Synthesis of Pyridoxine by a Pyridoxal Auxotroph of *Escherichia coli*¹

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ABSTRACT

DEMPSEY, WALTER B. (University of Florida, Gainesville). Synthesis of pyridoxine by a pyridoxal auxotroph of *Escherichia coli*. J. Bacteriol. 92:333-337. 1966.—A pyridoxal auxotroph of *Escherichia coli* B produced pyridoxol and pyridoxol 5'-phosphate during starvation for pyridoxal. The identification of these compounds was made both by bioassay and by ion-exchange chromatography. Pyridoxol 5'-phosphate oxidase activity was absent in extracts of the auxotroph. The rate of synthesis of total pyridoxine by a pyridoxal-starved culture of this auxotroph was 6.0×10^{-6} moles per mg per hr. Cellular content of pyridoxine was constant at 4.0×10^{-10} moles/mg.

Escherichia coli B-B₆-2, one of eight types of pyridoxineless mutants described by Dempsey and Pachler (4), has characteristics expected of an organism blocked in the ultimate or penultimate step in pyridoxal 5'-phosphate (PALP) biosynthesis. These are: (i) the mutant grows in the presence of pyridoxal (PAL) but not pyridoxol (POL); and (ii) the mutant, in cross-feeding tests, feeds all of the other pyridoxineless mutants isolated. This mutant has now been studied further in an effort to elucidate the last steps of PALP biosynthesis in *E. coli*.

This report shows that this mutant is apparently a pyridoxine auxotroph because it lacks pyridoxol 5'-phosphate (POLP) oxidase activity. As a consequence of this lack, the organism accumulates POLP. In addition, this report shows that the rate of pyridoxine biosynthesis during starvation of the mutant for PAL increases to four times the rate of pyridoxine biosynthesis found in wild-type *E. coli* B. [Pyridoxine is defined in this report as all of those compounds found in acid-hydrolyzed material which allow growth of *Saccharomyces carlsbergensis* in the bioassay medium of Atkin et al. (1).]

MATERIALS AND METHODS

Organism. The isolation of the pyridoxine auxotroph, *E. coli* B-B₆-2, from *E. coli* B has been described (4). This mutant was unable to use POL to meet its nutritional requirement, but grew well in media sup-

plemented with PAL. Since spontaneous prototrophs from *E. coli* B-B₆-2 occasionally occurred, the purity of all cultures used was tested by examining 800 to 1,000 clones by the replica plating method described previously (4). Only cultures with no revertants by this test were used in these experiments. Amino acid auxotrophs of *E. coli* B-B₆-2 were isolated by a standard penicillin enrichment method after ultraviolet irradiation. (8).

Miscellaneous. Media, growth conditions, measurements of optical density, and dry cell mass were as previously described (3). All pyridoxine was measured by the yeast bioassay of Atkin et al. (1). Details and modifications of this bioassay in analysis of total, cellular, culture fluid, and nonesterified pyridoxine have been described previously (3).

Chromatography. Cell-free culture fluid for chromatography was prepared by centrifuging cultures at $500 \times g$ for 10 min. The supernatant fluid was then passed through a 0.45- μ membrane filter. Chromatography was effected by diluting 3.2-ml samples of cell-free culture fluid with water, adjusting the resultant mixture to pH 4.25 with formic acid, and quantitatively applying the sample to the top of a mixed Dowex 1-Dowex 50 ion-exchange column. Preparation of this column and the method of operating it have been described by Bain and Williams (2). The average recovery of the sample applied was 90 to 100%. Cell extracts for chromatography were prepared by first centrifuging cultures at $1,000 \times g$ for 30 min at 20 C. The centrifuged cells were washed with 30 ml of 0.9% NaCl, suspended in 15 ml of distilled water at 0 C, and triturated in 15 ml of 2 M HClO₄ at 0 C for 3 min. The pH was raised to 4.25 with concentrated KOH while the temperature was maintained below 10 C. The slurry was centrifuged at 0 C for 30 min at $40,000 \times g$. The supernatant solution was chromatographed directly by the above-mentioned procedure.

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Cellular pyridoxine content of *E. coli* B-B₆-2 after PAL starvation. One liter of *E. coli* B-B₆-2 growing exponentially in minimal medium containing PAL at 2×10^{-7} M was centrifuged, and the bacteria were suspended in 2 liters of minimal medium without PAL. This culture was divided into two equal portions. One was immediately centrifuged, and the pellet was extracted with cold 1 M HClO₄, as described above. The total extract was subjected to the chromatographic analysis described above. The other portion was treated in an identical manner after it had shaken at 30 C for 4.5 hr. A similar extract of exponentially growing wild *E. coli* B was made, to test the efficiency of the column.

Assay of POLP oxidase. *E. coli* B-B₆-2 and *E. coli* B wild type were each grown in 1 liter of glycerol minimal medium (3) containing 1 mg of pyridoxal·HCl. Both cultures were centrifuged at $5,000 \times g$ at 15 C for 30 min as they entered stationary phase. The cell pastes were suspended in 35 ml of 0.9% NaCl, stored 12 hr at 5 C, and then centrifuged at $40,000 \times g$ for 30 min at 5 C. The supernatant fluid was discarded, and the paste was suspended in 20 ml of 0.05 M potassium phosphate (pH 8.0). The suspensions were disrupted at 20 kc for 3 min in a 60-w ultrasonic oscillator (Measuring & Scientific Equipment, Ltd., London, England). The debris was removed by centrifugation at $40,000 \times g$ for 30 min at 2 C. The supernatant solution was then used directly in the assay. The assay was made as described (6) at 37 C at pH 8.0 in 0.05 M potassium phosphate (pH 8.0) containing MgSO₄ at 2 mM. POLP was used at 2×10^{-5} M and POL at 2.5×10^{-5} M (6). Control reactions were made by adding trichloroacetic acid to the assay mixture before addition of bacterial extract.

RESULTS

Synthesis of POLP and POL by *E. coli* B-B₆-2. During starvation of *E. coli* B-B₆-2 for PAL, the total pyridoxine in the cultures increased for about 3 hr. In these experiments, the mutant organism, growing exponentially in minimal medium with PAL, was centrifuged and suspended in either identical medium or in a medium without PAL. A typical experiment is shown in Fig. 1. The initial increase in cell mass during the coenzyme starvation, which can be seen in Fig. 1, has been shown by Wilson and Pardee to be a general phenomenon of coenzyme starvation (10).

The distribution of the pyridoxine in the whole culture was measured during the above type of experiment by filtering portions of the cultures through membrane filters and assaying the two resulting fractions separately (Fig. 2). This measurement determined that the bulk of the newly synthesized pyridoxine appeared in the medium just as it did in amino acid starvation (3). Figure 2 also shows that the cellular content of pyridoxine is constant for the PAL-starved culture but increases for the PAL-fed culture.

FIG. 1. Synthesis of pyridoxine by pyridoxal-starved cultures of *Escherichia coli* B-B₆-2. Cultures of *E. coli* B-B₆-2 growing exponentially at 30 C in minimal medium, 6×10^{-7} M in pyridoxal were centrifuged and suspended at time zero in fresh identical minimal medium with or without pyridoxal. Samples were withdrawn every 30 min for analyses. Total pyridoxine was measured by a yeast bioassay after acid hydrolysis of the samples. (A) Total pyridoxine per milliliter of culture, including any pyridoxal initially added to the medium. (B) Optical density of the cultures, measured at 650 mμ.

The latter perhaps occurs by simultaneous accumulation of PAL and de novo synthesis of pyridoxine.

The data in Fig. 2 are normalized to dry cell mass. The rate of synthesis of pyridoxine by the starved mutant can be calculated from the data of Fig. 2B to be 6×10^{-10} moles per mg per hr, or 4.5 times the wild rate of 1.3×10^{-10} moles per mg per hr (3).

This rate of synthesis was confirmed in one of two amino acid auxotrophs derived from *E. coli* B-B₆-2 and starved for both PAL and amino acid at the same time. The experiments were conducted as above with both a tryptophanless and a threonineless mutant (Fig. 3). These observations eliminated the necessity for significant correction for cell density during starvation. The

FIG. 2. Distribution of pyridoxal in cultures of *Escherichia coli* B-B₆-2. Details as in Fig. 1. Cells were removed from the culture by filtration upon 0.47- μ membrane filters. Filters with cells and the filtrate were then hydrolyzed separately, and total pyridoxine was measured in each by a yeast bioassay.

data with the tryptophanless mutant suggested that this amino acid may be a precursor of pyridoxine in *E. coli*. In both of these starvations, all of the newly synthesized pyridoxine was found in the medium.

Identification of POLP and POL. When samples of cell-free culture fluid from PAL-starved cultures were applied to the anionic-cationic exchange resins of the Bain and Williams method (2), 90% of the total pyridoxine was accounted for as POL and POLP. The identification of these compounds was based both upon their elution pattern and the ability of the hydrolyzed, eluted material to feed the bioassay organism, *S. carlsbergensis*. The data from two analyses done upon fluid from the experiment shown in Fig. 1

FIG. 3. Pyridoxine synthesis by two amino acid auxotrophs of *Escherichia coli* B-B₆-2. Cultures of the organisms growing exponentially at 30°C in minimal medium containing 0.1 mg/ml of respective amino acids and 6×10^{-7} M pyridoxal were centrifuged when density reached 0.25 mg of cells/ml, and were suspended at that density in fresh minimal medium. Samples were withdrawn every 30 min, and total pyridoxine was measured. Both cultures grew to 0.28 mg/ml (dry cell weight) during the 3-hr period.

are shown in Fig. 4. Ten analyses done upon culture fluid obtained during other PAL starvations of *E. coli* B-B₆-2 also showed that only POLP and POL were accumulated.

To rule out the possibility that these two compounds accumulate in any starvation of *E. coli*, the cell-free culture fluid of a threonineless *E. coli* culture starved for threonine was also analyzed as above. The predominant form of accumulation in this culture was pyridoxamine 5'-phosphate (PAMP). A control on this control experiment was made by simultaneously starving a threonineless derivative of *E. coli* B-B₆-2 for both PAL and threonine (Fig. 4). The principal compounds accumulated in this starvation were POLP and POL.

Cellular extracts of PAL-starved *E. coli* B-B₆-2 contained principally POLP. These extracts were prepared by triturating centrifuged, starved cells in cold perchloric acid. The entire neutralized extracts were analyzed by the same chromatographic procedure described above. The results for *E. coli* B-B₆-2 starved for 4.5 hr are shown in Table 1.

Inability of extracts of *E. coli* B-B₆-2 to convert POLP to PALP. The identification of POLP as the pyridoxine compound accumulated by *E. coli* B-B₆-2, together with the requirement of the mutant for PAL, suggested that the defect in the mutant was an inability to oxidize the 4-methylol substituent of either POL or POLP to a 4-formyl substituent. Wild-type *E. coli* B and *E. coli* B-B₆-2

FIG. 4. Identification of pyridoxol and pyridoxol 5'-phosphate. Samples of cell-free culture fluid were applied to a Dowex 1-Dowex 50 ion-exchange chromatographic column and eluted in fractions of 10 ml. Phosphate esters (tubes 1 through 35) were acid-hydrolyzed before being analyzed for pyridoxine by yeast bioassay. The standards shown represent the elution patterns of the principal component of the best commercially available pyridoxine compounds when 0.6 to 1.2 μ moles of each compound was applied to the column. In each part of this figure, 1 refers to the duration of starvation for the particular metabolite, 2 to the cell mass at the time of analysis, and 3 to the pyridoxine content of the cell-free culture fluid at the time of analysis.

were both grown in minimal medium, 6×10^{-6} M in PAL, and were then extracted and assayed for their ability to convert POLP to PALP. The general conditions adopted for the incubation were those used by Henderson (6). The results of these tests showed that extracts of wild *E. coli* converted POLP to PALP at 1.3 nmols/mg of protein in 15 min. This conversion could not be shown with extracts of the mutant. The oxidation of POL to PAL could not be shown with extracts of either organism.

DISCUSSION

The data presented above show that POLP and POL are synthesized in a mutant of *E. coli* which cannot oxidize the former compound to PALP. In addition, the data show that the organism grows well in a minimal medium supplemented with PAL. These facts can be reconciled with the biosynthetic pathway proposed below.

TABLE 1. Cellular pyridoxine content of *Escherichia coli* B-B₆-2 after pyridoxal starvation

Analysis of	Total nmols of pyridoxine ($\times 10^{-1}$)		
	<i>E. coli</i> B-B ₆ -2 without pyridoxal		<i>E. coli</i> B wild type, logarithmic
	0 hr	4.5 hr	
Whole culture.....	NT ^a	105	NT
Culture fluid.....	NT	87	NT
Cells.....	10	18	NT
Cell extract.....	10	18	NT
Total applied to column.....	9	17	8
Total recovered from column as pyridoxamine 5'-phosphate.....	7	16	8
as pyridoxol 5'-phosphate ..	2	1	3
as pyridoxal 5'-phosphate ..	4	14	0.5
as nonesters.....	1	1	5
	NT	0.2	NT

^a NT, no test.

Common metabolite $\rightarrow \dots \rightarrow$

Although rigorous definition of the pathway clearly awaits careful studies with purified enzymes, this particular sequence is considered to be more probable than others because it is consistent both with the above data and with the meager available information about the activities of related enzymes in *E. coli*. This information is: (i) A positive demonstration of POLP oxidase activity in wild-type *E. coli* (6). This activity was confirmed in wild *E. coli* above. (ii) A striking ability of *E. coli* extracts at pH 7.5 to phosphorylate 10^{-6} M POL to POLP rapidly and efficiently. Under the same conditions, PAL remains unphosphorylated (Kenny and Dempsey, *in preparation*). (iii) A general lack of any demonstrable POL oxidase activity in microorganisms (5). This was supported by one experiment above. (iv) A positive demonstration of PAL kinase activity in *E. coli* (7). The identify of this enzyme with the activity in *E. coli* extracts which catalyzes the phosphorylation of POL to POLP has yet to be shown.

By this scheme, the block in *E. coli* B-B₆-2 is at point A. This block then prevents the de novo synthesis of PALP, but allows the accumulation of POLP and POL. PAL presumably allows the organism to grow, because the organism is still competent at converting PAL to PALP.

The fourfold increased rate of pyridoxine biosynthesis in newly PAL-starved *E. coli* B-B₆-2

is thought to represent the effect of the removal of all or part of the feedback inhibition normally caused by PALP on the apparently constitutive pyridoxine synthetic system (3). In the absence of a repression type of control mechanism, this may be the maximal rate of synthesis possible in this organism.

The eventual stoppage of pyridoxine biosynthesis in cultures of the PAL-starved mutants may be explainable by the massive buildup of POLP in the cell (Table 1). Assuming that PALP is the normal effector of control, then it is reasonable to assume further that POLP at high enough concentrations might also be able to inhibit further synthesis. According to this hypothesis, anything which decreased the intracellular POLP should allow synthesis of pyridoxine to resume. This is currently being tested.

Continued synthesis of pyridoxine by the PAL-fed mutant (Fig. 1 and 2) is thought to arise from the inability of the cell to build the PALP pool high enough to satisfy enzyme needs and to stop the synthesis of pyridoxine. This may be because the cells cannot bring in enough PAL, or perhaps because the blocked enzyme might also normally participate in maintaining the cellular PALP pool by oxidizing back to PALP any PAMP which has dissociated from transaminases (9).

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