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Growth response to 4-hydroxy-L-threonine of *Escherichia coli* mutants blocked in vitamin B_6 biosynthesis

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Mutants of *Escherichia coli* (pdx B and pdx C) which are blocked in the biosynthesis of pyridoxol (vitamin B_6) showed a growth response to 4-hydroxy-L-threonine. This observation constitutes the first direct evidence in support of the view that 4-hydroxy-L-threonine is implicated in the biosynthesis of vitamin B_6. 1-Aminopropan-2,3-diol, the decarboxylation product of 4-hydroxy-L-threonine, does not support the growth of these mutants. Deuterium from deuterium-labelled 1-aminopropan-2,3-diol was not incorporated into pyridoxol.

Biosynthesis; *Escherichia coli*; Vitamin B_6; Pyridoxol; 4-Hydroxy-L-threonine; 1-Aminopropan-2,3-diol

1. INTRODUCTION

The C_3-unit, C-5′,-5,-6, of pyridoxol (vitamin B_6 (**2**, Scheme 1, originates from two independent sources: it is generated either from glycerol via glyceraldehyde phosphate [1–3] or, alternatively, from glycolaldehyde plus glycine [4–6]. In order to rationalize this dichotomy of origin in terms of a common intermediate, we suggested [7] that 1-aminopropan-2,3-diol (**4**; Scheme 1) might lie on the pathway. This compound is an attractive candidate for intermediacy, since it could arise, on the one hand, from glyceraldehyde phosphate by transamination followed by phosphate ester hydrolysis, and on the other hand from 4-hydroxy-L-threonine* (**3**, Scheme 1) by decarboxylation [7]. 4-Hydroxy-L-threonine, whose origin, by aldolase type condensation of glycolaldehyde and glycine, closely parallels the origin of L-serine from formaldehyde and glycine, has been detected in *Streptomyces fradiae* [8] and *Streptomyces* sp. #1–1 (HLR 330 A) [9].

We now report the results of experiments designed to test the possible role of 1-aminopropan-2,3-diol (**4**, Scheme 1) and 4-hydroxy-L-threonine (**3**, Scheme 1) in the biosynthesis of vitamin B_6. The evidence here presented supports the view that 4-hydroxy-L-threonine is indeed an intermediate of pyridoxol biosynthesis, whereas 1-aminopropan-2,3-diol is not.

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*synonyms: L-threo-3-hydroxyhomoserine, 2-amino-2-deoxy-D-threonic acid, (2-*S*,3-*S*)-2-amino-3,4-dihydroxybutanoic acid, L-threo-α-amino-β, γ-dihydroxybutyric acid.

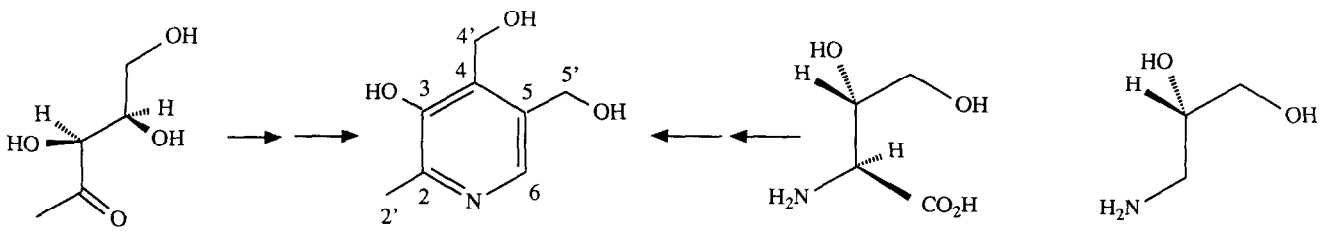
2. MATERIALS AND METHODS

2.1. *Growth of mutants in the presence of pyridoxal or 4-hydroxy-L-threonine (3, Scheme 1) or 1-aminopropan-2,3-diol (DL-4, Scheme 1)*

Mutants of linkage groups pdx A (WG 25 [10]), pdx B (WG 3 [10], WG 1012 [11]), pdx C (WG 1145 [12], AT 3143 [13]) and pdx J (AT 3208 [13]) were grown in 50 ml M9 minimal medium (6.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 20 ml 20% glucose solution, 1 ml $MgSO_4$ (1 M), 1 ml $CaCl_2$ (0.1 M), 1 mg pyridoxal-5′-phosphate in 1 litre of H_2O (pH 7.0) containing the following additives:

WG	3	(pdx B):	no additives
WG	1012	(pdx B):	20 μg/ml histidine
WG	1145	(pdx C):	20 μg/ml serine
AT	3143	(pdx C):	20 μg/ml each of histidine, proline, methionine, isoleucine, valine and serine
WG	25	(pdx A):	no additives
AT	3208	(pdx J):	1 μl/ml thiamine

The bacteria were grown up to the late logarithmic growth phase and collected by low speed centrifugation at room temperature. The pellet was washed twice with an aqueous NaCl solution (0.14 M). The cells were suspended in 20 ml M9 medium (50°C) without pyridoxal-5′-phosphate containing agar (1.6%) and triphenyltetrazolium dichloride (2.0 mg) and the appropriate additive for each strain (see above). The suspension was poured into a Petri dish and, after solidification, wells (9 mm diameter) were pierced into the agar with a sterile cork borer. Aqueous solutions (100 μl) of either DL-1 aminopropan-2,3-diol (1, 5, 10, 50, 100 nmol) or 4-hydroxy-L-threonine (1, 5, 10, 50, 100 nmol) or pyridoxal (1, 5, 10, 50 nmol) were placed into individual wells. The growth zone (distance between rim of the well and periphery of the growth circle) was determined after incubation at 37°C for 12 h. Each of the growth curves presented in Fig. 1 represents the mean of three independent experiments.



Scheme 1. D-1-Deoxyxylulose (**1**) and 4-hydroxy-L-threonine (**3**), precursors of pyridoxol (**2**). 1-Aminopropan-2,3-diol (**4**), the decarboxylation product of **3**, is not a precursor of pyridoxol.

2.2. *Synthesis of* DL-*[1,1,2,3,3-$^2H_5$]-1-aminopropan-2,3-diol (**9**, Scheme 2)*

2.2.1. DL-[4,4′,4′,5,5-$^2H_5$]-4-Hydroxymethyl-2,2-dimethyl-1,3-dioxolane

Synonyms: DL-[1,1,2,3,3-$^2H_5$]-1,2-*O*-isopropylideneglycerol, [4,4′-,4′,5,5-$^2H_5$]solketal) (**6**, Scheme 2).

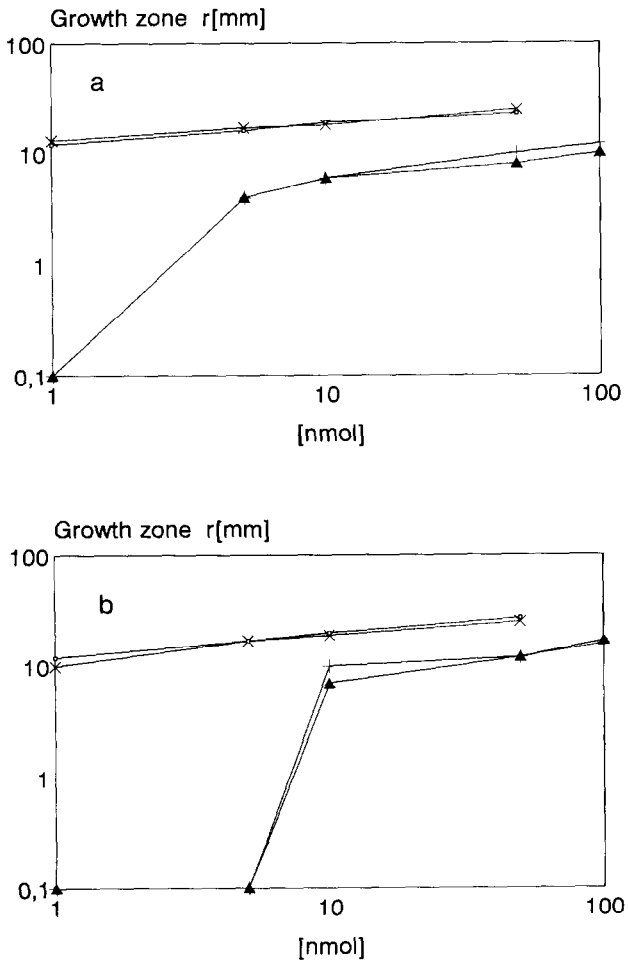


Fig. 1. Growth response to pyridoxal and to 4-hydroxy-L-threonine of (a) mutants of linkage groups pdx B (WG 3, WG 1012) and (b) mutants of linkage group pdx C (WG 1145, AT 3143). (a) □, WG 3 with pyridoxal; +, WG 3 with 4-hydroxy-L-threonine; x, WG 1012 with pyridoxal; ▲, WG 1012 with 4-hydroxy-L-threonine. (b) x, WG 1145 with pyridoxal; ▲, WG 1145 with 4-hydroxy-L-threonine; □, AT 3143 with pyridoxal; +, AT 3143 with 4-hydroxy-L-threonine.

To a solution of [1,1,2,3,3-$^2H_5$]glycerol (97.7 atom % ^2H, MSD Isotopes) (2.5 g, 25.8 mmol) (**5**, Scheme 2) in acetone (25 ml) was added 2,2-diethoxypropane (3.75 ml), followed by *p*-toluenesulfonic acid monohydrate (63 mg). The mixture was stirred vigorously at room temperature for 1 h during which time the progress of the reaction was monitored by thin layer chromatography (TLC) (SiO_2, ether). When the conversion appeared to be complete the reaction was stopped by addition, to neutrality, of a solution of sodium methoxide in dry methanol. (Prolonged reaction leads to isomerization to the undesired 2,2-dimethyl-5-hydroxy-1,3-dioxane, i.e. 1,3-*O*-isopropylideneglycerol (**10**, Scheme 2). The reaction mixture was evaporated in vacuo and the residue purified by bulb-to-bulb distillation at 130°C and 11 mm. Yield of **6**, Scheme 2: 3.44 g, 95%.

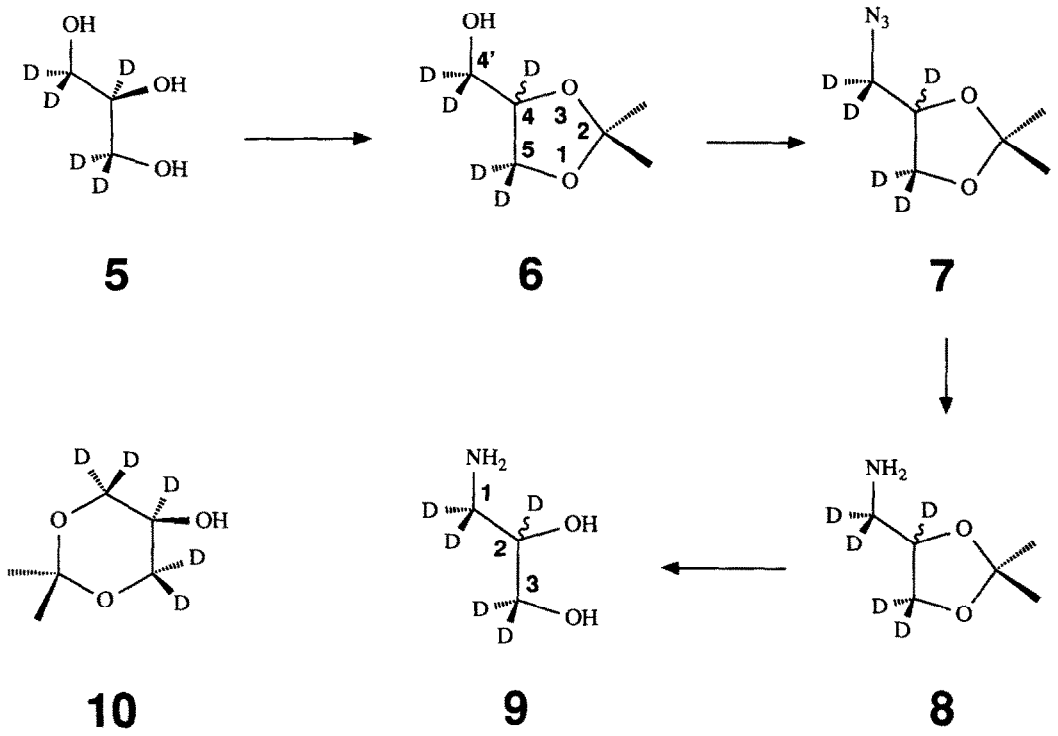
2.2.2. DL-[4,4′,4′,5,5-$^2H_5$]-4-Aminomethyl-2,2-dimethyl-1,3-dioxolane (**8**, Scheme 2)

The above compound (**6**, Scheme 2) (3.43 g, 25 mmol) was dissolved in dry benzene (30 ml) in a 250 ml flask. Triphenylphosphine (7.2 g, 27.5 mmol, 1.1 equivs.) was added, followed by a solution of hydrazoic acid (**caution, highly toxic**) in dry benzene (30 ml, 1.32 mmol HN_3 per ml). The mixture was cooled to 0°C under N_2 gas and a solution of diethyl azodicarboxylate (5.2 g, 30 mmol, 1.2 equivs.) in dry benzene (30 ml) was added with stirring under N_2 at 0°C. After the vigorous evolution of gas had subsided, the cooling bath was removed and the solution was stirred at room temperature overnight. The solution was concentrated in vacuo and the semi-solid residue applied to a silica column. The product was eluted with light petroleum/ether 1:1, the combined fractions containing the product were evaporated in vacuo and the residual oily azide (**7**, Scheme 2) used without further purification.

The crude azide (**7**, Scheme 2) was dissolved in dry ether (15 ml) and the solution was added dropwise to a stirred suspension of $LiAlH_4$ (2 g) in dry ether (125 ml). The mixture was refluxed 2.5 h, stirred at room temperature overnight and then decomposed, by addition of water (2 ml) followed by aqueous sodium hydroxide (15%, 2 ml) and water (6 ml). The mixture was boiled under reflux for 15 min, the supernatant was decanted and the solid residue extracted twice under reflux for 15 min with dry ether (2 × 80 ml). The combined extracts were dried (anhydrous Na_2SO_4) and evaporated. The residue was dissolved in CH_2Cl_2 (50 ml), dried (anhydrous Na_2SO_4) and evaporated, and the residue distilled at 150°C and 10 mm. Yield of **8**, Scheme 2: 1.95 g, 57.4%.

2.2.3. DL-[1,1,2,3,3-$^2H_5$]-1-Aminopropan-2,3-diol (**9**, Scheme 2)

The isopropylidene derivative (**8**, Scheme 2) was dissolved in tetrahydrofuran (5 ml), the solution was cooled to 0°C and conc. HCl (2 ml) was added. The mixture was refluxed 30 min, cooled to room temperature, neutralized by addition of solid K_2CO_3 and evaporated in vacuo. The residual oil was extracted with hot absolute ethanol (5 × 3 ml), the extracts were combined, cooled, filtered and evaporated. The product (**9**, Scheme 2) was obtained as an oil which was used without further purification.



Scheme 2. Synthesis of deuterium-labelled 1-aminopropan-2,3-diol (**9**).

2.3. *Incorporation studies with* DL-*[1,1,2,3,3-$^2H_5$]-1-aminopropan-2,3-diol (**9**, Scheme 2)*

A culture of *Escherichia coli* B WG2 was incubated, as described earlier [1], with D-glucose as the general carbon source, in the presence of DL-[1,1,2,3,3-$^2H_5$]-1-aminopropan-2,3-diol (**9**, Scheme 2). Pyridoxol hydrochloride was isolated, after addition of natural abundance pyridoxol hydrochloride as carrier, and was purified by column and TLC, followed by high vacuum sublimation [1]. The ^2H NMR spectrum of the isolated sample of pyridoxol hydrochloride (ca. 2.0 mg in 60 μl of methanol, saturated solution) was recorded on a Bruker AM 500 spectrometer. No deuterium enrichment was detected.

3. RESULTS AND DISCUSSION

A culture of *E. coli* B WG2 was incubated with a sample of DL-1-aminopropan-2,3-diol (**9**, Scheme 2), in which all non-exchangeable hydrogen atoms had been replaced by deuterium. An attempt to detect incorporation of deuterium from this substrate, into the C_3-unit, C-5′, -5, -6, of pyridoxol, was unsuccessful. No deuterium was detected by NMR spectrometry in the sample of pyridoxol hydrochloride that was isolated from the culture. Whilst it is unwise to draw definitive inferences from negative results, we nevertheless conclude that 1-aminopropan-2,3-diol is not an intermediate of pyridoxol biosynthesis since, in a parallel experiment, under similar experimental conditions, a deuterium-labelled sample of D-1-deoxyxylulose (**1**, Scheme 1) had yielded pyridoxol hydrochloride which showed the predicted non-random distribution of deuterium [14], indicating that D-1-deoxyxylulose had indeed served as the precursor of the other fragment of pyridoxol, the C_5-unit, C-2′,-2,-3,-4,-4′.

Confirmatory evidence for the non-incorporation of DL-1-aminopropan-2,3-diol into pyridoxol came from another experiment in which mutants blocked in pyridoxal biosynthesis were grown in the presence of this compound. The mutants tested represented four linkage groups (pdx A, pdx B, pdx C (i.e. ser C), and pdx J) coding for enzymes involved in pyridoxal biosynthesis [15]. DL-1-Aminopropan-2,3-diol did not support growth of any of these mutants. Thus, another rationalization of the two modes of derivation of the C_3-unit, C-5′,-5,-6, of pyridoxol must be sought.

A parallel set of growth experiments in which 4-hydroxy-L-threonine (**3**, Scheme 1) was tested as the substrate led to entirely different results. The compound was found to support growth of two mutants belonging to the pdx C linkage group (*E. coli* AT 3143 and *E. coli* WG 1145) and of two mutants belonging to the pdx B linkage group (*E. coli* WG 3 and *E. coli* WG 1012). By contrast, no growth response to 4-hydroxy-L-threonine was observed with mutants of the pdx A (WG 25) and pdx J (AT 3208) linkage groups.

The results of these growth experiments are presented in Fig. 1, where they are compared to growth in the presence of pyridoxal. The data are plotted on a log/log scale. When pyridoxal served as the substrate, the growth curves were essentially linear, wheras they were non-linear with 4-hydroxy-L-threonine. This may indicate that the growth response of *E. coli* to this amino

acid reflects a balance between the precursor function and its anti-metabolic properties [9].

The finding that 4-hydroxy-L-threonine sustains the growth of several pdx mutants of *E. coli* is the first direct evidence in support of the view [7] that this amino acid serves as a precursor of vitamin B_6. Furthermore, the finding is in agreement with the observation derived from genetic experiments [16] which led to the conclusion that genes of the linkage groups pdx B and C are implicated in the synthesis of 4-hydroxy-L-threonine, whereas genes of the linkage groups pdx A and pdx J are involved in the reactions that lead to the formation of the pyridine ring of vitamin B_6.

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