to independent experimental test. Thus, if glycolaldehyde were a mandatory intermediate, its addition to the culture medium of *E. coli* B mutant WG2 should lead to a change in the distribution of label in pyridoxol derived from [2-^{14}C]-glycerol. Incorporation of label into C-5 should be suppressed, leading, in the limit, to a sample of pyridoxol containing 50% of its molar specific activity at each of C-2 and C-4, and 0% at C-5. If incorporation of glycolaldehyde into pyridoxal in mutant WG3 is mutation-induced, addition of carrier glycolaldehyde to the culture medium of WG2 would leave the distribution of label in pyridoxol, derived from [2-^{14}C]glycerol, unchanged, with 33% of the molar specific activity at each of C-2, C-4, and C-5.

This, and other experiments designed to clarify the function of glycolaldehyde in pyridoxal biosynthesis are under way.

EXPERIMENTAL

The conditions for bacterial growth, and the procedures for the isolation of pyridoxol hydrochloride from *E. coli* B strain WG3 after incubation with [^{14}C]glycolaldehyde [8,15] and from *E. coli* B strain WG2 after incubation with glycerol [3] have been described, as have the methods employed in the systematic degradation of ^{14}C-labelled samples of pyridoxol, permitting assay of radioactivity at individual carbon atoms (C-2′, C-2, C-4, C-4′, C-5′).[3] A new method, permitting determination of label at C-5 of pyridoxol, is now reported. Radioactivity was assayed by liquid scintillation counting (Mark 1 liquid scintillation computer, model 6860 Nuclear Chicago). Samples were dissolved in water or methanol and dispersed in Aquasol (New England Nuclear). Triplicate samples of each compound were counted under comparable conditions of quenching. Confidence limits shown in the Tables are standard deviations of the mean.

Isolation of Carbon Atoms 5 and 5′ of Pyridoxol as Phthaloylglycine (*Scheme* 1).—5-*Chloromethyl*-2,2,8-*trimethyl*-4H-m-*dioxino*[4,5-c]*pyridine hydrochloride* (III).[16] Thionyl chloride (0.055 ml) was added to a cold (0 °C) stirred suspension of 3,4′-*O*-isopropylidenepyridoxol hydrochloride (II) [3] (70 mg) in chloroform (1.0 ml). A clear solution was obtained after stirring for *ca.* 5 min at room temperature. Stirring was continued for a further 1 h. When the solution was evaporated under reduced pressure, the hydrochloride (III) was obtained as a white crystalline solid (73 mg, 98%), m.p. 186—189° (lit.,[16] 191—192°). To obtain the corresponding free base the hydrochloride, dissolved in water, was basified with solid sodium hydrogen carbonate and extracted with ether (3 × 8 ml). When the extract was dried (Na_2SO_4) and evaporated the product was obtained as a pale yellow oil.

2,2,8-*Trimethyl*-5-*phthalimidomethyl*-4H-m-*dioxino*[4,5-c]-*pyridine* (*isopropylidenephthaloylisopyridoxamine*) (IV). The free base obtained from (III) (73 mg) was dissolved in dry dimethylformamide (1 ml). *N*-Potassiophthalimide (60 mg) was added and the solution was heated with stirring at 130—140 °C for 2 h and then allowed to cool to room temperature. When water (10 ml) was added, and the solution stirred at room temperature, the *phthalimido-derivative* (IV) crystallized (75 mg, 80%); m.p. 140—142°. Recrystallization from 95% ethanol gave a sample of m.p. 147—148° (Found: C, 67.7; H, 5.35; N, 8.25. $C_{19}H_{18}N_2O_4$ requires C, 67.45; H, 5.35; N, 8.3%); $\nu_{max.}$ 1 777 and 1 715 cm^{-1}; δ ($CDCl_3$) 1.55 (6 H, s), 2.37 (3 H, s), 4.67 (2 H, s), 5.08 (2 H, s), 7.75 (4 H, m), and 8.11 (1 H, s).

Phthaloylglycine (V) *by Oxidation of the Phthalimido-derivative* (IV). The phthalimido-derivative (IV) (65 mg) was dissolved in dilute sulphuric acid (3 ml; 1N); the solution was heated on a steam-bath for 20 min and then allowed to cool to room temperature. Potassium permanganate (150 mg) was added in small portions with stirring over 1 h, and stirring was then continued for a further 1 h. Precipitated manganese dioxide was dissolved by addition of sodium hydrogen sulphite and the resulting turbid solution was extracted with ethyl acetate (3 × 8 ml). The extract was dried (Na_2SO_4) and evaporated under reduced pressure to give a cream-coloured residue (40 mg). This was dissolved in saturated aqueous sodium hydrogen carbonate (1 ml), the solution was washed with chloroform (2 × 1 ml), and the aqueous layer was acidified with a few drops of 6N-hydrochloric acid. The phthaloylglycine (21 mg, 53%) which crystallized was sublimed at 10^{-2} mm Hg and 120 °C; m.p. 193—194° (lit.,[17] 191—192°). The i.r. spectrum was identical with that of an authentic sample obtained by fusion of glycine with phthalic anhydride.[17]

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