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APPARENT PYRIDOXINE TRANSPORT MUTANTS OF *ESCHERICHIA COLI* WITH PYRIDOXAL KINASE DEFICIENCY

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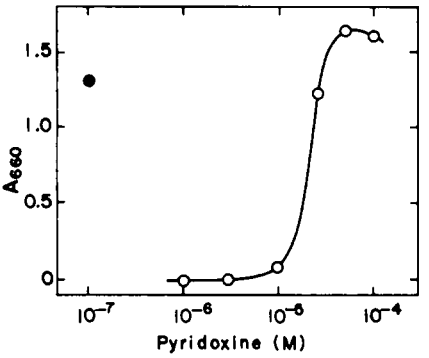
Summary

By nitrosoguanidine treatment of a vitamin B-6 auxotroph (KG980) of *Escherichia coli*, mutants were isolated that require for growth markedly higher concentrations of pyridoxine than the parent strain. One of the mutants, strain HN1, exhibited a severely reduced ability to take up extracellular pyridoxine. Besides, cell-free extracts of HN1 showed an extremely low activity to phosphorylate pyridoxine compared to that of KG980. These findings together with other results suggest that phosphorylation of pyridoxine is essential for the concentrative uptake of the vitamin.

It has been shown that *Escherichia coli* accumulates extracellular pyridoxine by an energy- and temperature-dependent process [1]. However, little is known about the mechanism of pyridoxine uptake, except that most of the intracellular vitamin accumulated is present as pyridoxal phosphate and pyridoxamine phosphate [2] and also the uptake does not display typical saturation kinetics [3]. It remains unclear whether the intracellular metabolism of pyridoxine is essential for the concentrative uptake of the vitamin.

For use in studies of the uptake mechanism we attempted to obtain pyridoxine transport mutants of *E. coli*, since mutants with altered transport properties proved to be quite useful in the clarification of various bacterial transport systems. We utilized a vitamin B-6 auxotroph, strain KG980 [1], derived from wild strain K12 as the parent strain, treated this auxotroph with nitrosoguanidine and penicillin according to the procedure for the isolation of vitamin B-12 transport mutants [4] with minor modifications suitable to obtain mutants which would not grow on 0.1 μM pyridoxine but require at



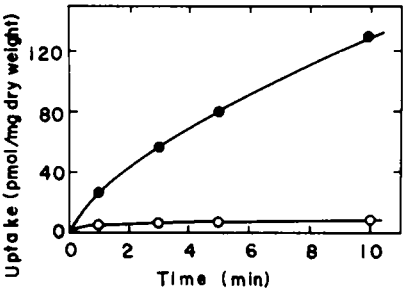


Fig. 1. Effects of pyridoxine concentrations on the growth of HN1 and KG980. Cells were grown aerobically at 37°C for 20 h after inoculation on the minimal medium of Davis and Mindogioli [5] supplemented with 0.2% glucose and various concentrations of pyridoxine. Growth was measured turbidimetrically at 660 nm. ○—○, HN1; ●, KG980. One experiment typical of three is shown.

Fig. 2. Time course of [G-^{3}H]pyridoxine uptake by HN1 and KG980 cells. Experiments were conducted as previously described [3] at 37°C, aerobically with 1 μM [G-^{3}H]pyridoxine added to cell suspensions (1 mg dry wt./ml). ○—○, HN1; ●—●, KG980. One experiment typical of five is shown.

least 10 μM concentration of the vitamin for growth, and finally isolated five mutants with the expected properties.

Fig. 1 shows the growth response of one of the mutants, strain HN1, to various concentrations of pyridoxine. The mutant grew only a little with 10 μM pyridoxine and required 50 μM of the vitamin for maximal growth, whereas the parent strain KG980 grew well with 0.1 μM pyridoxine. Thus, pyridoxine requirement for growth of HN1 was greater than 100-fold that of KG980.

The ability of HN1 cells grown with 50 μM pyridoxine to take up [G-^{3}H]pyridoxine was examined in comparison with that of KG980 grown with 0.1 μM vitamin. Fig. 2 illustrates the time course of uptake when the extracellular labeled pyridoxine concentration was 1 μM. The results demonstrated that HN1 cells had an extremely reduced ability of pyridoxine uptake as expected from the enhanced pyridoxine requirement for growth described above. It may be further noted that the ratio of the uptake level in HN1 to that in KG980 decreased with longer incubation periods. The finding suggested that the defect might not be in the initial process of uptake across the cell membrane, but in some later processes of intracellular metabolism.

To obtain more information on the defective process, intracellular labeled compounds were analyzed after HN1 and KG980 cells were allowed to take up the labeled pyridoxine for 2 min. As shown in Table I, [G-^{3}H]-pyridoxine content was higher in HN1 cells than in KG980, whereas the content of phosphorylated forms of the vitamin was enormously greater in KG980. The results indicate that the entry of pyridoxine is unlikely to be impaired, but that the defect is likely to be in the conversion of intracellular pyridoxine to phosphate forms of the vitamin.

Of the three phosphate forms of vitamin B-6, pyridoxal phosphate is the main species accumulated when KG980 cells are exposed to a solution of pyridoxine for a short period [1]. It is also established that the conversion of pyridoxine to pyridoxal phosphate in *E. coli* involves the formation of

TABLE I

INTRACELLULAR FORMS OF [G-^{3}H] VITAMIN B-6

1-ml aliquots of cell suspensions (3 mg dry wt./ml) of HN1 or KG980 were filtered after 2-min incubation with 1 μM [G-^{3}H]pyridoxine (50 Ci/mol) and the intracellular labeled compounds were extracted as previously described [2]. The extracts, after pH adjustment to 4.7, were applied to a column (0.6 × 3 cm) of Dowex 50W X-4 equilibrated with 10 mM sodium acetate buffer (pH 5.5), and the column was washed with the same buffer (20 ml), 50 mM sodium acetate buffer, pH 6.0 (10 ml), and 100 mM sodium phosphate buffer, pH 7.0 (10 ml), successively to elute vitamin B-6 phosphates, pyridoxal and pyridoxine, respectively. Radioactivity associated with unlabeled vitamin B-6 phosphates and pyridoxine, which were cochromatographed and detected spectrophotometrically, was counted. Values are the means from two experiments.

Strain	Vitamin B-6 (pmol/mg dry wt.)		
	Pyridoxine	B-6 phosphates	Total
HN1	1.7	2.7	4.5
KG980	0.8	46.1	48.6

pyridoxine phosphate as an intermediate [6]. Accordingly, if the production of vitamin B-6 phosphates is blocked in HN1, the defect is probably at the step of phosphorylation of pyridoxine or oxidation of pyridoxine phosphate. Activities of pyridoxal kinase (EC 2.7.1.35) and pyridoxaminephosphate oxidase (pyridoxinephosphate oxidase, EC 1.4.3.5) in the cell-free extracts of HN1 were, therefore, examined in comparison with those of KG980 with the use of pyridoxine and pyridoxine phosphate, respectively, as a substrate for each of the enzymes. The data given in Table II indicate that pyridoxal kinase activity of HN1 is severely reduced. It was less than 1% of that of KG980, although it was still significant and reproducible. On the other hand, there was no difference in the pyridoxinephosphate oxidase activity between the two strains. Thus, it is clear that HN1 is deficient in the phosphorylation of pyridoxine and the HN1 cells apparently failed to accumulate vitamin

TABLE II

PYRIDOXAL KINASE AND PYRIDOXINEPHOSPHATE OXIDASE ACTIVITY

The reaction mixture for the assay of pyridoxal kinase contained the dialyzed cell-free extract of either HN1 or KG980 cells (0.2—1 mg protein), 5 nmol of [G-^{3}H]pyridoxine, 0.5 μmol of ATP, 0.25 μmol of $ZnSO_4$ and 5 μmol of potassium phosphate buffer (pH 6.0) in a total volume of 0.5 ml. After 15-min incubation at 37°C, the mixture was boiled for 5 min, its pH was adjusted to 4.25 with acetic acid and the precipitate formed was removed by centrifugation. The supernatant was applied to a Dowex 50W X-4 column (0.6 × 3 cm) equilibrated with 0.05 M sodium acetate buffer, pH 4.25, and the column was washed with 2.5 ml of the same buffer. The eluate, which had been confirmed to contain more than 95% of pyridoxine phosphate formed but no pyridoxine, was counted. The reaction mixture for the assay of pyridoxinephosphate oxidase was similar to that used by Henderson [6] and contained the cell-free extract (3—6 mg protein), 90 nmol of pyridoxine phosphate, 6 μmol of $MgSO_4$ and 0.15 nmol of sodium carbonate buffer, pH 10, in a total volume of 3 ml. After incubation for 1 h at 37°C, pyridoxal phosphate formed was determined by using the method of Adams [7]. Values are the means from three experiments.

Strain	Activity (pmol/min per mg protein)	
	Pyridoxal kinase	Pyridoxinephosphate oxidase
HN1	4.97	31.7
KG980	630	29.0

B-6 phosphates because of this deficiency. The markedly reduced uptake of pyridoxine in the mutant seems to demonstrate the essential role of phosphorylation for the concentrative uptake of the vitamin similarly as shown in pyridoxine uptake in *Salmonella typhimurium* [8], although the unlikely possibility that the entry of pyridoxine is also impaired in HN1 still exists.

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