Role of Pyridoxal Kinase in Vitamin B₆ Uptake by *Escherichia coli*

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(Received November 4, 1980)

Summary *Escherichia coli* KG980, a vitamin B₆ auxotroph derived from wild strain K12, concentrated exogenous pyridoxal in an energy-dependent manner, and the effects of energy sources and inhibitors on pyridoxal uptake, compared with those on proline uptake indicated that the energy required was in the form of phosphate bonds and not of membrane potential. The vitamin taken up was primarily present as pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate intracellularly, and energy depletion decreased the accumulation as the phosphorylated derivatives but not as unaltered pyridoxal itself. This finding suggested that the intracellular phosphorylation, which was known to require ATP, was essential for the concentrative uptake of the vitamin. The suggestion was confirmed by the following evidence. 1) Pyridoxal oxime inhibited pyridoxal uptake by decreasing the intracellular phosphorylation without affecting the entry of pyridoxal across the cell membrane. 2) A pyridoxal-kinase deficient mutant (HN1) derived from the strain KG980 showed a low ability to take up pyridoxal because of the failure to accumulate it effectively as phosphorylated derivatives. The carrier-mediated nature of pyridoxal uptake, previously suggested by saturation kinetics, was further supported by the present finding that 4'-deoxypyridoxine inhibited pyridoxal uptake competitively, decreasing the intracellular appearance of unmetabolized pyridoxal. It is therefore most likely that pyridoxal enters the cells by facilitated diffusion and is accumulated by conversion to phosphorylated derivatives. Similar results on the uptake of pyridoxine and pyridoxamine are also presented.

Key Words vitamin B₆ uptake, pyridoxal uptake, pyridoxal kinase, inhibitors of pyridoxal kinase, pyridoxal kinase deficiency, *E. coli*

The uptake systems for vitamin B₆ in several different microorganisms appear to be quite different in substrate specificity and mechanism of transport. *Saccharomyces carlsbergensis* has two active transport systems for pyridoxine,

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pyridoxal and pyridoxamine, but not for their phosphorylated derivatives (1). In contrast, *Lactobacillus delbrueckii* accumulates pyridoxal 5'-phosphate, pyridoxamine 5'-phosphate and pyridoxine 5'-phosphate apparently by active transport, but not nonphosphorylated forms of the vitamin (2). *Salmonella typhimurium* (3) and a few lactic acid bacteria (2) concentrate one or two of the nonphosphorylated forms of vitamin B₆. It has been proposed that the vitamin enters the cells of these bacteria *via* a facilitated diffusion mechanism and is then trapped through the action of pyridoxal kinase (3).

Escherichia coli takes up concentratively all three forms of nonphosphorylated vitamin B₆ but does not the phosphorylated forms (4). Thus the bacterium is similar to *S. carlsbergensis* in substrate specificity. However, it is unknown whether or not *E. coli* also has active transport systems for the vitamin. Limited information available on the transport mechanism includes that pyridoxine taken up by *E. coli* cells is mostly present as pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate (5), and that a mutant recently isolated deficient in the phosphorylation of pyridoxine has a very low pyridoxine uptake ability (6). These findings do not support an active transport mechanism but rather suggest that intracellular phosphorylation is essential for the accumulation of the vitamin as proposed in the case of *S. typhimurium*.

In the present work we have attempted to obtain conclusive evidence for the role of pyridoxal kinase in the uptake of vitamin B₆. We have chosen the uptake of pyridoxal for the study rather than that of pyridoxine, since pyridoxal uptake displays typical saturation kinetics (4), suggesting the carrier-mediated nature of the system, while pyridoxine uptake is kinetically more complicated (4) and therefore appears less suited to our purpose. The experimental results reported herein indicate that pyridoxal uptake is energy dependent at the step of intracellular phosphorylation and that the inhibition of pyridoxal kinase with suitable inhibitors or deficiency of the enzyme abolishes the accumulation of the vitamin. Some of the results also added evidence on the carrier-mediated nature of pyridoxal uptake.

MATERIALS AND METHODS

Chemicals. Pyridoxal oxime and *N,N'*-bispyridoxylhydrazine were prepared by the procedure described by McCormick and Snell (7). 5'-Deoxypyridoxal was prepared according to the method of Iwata (8), with a modification that 5'-deoxypyridoxamine was used in place of 5'-deoxypyridoxine as the starting material. The crystallized product was identified on the basis of the melting point (107–109°C) and the absorption spectra at various pH. 5'-Deoxypyridoxamine was prepared from pyridoxamine by the method of Kuroda (9). 5'-Deoxypyridoxine was prepared by the reduction of 5'-deoxypyridoxal with NaBH₄ and purification by chromatography on Dowex 50W-X4 using sodium acetate buffers of increasing pH (4.25–7.5) and concentration (0.1–0.5 M) for elution. The product was freed from the buffer components by chromatography on Dowex 1-X4; 5'-deoxypyridoxine was

bound to the resin at pH 10, and after washing with water, it was eluted with 0.1 M acetic acid, then concentrated under reduced pressure at 35°C. The other non-radioactive chemicals including vitamin B₆ compounds and their analogs were obtained from commercial sources.

The preparation of [³H]pyridoxine, [³H]pyridoxal and [³H]pyridoxamine has been described previously (10–12). In addition, [³H]pyridoxal of higher specific radioactivity was prepared as follows: [³H]pyridoxal 5'-phosphate was prepared and purified as previously described (13). The product was then dephosphorylated by incubation with bovine mucosal alkaline phosphatase (Sigma), and [³H]pyridoxal thus formed was purified as described above for the purification of 5'-deoxypyridoxine. A representative preparation of [³H]pyridoxal thus obtained had a specific radioactivity of 252 dpm/pmol. [³H]5'-Deoxypyridoxine was prepared by the reduction of 5'-deoxypyridoxal with [³H]NaBH₄ (Radiochemical Centre, Amersham) and the product was purified as in the purification of unlabeled 5'-deoxypyridoxine described above. [³H]5'-Deoxypyridoxal was prepared by oxidation of [³H]5'-deoxypyridoxine according to the method of Iwata (8). The product was purified by thin layer chromatography on Silica Gel 60_{F254} (Merck) with a mixture of *n*-butyl alcohol, acetic acid and water (3:1:1, v/v) as solvent, and then extracted with chloroform. The preparations of [³H]5'-deoxypyridoxine and [³H]5'-deoxypyridoxal were appropriately diluted with unlabeled compounds to give specific radioactivities of 73.6 and 57.6 dpm/pmol, respectively.

Cell growth. *E. coli* KG980 was grown as previously described (4) on the minimal medium of Davis and Mingioli (14) containing 0.2% glucose supplemented with 0.1 μM pyridoxine and was harvested when the optical density at 660 nm was about 1.0. The mutant strain HN1 was grown in the same way except that the medium was supplemented with 0.1 μM pyridoxal in place of pyridoxine.

Starvation of cells. According to the method of Berger and Heppel (15), cells suspended in the minimal medium were incubated with 5 mM 2,4-dinitrophenol for 15 hr.

Assay of uptake. Uptake experiments were usually conducted as previously described (4) using cell suspensions (about 1 mg dry weight/ml) in the minimal medium containing 0.4% glucose. The previously employed method of filtration through two membrane filters, one placed on the other (4), was also used, to obtain the control value from the radioactivity of the lower filter. For particular experiments using the starved cells, the cells treated with 2,4-dinitrophenol were washed three times with 0.9% NaCl before suspension in the minimal medium containing 0.4% glucose; when the effects of arsenate were examined, the cells were washed with 25 mM Tris-HCl (pH 7.5) and were suspended in Medium B of Berger and Heppel (15). For experiments to determine the form of intracellular labeled vitamin B₆, cells (1.5–2 mg) in 0.5 ml of the medium were incubated with [³H]pyridoxal of higher specific radioactivity described above, then filtered and washed with 10 ml of the minimal medium. The upper filter was immediately dipped into 10 ml of ice-cold 2% HClO₄ and, after standing for 30 min and centrifugation,

the supernatant was analyzed for the labeled compounds. The lower filter was treated in the same way to give control values for the compounds determined.

Analysis of the forms of labeled vitamin B₆. Vitamin B₆ compounds were separated on a column of Dowex 50W-X4 (0.5×40 cm) and radioactivity was determined as previously described (4). A simplified method with a smaller column (0.6×3 cm) (6) was also employed to assay the amount of [³H]pyridoxal and the total amount of phosphorylated derivatives. The total of labeled vitamin B₆ compounds was usually determined from the radioactivity of samples before chromatography.

RESULTS

Energy dependence of uptake and metabolism of pyridoxal

Although pyridoxine uptake has been shown to be energy dependent (16), this cannot be simply applied to pyridoxal uptake, since different forms of vitamin B₆ may be taken up by different systems existing in several microorganisms (1, 2). In addition, pyridoxine uptake in *S. typhimurium* (3) is reported neither to be stimulated by added glucose nor inhibited by various metabolic inhibitors. Therefore, experiments were conducted to establish clearly the energy dependence of pyridoxal uptake. Attempts were also made to determine whether the form of energy required is a membrane potential or high-energy phosphate bond. As shown in Fig. 1A, pyridoxal uptake by starved cells was markedly stimulated by exogenously added glucose or D-lactate, a substrate of respiratory oxidation, indicating that both compounds were effectively utilized as energy sources. Addition

Fig. 1. Effects of added energy sources and arsenate on uptake of pyridoxal (A) and proline (B) by starved cells of *E. coli* KG980. Starved cells in Medium B (15) were incubated at 37°C for 15 min with, 10 mM glucose (○), 10 mM glucose plus 0.5 mM sodium arsenate (●), 20 mM lithium D-lactate (△), 20 mM lithium D-lactate plus 0.5 mM sodium arsenate (▲), or none (□) before the addition of [³H]pyridoxal (1 μM) or [³H]proline (10 μM).

of arsenate, which causes a depletion of intracellular ATP levels in *E. coli* cells (15), clearly inhibited the uptake when either of the energy sources was used. For comparison, uptake of proline was examined, since this amino acid has been reported to be actively transported by some strains of *E. coli*, depending on energy in the form of membrane potential (17). Proline was effectively taken up with either of the added energy sources, but the uptake was not inhibited by arsenate, which appeared rather stimulatory when glucose was the energy source (Fig. 1B). This finding suggests that the form of energy required for pyridoxal uptake is not the membrane potential as in the case of proline uptake but some phosphate-bond energy as in ATP. The suggestion was supported by the effects of anaerobiosis and azide on the glucose-dependent uptake of pyridoxal and proline. Pyridoxal uptake during the initial 2 min in a nitrogen atmosphere or in the presence of NaN₃ (30 mM) was not significantly affected, and was 99% or 88% of that of the control, respectively, whereas proline uptake was markedly reduced to 63% under the anaerobic conditions and was more severely inhibited by NaN₃ to 4% of the control. The insensitivity of pyridoxal uptake to the respiratory inhibition thus shown is consistent with the dependence on phosphate-bond energy suggested above, since ATP can be generated by glycolysis. In fact, pyridoxal uptake was markedly inhibited to 30%, when a glycolytic inhibitor, NaF (30 mM), was present in addition to NaN₃ (Fig. 6).

Energy in the form of phosphate bond required for pyridoxal uptake may be used at the process of transport across the cell membrane as in the case of glutamine uptake (16) or at the step of intracellular phosphorylation to pyridoxal 5'-phosphate as proposed in *S. typhimurium*. In order to obtain information on this point, the fate of pyridoxal taken up by cells was examined. Figure 2 gives a typical

Fig. 2. Dowex 50W-X4 column chromatography of [³H]compounds extracted from *E. coli* KG980 cells incubated with 1 μ M [³H]pyridoxal for 3 min. Experiments were conducted as described under MATERIALS AND METHODS using cells in the minimal medium containing 0.4% glucose. Arrows show the positions of unlabeled vitamin B₆ compounds cochromatographed and detected spectrophotometrically.

example of analysis of intracellular labeled vitamin B₆ compounds after uptake of [³H]pyridoxal for a short period. The major components were obviously pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate (Fig. 2). Unphosphorylated compounds found were small amounts of pyridoxal and pyridoxamine. Pyridoxal content was estimated to be 3.5 pmol/mg dry cells. Assuming that 1 g dry weight of *E. coli* cells is equivalent to 2.73 ml of cell water as shown by Winkler and

Table 1. Effects of starvation and glucose refeeding on the intracellular [³H]vitamin B₆ compounds in *E. coli* KG980 after uptake of [³H]pyridoxal.

Cells	Intracellular [³ H]vitamin B ₆ ^a (pmol/mg dry weight)		
	Pyridoxal	B ₆ phosphates	Total
Starved	3.3	13.4	24.6
Glucose refed	3.7	87.9	91.6

^a Starved cells were incubated for 15 min in the minimal medium with or without 0.4% glucose before the addition of [³H]pyridoxal (1 μM). After 2 min, intracellular [³H]-compounds were extracted and analyzed by the simplified method of chromatography described in MATERIALS AND METHODS.

Table 2. Effects of possible inhibitors for pyridoxal kinase on the phosphorylation of pyridoxal, pyridoxine and pyridoxamine by cell extract of *E. coli* KG980.^a

Inhibitor	Relative phosphorylation ^b (%)		
	Pyridoxal	Pyridoxine	Pyridoxamine
None	100	100	100
Pyridoxal oxime (20 μM)	20	<1	<1
4'-Deoxypyridoxine (25 μM)	58	51	— ^d
<i>N,N'</i> -Bispyridoxylhydrazine (25 μM)	65	46	—
Toxopyrimidine ^c (25 μM)	108	98	—
Sodium arsenite (25 mM)	87	106	—
5'-Deoxypyridoxal (100 μM)	45	32	—
5'-Deoxypyridoxine (100 μM)	40	77	—

^a Assay of phosphorylation of pyridoxine was conducted with dialyzed cell-free extract and [³H]pyridoxine as previously described (6). Phosphorylation of pyridoxal and pyridoxamine was assayed similarly using ³H-labeled substrates except that 0.05 M sodium phosphate buffer, pH 7 was used for chromatography to separate [³H]-pyridoxamine 5'-phosphate from [³H]pyridoxamine. ^b Control values for phosphorylation of 10 μM pyridoxal, pyridoxine and pyridoxamine were 55.4, 742 and 111 pmol/min/mg protein, respectively. Apparent *K_m* values for phosphorylation of pyridoxal, pyridoxine and pyridoxamine were determined to be 29, 1.3 and 118 μM, respectively. ^c 2-Methyl-4-amino-5-hydroxymethylpyrimidine. ^d Not examined.

Wilson (18), the intracellular pyridoxal concentration was calculated to be 1.28 μM , which was nearly equal to the extracellular pyridoxal concentration of 1 μM , but also a little higher than the latter. The fate of pyridoxal taken up was further examined with starved cells to clarify the effects of glucose added. The data shown in Table 1 indicate that the starvation markedly decreased the intracellular phosphorylated vitamin B₆ content but not the pyridoxal content.

From these results it appears likely that energy for pyridoxal uptake is required at the step of intracellular phosphorylation of the vitamin but not of its entry across the cell membrane. However, it is still possible that energy is required for both steps, and that pyridoxal is actively transported and further rapidly phosphorylated.

Effects of inhibitors of pyridoxal kinase on uptake and metabolism of pyridoxal

In an attempt to clarify the significance of intracellular phosphorylation in the uptake of pyridoxal, effects of inhibitors of pyridoxal kinase were examined. To select effective inhibitors, several compounds were first tested for the inhibitory effect on the enzyme in cell extracts of *E. coli* KG980. As shown in Table 2, pyridoxal oxime was the most potent inhibitor among the compounds tested. *N,N'*-Bispyridoxylhydrazine and 4'-deoxypyridoxine were also strongly inhibitory, whereas sodium arsenite which has been shown to inhibit pyridoxal kinase of *S. typhimurium* (3) was not effective in the present experiment. When the effect of these compounds on pyridoxal uptake was examined, pyridoxal oxime was found to inhibit the uptake most strongly. *N,N'*-Bispyridoxylhydrazine and 4'-deoxypyridoxine were also effective inhibitors (Fig. 3). 5'-Deoxypyridoxal was inhibitory for a short initial period only when this analog was added to the uptake medium

Fig. 3. Effects of some pyridoxal kinase inhibitors on pyridoxal uptake by *E. coli* KG980. Cells in the minimal medium containing 0.4% glucose were incubated for 15 min with, 20 μM pyridoxal oxime (●), 50 μM 4'-deoxypyridoxine (△), 25 μM *N,N'*-bispyridoxylhydrazine (□), or none (○), before the addition of [³H]pyridoxal (1 μM).

simultaneously with pyridoxal (Fig. 6B), but did not affect pyridoxal uptake when added previously. The other compounds in Table 2 exhibited only weak or insignificant inhibition. The effect of pyridoxal oxime on the uptake of pyridoxine and pyridoxamine was also examined, and strong inhibition was observed similarly to the case of pyridoxal uptake.

Although the marked inhibition by pyridoxal oxime of both phosphorylation and uptake of pyridoxal supports the importance of phosphorylation in the uptake process, it remains possible that the observed reduction of uptake was mainly due to the inhibition of the entry of the vitamin across the cell membrane by competition for the probable carrier. To check this possibility two experiments were conducted. In the first experiment, cells were first preloaded with pyridoxal oxime by incubation with the inhibitor as in Fig. 3, then, after filtration and washing by the usual method, the cells were allowed to take up labeled pyridoxal by immediate dipping of the filters with the cells into the medium containing the vitamin at $1\ \mu\text{M}$. The uptake during 2 min and 5 min of incubation was found to be about 60% of that of control cells which were preincubated without pyridoxal oxime. The results indicate that uptake is inhibited without the extracellular presence of excess inhibitor. In the second experiment, intracellular labeled pyridoxal and phosphorylated forms of the vitamin were determined after uptake of labeled pyridoxal in the presence of various concentrations of pyridoxal oxime. The results given in Fig. 4 indicate that

Fig. 4. Effects of various concentrations of pyridoxal oxime on the intracellular content of $[^3\text{H}]$ vitamin B_6 phosphates and $[^3\text{H}]$ pyridoxal in *E. coli* KG980 cells after 2-min incubation with $1\ \mu\text{M}$ $[^3\text{H}]$ pyridoxal. Cells in the minimal medium containing 0.4% glucose were incubated for 15 min with various concentrations of pyridoxal oxime before the addition of $[^3\text{H}]$ pyridoxal ($1\ \mu\text{M}$). Extraction and analysis of $[^3\text{H}]$ compounds in the cells were performed as described in MATERIALS AND METHODS using the simplified method of chromatography.

Table 3. Effects of 4'-deoxypyridoxine or pyridoxal kinase deficiency on the intracellular [³H]vitamin B₆ after uptake of [³H]pyridoxal.

Strain	Inhibitor	Intracellular [³ H]vitamin B ₆ ^a (pmol/mg dry weight)		
		Pyridoxal	B ₆ phosphates	Total
KG980	None	3.2	41.1	44.5
KG980	4'-Deoxypyridoxine (50 μM)	1.3	15.2	17.7
HN1	None	3.1	17.9	21.8

^a Cells were incubated for 15 min in the minimal medium containing 0.4% glucose with or without added 4'-deoxypyridoxine before addition of [³H]pyridoxal (1 μM). After 2 min, intracellular [³H] compounds were extracted and analyzed by the simplified method of chromatography as described in MATERIALS AND METHODS.

Fig. 5. Kinetics of inhibition of pyridoxal uptake by 4'-deoxypyridoxine and pyridoxal oxime. Various concentrations of [³H]pyridoxal together with 50 μM 4'-deoxypyridoxine (△), 20 μM pyridoxal oxime (□), or none (○) were added to suspensions of *E. coli* KG980 which had been preincubated for 15 min in the minimal medium containing 0.4% glucose. Uptake was measured after 1-min incubation.

the phosphorylated vitamin decreased with the increase of inhibitor concentration, whereas the content of pyridoxal was relatively constant at around 3 pmol/mg dry weight. This finding shows that intracellular pyridoxal concentration was almost the same as the extracellular one at various concentrations of pyridoxal oxime added. Accordingly, it seems certain that pyridoxal oxime did not significantly prevent pyridoxal from entering the cells but interfered with the accumulation of the vitamin *via* the inhibition of pyridoxal kinase.

Among the other inhibitors tested, 4'-deoxypyridoxine seemed to differ from pyridoxal oxime in the manner of inhibition, since the intracellular content of

pyridoxal itself, as well as that of phosphorylated derivatives, was clearly reduced by the addition of this analog (Table 3). This finding indicates that the entry of pyridoxal across the cell membrane is inhibited by 4'-deoxypyridoxine, and supports the idea of the presence of a specific membrane carrier which has been suggested by saturation kinetics (4). In agreement with this finding, kinetic experiments showed that 4'-deoxypyridoxine inhibited pyridoxal uptake competitively (Fig. 5), while pyridoxal oxime appeared to act differently.

Uptake of 5'-deoxypyridoxal and 5'-deoxypyridoxine

To obtain further information on the participation of pyridoxal kinase, uptake of pyridoxal analogs which lack the 5'-OH group to be phosphorylated was examined. No significant uptake of 5'-deoxypyridoxine was observed, as illustrated in Fig. 6A. This finding suggests that this analog may be unable to enter the cells. On the other hand, a considerable amount of 5'-deoxypyridoxal was taken up during the short initial period. This uptake differed from pyridoxal uptake in that it was not affected by the conditions which markedly inhibited the uptake of the vitamin. As shown in Fig. 6, the uptake of 5'-deoxypyridoxal was not reduced by the presence of NaN_3 and NaF in the absence of glucose, or by the pretreatment of cells with HgCl_2 , whereas pyridoxal uptake was obviously lowered under these conditions.

Fig. 6. Uptake of 5'-deoxy-pyridoxal and -pyridoxine (A) compared with that of pyridoxal (B) under various conditions. Cells were incubated for 15 min in the minimal medium containing 0.4% glucose before the addition of [^3H]5'-deoxypyridoxal ($\circ$ in A), [^3H]5'-deoxypyridoxine ($\bullet$ in A) or [^3H]pyridoxal ($\circ$ in B) at $1\ \mu\text{M}$ concentration, and also before the addition of $10\ \mu\text{M}$ 5'-deoxypyridoxal together with $1\ \mu\text{M}$ [^3H]pyridoxal (∇ in B). Cells were incubated similarly but with $30\ \text{mM}$ NaN_3 and $30\ \text{mM}$ NaF and without glucose before the addition of $1\ \mu\text{M}$ [^3H]5'-deoxypyridoxal ($\square$ in A) or [^3H]pyridoxal ($\square$ in B). Cells were incubated with $1\ \text{mM}$ HgCl_2 in 0.9% NaCl at 37°C for 30 min, then washed and incubated in the minimal medium with 0.4% glucose before the addition of $1\ \mu\text{M}$ [^3H]5'-deoxypyridoxal ($\triangle$ in A) or [^3H]pyridoxal ($\triangle$ in B).

Pyridoxal uptake and metabolism by pyridoxal kinase-deficient mutant of E. coli

We previously isolated a mutant of *E. coli*, strain HN1, which requires a concentration of pyridoxine more than 200-fold higher than does the parent strain KG980 for growth (6). The mutant has been found to have a very low activity to phosphorylate pyridoxine and also to take up extracellular pyridoxine. Further investigation revealed that the mutation did not alter the requirement for pyridoxal and pyridoxamine simply in parallel with that for pyridoxine. As shown in Fig. 7, HN1 utilized pyridoxal for growth as well as did KG980, but required 40-fold higher concentration of pyridoxamine than the parent strain. When pyridoxal kinase activity of HN1 cell extract was assayed with pyridoxal and pyridoxamine as substrates, the results were roughly in agreement with the pattern of requirements described above (Table 4). Pyridoxal was phosphorylated more slowly than by KG980 cell extract, but still at a significant rate. Phosphorylation of pyridoxamine

Fig. 7. Effects of pyridoxal and pyridoxamine on the growth of *E. coli* HN1 and KG980. Growth of HN1 on pyridoxamine (○) or pyridoxal (Δ), and that of KG980 on pyridoxamine (●) or pyridoxal (▲) were measured turbidimetrically at 660 nm after aerobic incubation (37°C, 20 hr) of the inoculated minimal medium supplemented with 0.2% glucose and various concentrations of pyridoxal or pyridoxamine.

Table 4. Phosphorylation of pyridoxal and pyridoxamine by cell extracts of *E. coli* KG980 and HN1.^a

Strain	Activity (pmol/min/mg protein)	
	Pyridoxal	Pyridoxamine
KG980	55.4	111
HN1	8.80	1.65

^a Experiments were conducted as described for Table 2.

Fig. 8. Uptake of pyridoxal (A) and pyridoxamine (B) by *E. coli* HN1 and KG980 cells. HN1 cells (○) or KG980 cells (●) were incubated for 15 min in the minimal medium containing 0.4% glucose before the addition of [^3H]pyridoxal or [^3H]pyridoxamine at $1\ \mu\text{M}$ concentration.

appeared nearly negligible, but it was less severely lowered than the phosphorylation of pyridoxine (6). These findings suggest that pyridoxal kinase of HN1 has undergone an alteration such that the behavior of the enzyme to the three forms of vitamin B₆ is differently affected.

Pyridoxal uptake by the mutant cells was clearly slower than that by the parent cells but was still comparable to the latter, while pyridoxamine uptake was more severely affected (Fig. 8). The results were apparently in agreement with the relatively mild or severe reduction of phosphorylation of pyridoxal or pyridoxamine respectively. In order to confirm that the difference in the uptake between HN1 and KG980 cells was due to the difference of phosphorylation, intracellular forms of pyridoxal taken up were examined. As shown in Table 3, the content of phosphorylated forms of the vitamin was, in fact, markedly lower in the mutant than the parent strain, while the amount of free pyridoxal was almost the same in both strains. The data also shows that the intracellular concentration of pyridoxal in HN1 cells was nearly the same as the extracellular pyridoxal concentration. From the relatively constant pyridoxal content, it also seems likely that the entry process of pyridoxal has not been affected in the mutant. As a test for this hypothesis, kinetic properties of pyridoxal uptake by the mutant were investigated. The uptake exhibited saturation kinetics and the apparent K_m value obtained was $1.1\ \mu\text{M}$, very close to that ($1.2\ \mu\text{M}$) previously obtained for pyridoxal uptake by the parent strain (4). Thus the results suggest that the mutant has a specific carrier for the transport of pyridoxal as does the parent strain.

DISCUSSION

The present results have shown that 4'-deoxypyridoxine inhibits pyridoxal uptake competitively, and also that this inhibitor interferes with the entry of pyridoxal across the cell membrane. From these findings together with the saturation kinetics demonstrated in the previous work (4), it is most likely that pyridoxal transport is a carrier-mediated process. In addition, it is consistent with the presence of a specific carrier, separate and distinct from pyridoxal kinase, that the measured K_m value of 29 μM (Table 2) for phosphorylation of pyridoxal by crude cell extract was radically different from the K_m value for the uptake (1.2 μM) (4).

The main objective of the present work was to clarify the significance of intracellular phosphorylation in the whole uptake process. Experiments on energy dependence have shown that pyridoxal uptake requires energy in the form of phosphate bond, and that the depletion of energy source mainly affects the intracellular appearance of transported pyridoxal as the phosphorylated derivatives but not as free pyridoxal itself. In addition, most of the pyridoxal taken up was usually found intracellularly as the phosphorylated derivatives and only a little present as pyridoxal, whose intracellular concentration was nearly equal to its extracellular concentration. From these results it appears that the phosphate bond energy is required only for the phosphorylation of pyridoxal, but not for the process of entry across the cell membrane. An alternative possible explanation of the results could be that pyridoxal is actively transported depending on energy, no accumulation of free pyridoxal being observed because it is rapidly phosphorylated and that energy depletion does not significantly change the pyridoxal content because transport and metabolism are both affected. However, the following findings go against the possibility that pyridoxal is accumulated by an active process: addition of pyridoxal oxime, which strongly inhibited pyridoxal kinase but did not significantly interfere with the entry of pyridoxal, prevented the accumulation of the vitamin as the phosphorylated forms in a dose-dependent manner, while no significant accumulation as free pyridoxal was observed despite the reduction of phosphorylation. Intracellular concentration of pyridoxal was nearly equal to the extracellular concentration at various concentrations of pyridoxal oxime added. Similarly, a pyridoxal kinase-deficient *E. coli* mutant HN1 did not significantly accumulate pyridoxal against a concentration gradient in spite of the low phosphorylation ability, although the K_m value for pyridoxal uptake by the mutant and the parent cells were almost the same. It is almost certain from these findings that no marked accumulation of pyridoxal would take place even if the intracellular phosphorylation was completely blocked.

The uptake of 5'-deoxypyridoxal having no hydroxyl group to be phosphorylated was not affected by the inhibition of energy metabolism (Fig. 6A); in other words, the uptake was independent of energy. This finding indicates that the hydroxyl group is essential for the energy-dependent uptake of pyridoxal and, therefore, supports the nonactive nature of pyridoxal uptake discussed above. The

observed energy-independent uptake of 5'-deoxypyridoxal was significant but was even less than the uptake of pyridoxal lowered by the inhibition of energy metabolism by the addition of NaN_3 and NaF in the absence of glucose. Binding of 5'-deoxypyridoxal to cellular proteins was probably involved in the uptake as previously demonstrated in *S. typhimurium* (1), since this compound with its free aldehyde group readily reacts with amino groups to form a Schiff's base.

The intracellular concentration of pyridoxal described above requires comment. It was calculated by assumption that the cells of *E. coli* contained an intracellular water space of 2.73 ml/g dry weight (18). The concentration was nearly equal to the extracellular pyridoxal concentration whenever examined but also was a little higher than the latter. It was, at highest, 136% of the extracellular concentration (from the data in Table 1) and did not appear high enough to be taken as evidence for active transport. The results would be best explained by the binding of pyridoxal to proteins, since pyridoxal kinase and a probable carrier protein discussed above should naturally bind pyridoxal, and other proteins are also likely to bind the vitamin relatively nonspecifically through the formation of a Schiff's base. It was recently reported that pyridoxal is accumulated against a concentration gradient in erythrocytes by binding *via* a Schiff's base formation to an intracellular protein which is probably hemoglobin (19). The probable binding of 5'-deoxypyridoxal to proteins discussed above also supports the similar binding of pyridoxal, which is relatively weak since pyridoxal with its aldehyde group mostly in the form of hemiacetal is less reactive than 5'-deoxypyridoxal. An alternative explanation of the results might be that the intracellular water space of the cells used in the present work is larger than that reported previously (18).

From the discussion described above it is most likely that pyridoxal enters the cells by a passive process, which is with little doubt carrier mediated, and that the intracellular phosphorylation is essential for the accumulation of the vitamin. As for the accumulation of pyridoxine and pyridoxamine, the essential role of phosphorylation is probably the same as in the case of pyridoxal, since the uptake was also markedly lowered by inhibition of pyridoxal kinase with pyridoxal oxime and by deficiency of the enzyme.

It remains to be seen whether the three forms of the nonphosphorylated vitamin use a common single membrane transport system. Preliminary experiments have suggested a somewhat complicated relation among the three forms. Moreover, the apparently nonsaturable component of pyridoxine uptake previously reported (4) poses a further question. Studies are in progress for the comprehensive clarification of these problems.

The authors thank vice-president Y. Nose of Fukui University of Medicine and Professor A. Iwashima of Kyoto Prefectural University of Medicine for their useful suggestions and discussions.

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