Synthesis of Vitamin B6 by a Mutant of *Escherichia coli* K12 and the Action of 4'-Deoxypyridoxine

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Mutants of *Escherichia coli* K12 blocked in the oxidation of pyridoxine 5'-phosphate ('Oxidase' mutants) excreted pyridoxine at an initial rate of 19 pmol h⁻¹ (10⁸ bacteria)⁻¹, i.e. 0.6 nmol h⁻¹ (mg dry wt)⁻¹, when starved for pyridoxal. Glycolaldehyde, L-phosphoserine, DL-serine and, to a lesser extent, L-leucine stimulated the rate of pyridoxine excretion, but there was no significant stimulation by 2'-hydroxypyridoxine. 4'-Deoxypyridoxine inhibited or stimulated growth of the 'Oxidase' mutant, depending on the relative concentrations of added pyridoxal and 4'-deoxypyridoxine. It was concluded that stimulation of growth by 4'-deoxypyridoxine was due to its conversion to pyridoxal.

INTRODUCTION

The only known intermediate of vitamin B6 biosynthesis in plants and bacteria is pyridoxine 5'-phosphate, which is oxidized to the active coenzyme pyridoxal 5'-phosphate (Dempsey, 1966). In the course of characterization of *pdx* mutations in *Escherichia coli* K12, we found that mutants blocked in the conversion of pyridoxine 5'-phosphate to pyridoxal 5'-phosphate ('Oxidase' mutants) could cross-feed mutants blocked at an earlier stage of vitamin B6 synthesis (Hockney & Scott, 1979). It was of interest to determine whether the excreted material responsible for the cross-feeding was pyridoxine 5'-phosphate or a derivative, possibly pyridoxine. Since the extent of excretion of this precursor was easily measurable by microbiological assay, it suggested a rapid and economical method for screening compounds for their ability to promote or inhibit vitamin B6 biosynthesis.

The vitamin B6 analogue 4'-deoxypyridoxine has been reported to inhibit the growth only of organisms that cannot synthesize vitamin B6, when the external supply of the vitamin is limiting (Rabinowitz & Snell, 1953). Recently, however, 4'-deoxypyridoxine at a concentration of 10 mg ml⁻¹ was reported to cause 22% inhibition of the growth of *Flavobacterium devorans* which is prototrophic for vitamin B6 (Picton, 1975).

The present work describes the identification of the pyridoxal 5'-phosphate precursor excreted by an 'Oxidase' mutant, the effect of various compounds on this excretion, and the effect of 4'-deoxypyridoxine on both the 'Oxidase' mutant and its parent strain.

METHODS

Organisms, growth and vitamin B6 production. *Escherichia coli* K12 'Oxidase' mutant BL-1 and its parent *E. coli* K12 CR63, described by Hockney & Scott (1979), were used. Strain BL-1 was grown in the glucose/salts (G/S) medium of Davis & Mingioli (1950) supplemented with 120 ng pyridoxal ml⁻¹, at 37 °C with shaking. Bacteria were harvested by centrifuging during exponential growth (5 × 10⁸ bacteria ml⁻¹). After resuspension at the same concentration in G/S medium, with (test) or without (control) the compound under investigation, the cultures were incubated with shaking for 3 h at 37 °C. *A*₅₄₀ was determined at intervals

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and after 3 h the pyridoxine concentration of the medium was determined by microbiological assay (Hockney & Scott, 1979).

Identification of vitamin B6 compounds. The column chromatographic technique of Tiselius (1972) using Dowex 50 (H⁺ form) with a combined pH and concentration gradient of ammonium formate separates all the vitamin B6 compounds. This method was used to identify vitamin B6 compounds of microbiological origin as described by Scott & Picton (1976). The 'Oxidase' mutant BL-1 was harvested by centrifuging during exponential growth and resuspended at the same density in G/S medium without vitamin B6 supplement. After incubation for 3 h at 37 °C, the bacteria were removed by centrifuging and the supernatant was assayed for vitamin B6 compounds (Scott & Picton, 1976).

Uptake of pyridoxine by E. coli CR63. The uptake of pyridoxine by *E. coli* CR63 was determined with [G-³H]pyridoxine (The Radiochemical Centre, Amersham). G/S medium (50 ml) containing 150 ng [G-³H]-pyridoxine ml⁻¹ (specific activity 0.24 Ci mmol⁻¹) and 6.0 mg 4'-deoxypyridoxine ml⁻¹ was inoculated with *E. coli* CR63 to a density of 5×10^7 bacteria ml⁻¹. A control culture without 4'-deoxypyridoxine was similarly prepared. Both cultures were incubated with shaking at 37 °C for 6 h and *A*₅₄₀ was measured at intervals. Uptake of [G-³H]pyridoxine was determined (Scott & Picton, 1976) after collecting and washing the cells on membrane filters (Oxoid 25 mm, 0.45 µm pore size).

Vitamin B6 compounds in cultures after incubation in the presence of 4'-deoxypyridoxine. The 'Oxidase' mutant BL-1 was incubated in 80 ml G/S medium supplemented with 120 ng pyridoxal ml⁻¹ until there were 5×10^8 bacteria ml⁻¹. The organisms were collected by centrifuging and divided equally between 40 ml G/S medium and 40 ml G/S medium containing 2.0 mg 4'-deoxypyridoxine ml⁻¹. After 3 h at 37 °C, the cultures were made 0.05 M with respect to H₂SO₄ and heated at 121 °C for 3 h. This treatment hydrolysed the phosphates of vitamin B6 compounds into the non-phosphorylated derivatives. (The microbiological assay organism, *Kloekera apiculata*, does not respond to phosphorylated vitamin B6 compounds.) Insoluble debris was removed by centrifuging and the supernatant was analysed for vitamin B6 compounds (Scott & Picton, 1976). Direct microbiological assay of the hydrolysed culture was not possible owing to the presence of the inhibitor 4'-deoxypyridoxine, but this was separated from the other vitamin B6 compounds by the column chromatography.

RESULTS

Excretion of vitamin B6. When strain BL-1 was starved for pyridoxal, vitamin B6 (subsequently shown to be entirely pyridoxine) was excreted into the medium at 19 pmol (10⁸ bacteria)⁻¹ during the first hour. Bacteria continued to excrete pyridoxine after 3 h, but the rate of excretion progressively decreased. The total pyridoxine excreted after 3 h was 38 pmol (10⁸ bacteria)⁻¹. The total vitamin B6 measured microbiologically in the culture supernatant could be accounted for by the pyridoxine detected chromatographically. Similar quantities of vitamin B6 were detected after the culture or the effluent from the analytical column had been hydrolysed. Significant quantities of other vitamin B6 compounds were not detected.

Pyridoxine production in supplemented G/S medium. The effect of adding various compounds to the G/S medium on the production of pyridoxine by strain BL-1 is shown in Table 1.

Growth of strain BL-1 and its parent CR63 in the presence of 4'-deoxypyridoxine. The rate of growth and final bacterial density of *E. coli* CR63 decreased when 4'-deoxypyridoxine was added to the G/S medium. In the range 0 to 10 mg 4'-deoxypyridoxine ml⁻¹, the inhibition of growth rate was directly proportional to the concentration of inhibitor; 50% inhibition was achieved with a concentration of 6.0 mg 4'-deoxypyridoxine ml⁻¹. Similar results were obtained when the 'Oxidase' mutant BL-1 was grown in G/S medium supplemented with excess pyridoxal (120 ng ml⁻¹); 50% inhibition was observed at a concentration of 6.7 mg 4'-deoxypyridoxine ml⁻¹. With 6.0 ng pyridoxal ml⁻¹, which is growth-limiting for BL-1, both the growth rate and the final bacterial density were either stimulated or inhibited by 4'-deoxypyridoxine depending on its concentration. The greatest stimulation occurred in response to 1.5 mg 4'-deoxypyridoxine ml⁻¹ (Fig. 1). At approximately 75 µg 4'-deoxypyridoxine ml⁻¹, the effects of growth inhibition and stimulation appeared to be balanced, and growth was the same as in the absence of 4'-deoxypyridoxine. At lower concentrations, growth was inhibited, the maximum inhibition being 50% caused by an inhibitor concentration of 5 µg ml⁻¹.

Table 1. *Effect of various compounds on the excretion of pyridoxine by the E. coli 'Oxidase' mutant BL-1*

Strain BL-1 was grown in G/S medium supplemented with 120 ng pyridoxal ml⁻¹, at 37 °C. Bacteria were harvested during exponential growth (5×10^8 bacteria ml⁻¹) and resuspended in G/S medium supplemented with different compounds as shown. After 3 h at 37 °C the bacterial density and the concentration of pyridoxine excreted into the medium were determined. The results show pyridoxine excretion [pmol (10⁸ bacteria)⁻¹] as a percentage of the total pyridoxine excreted in 3 h by strain BL-1 in the absence of added compounds [38 pmol (10⁸ bacteria)⁻¹]. Unless stated otherwise, each compound was added at 0.4 mM.

Compound added to G/S medium	Pyridoxine excretion (% of control)	Compound added to G/S medium	Pyridoxine excretion (% of control)
L-Alanine	96	Malonate (10 mM)	9
D-Alanine	108	D-Pantothenate	86
β -Alanine	110	L-Phosphoserine (6 μ M)	148
Dihydroxyacetone phosphate	101	L-Phosphoserine	288
D-Fructose	95	Pyruvate	59
Glyceraldehyde	106	DL-Serine (12 μ M)	88
D-Glucose (40 mM)	113	DL-Serine	185
Glycolaldehyde	173	DL-Serine (20 mM)	185
Glycerol	101	D-Xylulose	114
L-Glutamate	114	2'-Hydroxypyridoxine	107
L-Glutamine	93	Glycolaldehyde (0.4 mM) + DL-Serine (0.8 mM)	338
L-Histidine	79		
L-Leucine	149

Fig. 1. Growth of the 'Oxidase' mutant BL-1 in the presence of different concentrations of 4'-deoxyripyridoxine. Bacteria were grown in G/S medium supplemented with 6.0 ng pyridoxal ml⁻¹ and 4'-deoxyripyridoxine at the concentrations shown. Cultures were incubated at 37 °C with shaking. The final density of each culture was expressed as a percentage of the density of a control culture containing no 4'-deoxyripyridoxine. Each culture started at 5.0×10^5 bacteria ml⁻¹, and the final density of the control culture was 2.2×10^8 bacteria ml⁻¹.

Uptake of pyridoxine by strain CR63. The presence of 6.0 mg 4'-deoxyripyridoxine ml⁻¹ abolished the uptake of [G-³H]pyridoxine by strain CR63. In the absence of 4'-deoxyripyridoxine the rate of uptake of pyridoxine was approximately 3 pmol h⁻¹ (10⁶ bacteria)⁻¹ during exponential growth.

Vitamin B6 compounds in cultures of E. coli BL-1 after incubation in the presence of 4'-deoxypyridoxine. Cultures of strain BL-1 (2×10^{10} bacteria in 40 ml) contained 129 ng and 79 ng pyridoxal after incubation with or without 2.0 mg 4'-deoxypyridoxine ml⁻¹, respectively. The extent to which the pyridoxal was free or present as pyridoxal 5'-phosphate is not known because the cultures were hydrolysed before analysis. Each culture also contained large quantities of pyridoxine and traces of pyridoxic acid. Analysis for pyridoxamine was not performed.

DISCUSSION

Although pyridoxine 5'-phosphate is the immediate precursor of pyridoxal 5'-phosphate in *E. coli*, it is not surprising to find that an organism unable to catalyse this conversion excretes pyridoxine. A pyridoxine phosphate oxidase mutant of *E. coli* B was reported to excrete both pyridoxine and pyridoxine 5'-phosphate (Dempsey, 1966). It is assumed that 'Oxidase' mutants of *E. coli* K12 also tend to accumulate metabolically useless pyridoxine 5'-phosphate within the cell. Thus pyridoxine 5'-phosphate may be excreted with partial (*E. coli* B) or total (*E. coli* K12) dephosphorylation.

In the absence of pyridoxal, the 'Oxidase' mutant BL-1 cannot grow and synthesizes pyridoxine at a rate about three times that of the wild-type strain (Hockney & Scott, 1979), possibly due to relief from feedback inhibition by pyridoxal 5'-phosphate. Most of this pyridoxine is excreted, and any compound that stimulates the excretion of pyridoxine probably exerts its effect by stimulating the pathway of vitamin B6 biosynthesis.

A scheme for the biosynthesis of vitamin B6, involving glycolaldehyde, xylulose and 2'-hydroxypyridoxine has been proposed by Schroer & Frieden (1973). This scheme predicts that ¹⁴C from [¹⁴C]glycolaldehyde would be incorporated into vitamin B6 at C-4 and C-4'. Radiotracer experiments have shown, however, that the aldehyde and alcohol carbon atoms of glycolaldehyde are incorporated into C-5 and C-5' respectively (Hill *et al.*, 1975). Data in Table 1 support the role of glycolaldehyde in vitamin B6 biosynthesis but give no support to the role of xylulose or 2'-hydroxypyridoxine. The possibility that the stimulation of pyridoxine synthesis by glycolaldehyde was due to its conversion into serine was eliminated by the demonstration of additivity of stimulation with glycolaldehyde and a saturating concentration of serine. Phosphoserine has been suggested as a precursor of vitamin B6 (Dempsey, 1969) because strains unable to dephosphorylate it synthesize vitamin B6 at an elevated rate; our present results support this suggestion. Since the extracellular alkaline phosphatase of *E. coli* is repressed in G/S medium, phosphoserine was probably transported into the cell without dephosphorylation.

It has been suggested that the growth inhibition of micro-organisms unable to synthesize vitamin B6 by 4'-deoxypyridoxine is due to the formation of a phosphorylated derivative which competitively inhibits the binding of pyridoxal 5'-phosphate to various apoenzymes (Umbreit & Waddell, 1949). Our results extend these findings to include *E. coli* and to an inhibition of pyridoxine transport. The action of 4'-pyridoxine on *E. coli* is more complex than simple inhibition, however, because under certain conditions it can stimulate the growth of vitamin B6 auxotrophs. This stimulatory effect at certain concentrations might be explained as follows: 4'-deoxypyridoxine inhibits growth of strain BL-1, but when pyridoxal is limiting, a mechanism is available to convert the analogue into pyridoxal and pyridoxal 5'-phosphate. Although this mechanism becomes operative at a certain threshold concentration of 4'-deoxypyridoxine, there is a saturation level above which further conversion of 4'-deoxypyridoxine is no longer possible. Interestingly, in *Bacillus subtilis*, 4'-deoxypyridoxine 5'-phosphate is apparently an intermediate in vitamin B6 biosynthesis (Pflug & Lingens, 1978).

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