Transport and Metabolism of Vitamin B6 in Lactic Acid Bacteria*

JAMES H. MULLIGAN† AND ESMOND E. SNELL§

From the Department of Biochemistry, University of California, Berkeley, California 94720

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† Recipient of a United States Public Health Service predoctoral traineeship from Grant GM-00031.
§ Present address, Department of Microbiology, The University of Texas at Austin, Austin, Tex. 78712, where reprint requests should be sent.

Streptococcus faecalis 8043 concentrates extracellular \(^{3}H\)pyridoxal or \(^{3}H\)pyridoxamine primarily as the corresponding 5'-phosphates. Accumulation of pyridoxamine requires an exogenous energy source and is inhibited by glycolysis inhibitors. A membrane potential is not required for transport of pyridoxamine, and an artificially generated potential does not drive uptake in this organism. Based on this and other evidence, it is concluded that \(S.\ faecalis\) accumulates pyridoxamine by facilitated diffusion in conjunction with trapping by pyridoxal kinase. Pyridoxamine-P is not concentrated, but equilibrates with that provided externally. Lactobacillus casei 74699 concentrates radioactivity only from pyridoxal, which appears internally as pyridoxal-P, suggesting that it too absorbs the vitamin by facilitated diffusion plus trapping. The specificity of the growth requirement of \(S.\ faecalis\) and \(L.\ casei\) for vitamin B6 parallels the specificity of the transport systems for this vitamin in these organisms. Lactobacillus delbrueckii 74699 accumulates extracellular pyridoxine and pyridoxal-P, suggesting that it too absorbs the vitamin by facilitated diffusion plus trapping. The lack of parallels between uptake mechanisms in \(S.\ faecalis\) and \(L.\ casei\) for vitamin B6 by \(Salmonella\ typhimurium\) (11). This organism accumulated extracellular pyridoxine and pyridoxal as the phosphorylated derivatives, but did not accumulate pyridoxamine. On the basis of this and other evidence, we concluded that \(S.\ typhimurium\) accumulates vitamin B6 by facilitated diffusion, and that intracellular vitamin was trapped as phosphorylated derivatives by pyridoxal kinase (11). Whether \(E.\ coli\) actively transports vitamin B6 or employs facilitated diffusion and trapping is unknown.

The transport of vitamin B6 by \(S.\ carlsbergensis\) has entirely different characteristics than the systems in \(E.\ coli\) and \(S.\ typhimurium\). This organism concentrated pyridoxine, pyridoxal, and pyridoxamine intracellularly more than 100-fold by a process that is energy-dependent and sensitive to metabolic inhibitors. In contrast, neither of the intracellular vitamin was phosphorylated initially, and the authors concluded that \(S.\ carlsbergensis\) possesses an active transport system for vitamin B6 (12).

The lack of parallels between uptake mechanisms in \(S.\ typhimurium\) and \(S.\ carlsbergensis\) led us to inquire into the mechanism of vitamin B6 uptake in other types of microorganisms. Specifically, it would be interesting to know if pyridoxal kinase plays a recurring role as a means of trapping intracellular vitamin. We have chosen pyridoxamine uptake by \(S.\ faecalis\) for detailed study, and also report suggestive data concerning the mechanism of vitamin B6 uptake by \(L.\ casei\) and \(L.\ delbrueckii\). These three lactic acid bacteria are of additional interest because they require specific forms of vitamin B6 for growth (13-17); the present work also correlates the specificity of transport systems for vitamin B6 with the specificity of this growth requirement.

MATERIALS AND METHODS

Isotopically Labeled Compounds—The preparation and purification of \(^{3}H\)pyridoxine, \(^{3}H\)pyridoxal, \(^{3}H\)pyridoxamine, 5'-deoxy\(^{3}H\)pyridoxine, and 5'-deoxy\(^{3}H\)pyridoxal have been described.
The abbreviations used are: pyridoxine-P or PNP, pyridoxine 5'-phosphate; pyridoxal-P or PLP, pyridoxal 5'-phosphate; pyridoxamine-P or PMP, pyridoxamine 5'-phosphate; CCCP, carbonyl cyanide mchlorophenylhydrazone; DCDC, N,N'-dicyclohexylcarbodiimide; HOAc, acetic acid; NaOAc, sodium acetate.

FIG. 1. Uptake of vitamin B6 by Streptococcus faecalis (A), Lactobacillus casei (B), and Lactobacillus delbrueckii (C). Accumulation of [3H]labeled vitamin B6 was measured using the standard filter assay. The extracellular concentrations were: pyridoxal, 0.8 μM (PL); pyridoxamine, 1.1 μM (PM); pyridoxal-P, 1.1 μM (PMP); pyridoxal-P, 0.55 μM (PLP); pyridoxine-P, 0.42 μM (PNP). Where uptake of a specific compound by a given organism is not shown, the intracellular accumulation of that compound (at an extracellular concentration of 1 μM) was less than 0.2 μM after 60 min.

The uptake of [3H]pyridoxamine-P reaches equilibrium with that in the medium, while pyridoxal-P and pyridoxamine-P are apparently impermeable. L. casei concentrates radioactivity only from [3H]pyridoxal, while L. delbrueckii accumulates only the three phosphorylated forms of the vitamin.

Although not transported itself, pyridoxine is a competitive inhibitor (Kᵢ = 100 μM) of pyridoxal transport by S. faecalis (Fig. 2). Pyridoxine does not inhibit pyridoxamine accumulation when present at 200-fold molar excess. In addition, pyridoxal does not inhibit pyridoxamine uptake, and vice versa (data not shown), showing that these compounds are not transported by a common carrier. That pyridoxamine is not an illicit substrate of an uptake system for aromatic amino acids is shown by the fact that its uptake is not inhibited by a 200-fold molar excess of phenylalanine. The apparent Kᵢ values for uptake of pyridoxal and pyridoxamine by S. faecalis are about 0.44 μM and 0.06 μM, respectively.

A simple method for demonstrating active transport of a solute involves showing that the intracellular concentration of the free solute is higher than that in the medium. In the case of S. faecalis, transported pyridoxal and pyridoxamine appear primarily as the corresponding phosphorylated derivatives (Table I), and pyridoxamine-P is partially converted to pyridoxal-P. In L. casei, approximately 25% of the transported pyridoxal is free, and the balance is phosphorylated. Finally, in L. delbrueckii both pyridoxal-P and pyridoxamine-P are interconverted intracellularly, while transported pyridoxine-P appears unchanged except for some hydrolysis to pyridoxine, a result to be discussed later. Since neither S. faecalis nor L. casei maintained a concentration gradient of free vitamin, this criterion could not be used to show active transport. Accordingly, a more thorough investigation of the mechanism of pyridoxamine transport by S. faecalis was undertaken.

**General Characteristics of Pyridoxamine Accumulation by S. faecalis**—Fig. 3 shows that accumulation of pyridoxamine is maximal in a broad range above pH 5. The uptake of pyridoxamine shows a similar pH dependence, which is interesting in view of the fact that pyridoxal does not inhibit pyridoxamine uptake. Uptake of pyridoxamine in both whole cells and
Transport of pyridoxal was assayed after 10 min by the standard filter assay in the presence of unlabeled pyridoxine at concentrations shown on each curve. The pyridoxine used in this experiment contained less than 0.01% pyridoxal.

TABLE I

Transport and intracellular metabolism of vitamin B6 in lactic acid bacteria

Cells were suspended at 37°C in uptake medium containing 0.5% glucose and titrated vitamin B6 (0.73 μM PL, 1.17 μM PM, 0.89 μM PMP, 0.61 μM PLP, or 0.44 μM PNP). After incubation for 40 min (for Lactobacillus casei and Streptococcus faecalis) or 5 min (for Lactobacillus delbrueckii), five 0.5-ml samples from each incubation mixture were filtered, washed twice, and boiled with 5 ml of H2O for 8 min. These water extracts were then centrifuged, made 0.01 M in HCl, and analyzed as described elsewhere (19). Similar results were obtained after 1 min of uptake of [3H]PM by S. faecalis, except that less than 3% of the transported vitamin was unphosphorylated. Compounds whose intracellular forms were not analyzed (e.g. FM or PN in L. casei) are not accumulated by that organism. PIC and PICP are thought to arise by oxidation during hot water extraction and analysis, and not to occur naturally.

Organism and form of vitamin B6 transported

<table>
<thead>
<tr>
<th>Organism</th>
<th>PL</th>
<th>PM</th>
<th>PN</th>
<th>PMP</th>
<th>PLP</th>
<th>PNP</th>
<th>PIC</th>
<th>PICP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. faecalis</td>
<td>14.2</td>
<td>0</td>
<td>0</td>
<td>43.9</td>
<td>3.4</td>
<td>5.0</td>
<td>8.6</td>
<td>5.0</td>
<td>80</td>
</tr>
<tr>
<td>PM</td>
<td>9.2</td>
<td>2.8</td>
<td>0</td>
<td>31.0</td>
<td>22.6</td>
<td>2.1</td>
<td>6.0</td>
<td>4.0</td>
<td>70</td>
</tr>
<tr>
<td>PMP</td>
<td>9.2</td>
<td>0.4</td>
<td>0</td>
<td>31.4</td>
<td>23.4</td>
<td>1.7</td>
<td>9.1</td>
<td>5.0</td>
<td>82</td>
</tr>
<tr>
<td>L. casei</td>
<td>25.9</td>
<td>0.1</td>
<td>0</td>
<td>40.3</td>
<td>4.4</td>
<td>3.7</td>
<td>7.4</td>
<td>5.3</td>
<td>87</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>2.8</td>
<td>0.1</td>
<td>0</td>
<td>46.5</td>
<td>19.5</td>
<td>3.2</td>
<td>5.6</td>
<td>9.8</td>
<td>88</td>
</tr>
<tr>
<td>PLP</td>
<td>0.5</td>
<td>3.3</td>
<td>0</td>
<td>20.4</td>
<td>67.3</td>
<td>1.9</td>
<td>7.1</td>
<td>4.2</td>
<td>102</td>
</tr>
<tr>
<td>PNP</td>
<td>0</td>
<td>0</td>
<td>16.4</td>
<td>1.4</td>
<td>0.5</td>
<td>75.2</td>
<td>1.1</td>
<td>0.6</td>
<td>95</td>
</tr>
</tbody>
</table>

*Pyridoxal, pyridoxamine, and pyridoxine are abbreviated as PL, PM, and PN, respectively, and the corresponding 5'-phosphates as PLP, PMP, and PNP; PIC = 4-pyridoxic acid; PICP = 4-pyridoxic acid phosphate.

Fig. 2. Determination of kinetic parameters for the uptake of pyridoxal and its inhibition by pyridoxine in Streptococcus faecalis. Transport of [3H]pyridoxal was assayed after 10 min by the standard filter assay in the presence of unlabeled pyridoxine at concentrations shown on each curve. The pyridoxine used in this experiment contained less than 0.01% pyridoxal.

TABLE II

Effects of metabolic inhibitors and uncoupling agents on transport of pyridoxamine by Streptococcus faecalis

Uptake was measured using the standard assay, except that inhibitors were added to the noted concentrations 1 min before addition of 0.6 μM [3H]pyridoxamine.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ethylmaleimide (1 mM)</td>
<td>65</td>
</tr>
<tr>
<td>Iodoacetate (5 mM)</td>
<td>95</td>
</tr>
<tr>
<td>Sodium fluoride (3 mM)</td>
<td>100</td>
</tr>
<tr>
<td>Sodium arsenite (40 mM)</td>
<td>100</td>
</tr>
<tr>
<td>DCCD (0.79 mM)</td>
<td>0</td>
</tr>
<tr>
<td>CCCP (2 x 10^{-5} M)</td>
<td>0</td>
</tr>
<tr>
<td>Valinomycin (10^{-4} M)</td>
<td>0</td>
</tr>
<tr>
<td>Glucose omitted</td>
<td>70</td>
</tr>
</tbody>
</table>

Under the conditions shown. These results suggest that energy is required for uptake of pyridoxamine, but is not required in the form of a membrane potential.

Membrane Potential Is Not Required for Pyridoxamine Uptake—Although neither DCCD nor CCCP inhibited pyridoxamine uptake, it was necessary to show that these compounds did collapse the membrane potential under our conditions. Fortunately, Harold and co-workers have described the use of these reagents in a dissection of factors important for uptake of neutral amino acids (23). They found that either CCCP or DCCD completely inhibited threonine uptake by collapsing the membrane potential. Fig. 5 shows the results obtained on repeating those experiments, and results of similar trials with pyridoxamine under identical conditions. The fact that threonine uptake is abolished while pyridoxamine uptake is unaffected leads us to conclude that a membrane potential is not obligatory for pyridoxamine uptake.

Membrane Potential Cannot Drive Pyridoxamine Uptake—Despite the apparent lack of dependence of pyridoxamine transport on a membrane potential, the possibility still existed that under normal conditions pyridoxamine is actively transported at the expense of membrane potential, but is also rapidly trapped by phosphorylation. In the absence of a membrane potential, pyridoxamine uptake might enter via facilitated diffusion, and then be trapped. To investigate this possibility, an attempt was made to drive pyridoxamine transport with an artificially generated membrane potential in unenergized spheroplasts.
Fig. 5 (left). Effects of DCCD and CCCP on uptake of threonine and pyridoxamine by Streptococcus faecalis. Uptake was measured using the standard assay, except that CCCP (0.02 mM; C, △) or DCCD (0.79 mM; □, ▪) was added 1 min before adding substrate. Control experiments (●, □) contain no inhibitors. The concentration of [1H]pyridoxamine (PM) was 1.1 μM, while that of L-[1H]threonine was 50 μM.

Fig. 6 (center). Effect of valinomycin-induced potassium efflux on transport of threonine (A) and pyridoxamine (B) in energy-depleted cells of Streptococcus faecalis. Cells were grown overnight in Medium Q (with potassium citrate instead of sodium citrate, [K+] = 200 mM) supplemented with 3 pmol/ml of pyridoxamine (PM), then diluted into the same medium, and allowed to grow for 3 h. They were then harvested by centrifugation, washed once with ice-cold MgSO4 (2 mM), and resuspended at 2 mg/ml in ice-cold 0.1 M Tris/maleate (pH 6.5). For uptake experiments, cells were incubated at 25° for 1 min before addition of substrate. At the times noted, 0.5-ml portions were filtered and washed twice with 0.1 M Tris/maleate, pH 6.5. Control experiments (●, □) had no valinomycin added. In two separate attempts to drive transport (C, △), valinomycin (3 μM) was added to the cell suspension after 4 min of incubation with substrate. Concentrations were 30 μM L-[1H]threonine in A and 1 μM [1H]pyridoxamine in B.

Fig. 7 (right). Kinetics of inhibition of pyridoxamine uptake by 5'-deoxypyridoxamine. The standard uptake assay was used at various concentrations of [1H]pyridoxamine and 5'-deoxypyridoxamine, and data were obtained after 0, 2, 4, and 6 min of uptake. The data shown are rates of uptake as determined by linear regression analysis at concentrations of 5'-deoxypyridoxamine indicated on the curves.

**DISCUSSION**

Pyridoxal and pyridoxamine are accumulated and appear intracellularly in *S. faecalis* primarily as pyridoxal-P and pyridoxamine-P. In addition, intracellular levels of pyridoxamine-P rapidly reach the concentration of pyridoxamine-P supplied in the medium but do not rise above that level. Other forms of the vitamin (pyridoxine, pyridoxine-P, pyridoxal-P) are apparently unable to cross the cell membrane. These findings are in harmony with the fact that *S. faecalis* specifically requires either pyridoxal, pyridoxamine, or pyridoxamine-P for growth.

The lack of cross-inhibition between pyridoxal and pyridoxamine indicates that they are transported by different systems, although both have similar pH optima for transport. A carrier is involved for each since pyridoxine competitively inhibits pyridoxal uptake and 5'-deoxypyridoxamine competitively inhibits pyridoxamine uptake. The accumulation of pyridoxamine is sensitive to monovalent cations, being stimulated by either K+ or (CH3)3N+ and inhibited by Na+. These effects are similar in protoplasts and whole cells, and may be due to ion effects on membrane structure, as judged by differential binding of 8-anilinothalene sulfonate to protoplast membranes in the presence of sodium or potassium (data not shown).

The following data show that pyridoxamine is not accumulated by an active process in *S. faecalis*: (a) dissipation of the membrane potential by addition of DCCD or CCCP does occur, as shown by their inhibition of threonine transport. However, such dissipation has no effect on uptake of pyridoxamine. (b) An artificial membrane potential produced by valinomycin-induced potassium efflux does not drive pyridoxamine uptake, but is effective in promoting threonine transport. (c) 5'-Deoxypyridoxamine, a competitive inhibitor of pyridoxamine uptake, is not accumulated by *S. faecalis*. Since active transport is not involved in pyridoxamine uptake, and yet pyridoxamine is accumulated by *S. faecalis* in an energy-requiring process, it seems clear that this uptake involves facilitated diffusion followed by trapping of intracellular vitamin as phosphorylated derivatives, as found earlier for *S. typhimurium* (11). This explanation is also favored by the finding that less than 5% of the intracellular vitamin is unphosphorylated, even after only 1 min of uptake. The fact that pyridoxine is not accumulated by *S. faecalis* cannot be attributed to the specificity of the pyridoxal kinase from this organism, since the enzyme has a lower Kₘ value for pyridoxine than for pyridoxal.
(25); rather, it must reflect structural specificity of the membrane carriers.

An additional objective of the present work has been to determine whether growth requirements of various organisms for vitamin B6 are commonly dictated by the discrimination of membrane carriers or by limitations in metabolic machinery. In the case of Salmonella typhimurium mutants (11) and S. faecalis, the specificity of the growth requirement for vitamin B6 stems from discrimination by membrane carriers.

L. casei is even more fastidious than S. faecalis in its vitamin B6 requirement, requiring pyridoxal specifically for growth (13–15). In accordance with this specificity we found that L. casei accumulates only pyridoxal, and most of the intracellular vitamin is phosphorylated. These facts support the idea that specificity in growth requirement may be determined by an organism’s transport system. However, an important caveat is revealed by the knowledge (a) that a 100-fold molar excess of pyridoxine inhibits pyridoxal uptake by about 10%, and (b) that pyridoxal kinase from L. casei discriminates in favor of pyridoxal since its \( K_v \) value for this substrate is less than 0.01 that for pyridoxamine or pyridoxine (25). Thus, if accumulation of pyridoxal proceeds by facilitated diffusion with trapping the exclusion of pyridoxine might result from kinase activity alone, rather than by discrimination at the translocation level. This possibility appears unlikely for two reasons. First, a similar disparity in \( K_v \) values of pyridoxal kinase occurs in S. faecalis, where the \( K_v \) value for pyridoxamine is more than 100 times that for pyridoxal (25). Nonetheless, pyridoxamine is accumulated by facilitated diffusion and trapping more rapidly than pyridoxal. Therefore, one must conclude that there is excess pyridoxal kinase capacity in S. faecalis. Since the activity of pyridoxal kinase in L. casei is 4 times that in S. faecalis (25), L. casei should trap pyridoxine at least as efficiently as S. faecalis traps pyridoxamine, hence it appears that pyridoxine is not presented intracellularly to the kinase. Second, pyridoxine also inhibits pyridoxal uptake in L. casei; thus the carrier for pyridoxal, although fairly specific, does have some affinity for pyridoxine. It appears likely, therefore, that the basis for the specific growth requirement of L. casei for pyridoxal also lies in the specificity of a membrane carrier, and that the slight inhibition of pyridoxal uptake by pyridoxine stems from less than absolute carrier specificity.

A third pattern in specificity of nutritional requirements for vitamin B6 is presented by L. delbrueckii, which requires pyridoxamine-P or pyridoxal-P for growth, but cannot use pyridoxine-P or any of the unphosphorylated forms of vitamin B6 for this purpose (16, 17). The transport studies have shown that this organism cannot accumulate any of the free forms of the vitamin, but transports pyridoxine-P in addition to pyridoxamine-P and pyridoxal-P. In this case there exists a concentration gradient of unchanged transport substrate across the cell membrane. Ordinarily, such a gradient is prima facie evidence for an active transport mechanism, but that interpretation depends on the ability of the intracellular substrate to freely exchange with that in the medium. Certainly in the case of pyridoxal-P, which can bind both specifically to B6-dependent apoenzymes and nonspecifically to many other proteins, one could imagine that the intracellular vitamin might not be freely exchangeable. However, this interpretation is less likely for pyridoxamine-P, which would bind specifically only to the comparatively few apoenzymes (e.g., apotransaminases) that require it for activity, and the level of pyridoxamine-P employed in these experiments (as judged by the extracellular amounts required to support growth) is several orders of magnitude greater than that needed to cover such apoenzymes. Since transport of pyridoxal-P is inhibited by pyridoxamine-P and vice versa, and there is most probably a gradient of diffusible pyridoxamine-P, it appears likely that both compounds are actively transported by the same system. The situation for pyridoxine-P is more clear-cut. Here intracellular binding (except possibly as an inhibitor of pyridoxal-P-dependent apoenzymes) is not expected, and one may conclude that it also enters L. delbrueckii by active transport.

The finding that L. delbrueckii transports all three forms of phosphorylated vitamin B6 but uses only two of them for growth is unexpected. The disparity is easily explainable, however, in terms of cellular metabolic capabilities: analysis of the intracellular forms of the transported vitamin shows that while pyridoxal-P and pyridoxamine-P are readily interconverted, pyridoxine-P is not converted to these metabolically useful forms. Thus L. delbrueckii differs from the other organisms studied, in that the specificity of its growth requirement is dictated partially by intracellular metabolic capabilities.

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