Transport and Metabolism of Vitamin B6 in the Yeast Saccharomyces carlsbergensis 4228*

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Active transport of pyridoxine, pyridoxal, and pyridoxamine occurs in resting cells of Saccharomyces carlsbergensis 4228 and can lead to intracellular concentrations of free vitamin much higher than those supplied externally. The initial $K_m$ for pyridoxine uptake is $3.6 \times 10^{-7} M$ at $30^\circ$ and pH 4.5, which are optimum for growth. Transport is inhibited by many unphosphorylated vitamin analogs, the most effective being 5'-deoxypyridoxine, 5'-deoxypyridoxal, toxopyrimidine, 4'-deoxypyridoxine, and 3-amino-3-deoxypyridoxine. Two distinct uptake systems that differ in structural specificity and ionic requirements are present. One, with optimum pH of 3.5, transports pyridoxal effectively, but not pyridoxamine; the other (optimum pH, 6.0) transports pyridoxamine effectively, but not pyridoxal. Both systems transport pyridoxine, while neither transports pyridoxal 5'-phosphate. Other properties of these systems are similar, indicating that they share certain elements in common. An initial temperature optimum of $30^\circ$ is observed for pyridoxine transport and, at this temperature, an “overshoot” in intracellular vitamin levels, with subsequent decrease to a constant level, occurs with time. It appears that intracellular vitamin, or a derivative, activates the exit mechanism for the vitamin. Exit rates also depend on the resuspension buffer and are increased in the presence of glucose and decreased by azide. Above $30^\circ$ net uptake of pyridoxine drops initially, then rapidly increases to a second optimum at $50^\circ$; the uptake system is inactivated at about $55^\circ$. The optimum at $50^\circ$ apparently results from activation of inflow as exit is rapid and is accelerated by azide. No overshoot was detected at $50^\circ$, so it appears that the exit system is not regulated by intracellular vitamin at this temperature. A phase transition in membrane lipids occurs at $30^\circ$ and may be responsible for the change in properties of the inflow and exit mechanisms above this temperature.

Studies of vitamin B6 transport are complicated by the occurrence of this vitamin in several phosphorylated and non-phosphorylated forms. The process has been studied only to a small extent, mainly with pyridoxine as the vitamin source and in mammalian tissues (for a brief review, see Ref. 1). Concentration of the transported vitamin occurs in red blood cells, but the mechanism of uptake is not well understood (2–4). Concentration also occurs in blood platelets (5) and Ehrlich tumor cells (6), but was ascribed to metabolism or intracellular binding of the vitamin.

Various microorganisms are auxotrophic for vitamin B6 and sometimes use only one or two of the individual forms of this vitamin (7, 8). In some cases this nutritional specificity may result from inability of the organism to convert unphosphorylated forms of the vitamin to the active coenzymes, pyridoxal-P and pyridoxamine-P (9); in other instances specificity has been postulated to lie in the transport process (10, 11). In no case have these various possibilities been thoroughly studied.

The present work describes certain features of vitamin B6 transport in the yeast Saccharomyces carlsbergensis 4228. When thiamin is present in the culture medium, this organism requires an external supply of vitamin B6 for growth. Under these conditions pyridoxine, pyridoxal, and pyridoxamine are almost equally active in promoting growth, whereas pyridoxal-P and pyridoxamine-P have negligible activity (<4% that of the free forms) which is, at least in part, due to contamination with pyridoxal or pyridoxamine (12). In the absence of thiamin, the organism grows well without externally added vitamin B6 (13). We confirmed these relationships for the present culture, and have used it to study entry and exit mechanisms for vitamin B6 and the possible role of metabolism in uptake in this vitamin by nonproliferating cells.

MATERIALS AND METHODS

Isotopically Labeled Compounds—[14C]Pyridoxine hydrochloride (ring 14C, 6.29 mCi/mmol) was a generous gift from Hoffmann-La Roche. [14C]Pyridoxine hydrochloride (0.85 Ci/mol) prepared by an exchange procedure, was obtained from Amersham-Searle. More than 70% of the label was in position C6 and less than 10% at metabolically labile position C4 (14). [14C]Pyridoxine (1.04 Ci/mol). [14C]Pyridoxal (0.88 Ci/mol), 5'-deoxy[14C]pyridoxine (1.98 Ci/mol), and 5'-deoxy[14C]pyridoxal (1.98 Ci/mol) were prepared and purified as described by Mulligan and Snell (15) by appropriate
 modifications of procedures (16, 17) used previously for the corresponding unlabeled compounds.

Sources of other vitamin B6 analogs are indicated with the tables and figures.

Test Organism and Growth Conditions—Saccharomyces carlsbergensis 4228 was maintained on 2% agar slants containing 3% malt extract (Difco). Stock cultures were transferred to fresh slants every 2 weeks, incubated at 30°C for 20 hours, then stored at 4°C. For use in uptake studies, the organism was grown with rotary shaking at pH 4.5 in 1.5-liter Erlenmeyer flasks containing 300 ml of the medium of Atkin et al. (18) modified by the addition of 350 mg/ml of nicotinic acid (19). Sufficient pyridoxine-HCl (10 mg/ml) to allow maximum growth in the presence of thiamine. HCl (260 mg/ml) also was added to this medium (13, 19).

Determination of Total Vitamin B6 Uptake and Forms of Intracellular Vitamin—Yeast cells were harvested by centrifugation from growth media in early stationary phase (18 to 20 hours at 30°C), washed twice with water, resuspended in water to a known concentration (about 2 mg dry weight/ml), and stored for not longer than 5 hours at 0-4°C during the course of an experiment. Uptake properties remained unchanged during this storage period.

Unless indicated otherwise, the uptake mixture (designated as Salts M + 1% Glc) contained those salts present in the growth medium plus glucose. Its composition in grams per liter was: glucose, 10; KH₂PO₄, 0.56; (NH₄)₂SO₄, 1.88; KCl, 0.42; CaCl₂-2H₂O, 0.125; MgSO₄-7H₂O, 0.125; FeCl₂-4H₂O, 0.0026; MnSO₄-4H₂O, 0.0026. For convenience, this solution was usually kept at double these indicated concentrations, adjusted to pH 4.5 if necessary, and diluted with cells (final concentration, 1 mg (dry weight)/ml) and other added to the final volume. After incubation at 30°C with shaking for 30 min, labeled vitamin was added. Aliquot portions (usually 5 ml) were removed at intervals and filtered through 25-mm HA filters (Millipore Corp., 0.45 μm pore size), and washed with two 5-ml portions of ice water. Cells and filter were then transferred to counting vials. Ten milliliters of Triton X-100/1% tritiated water was added, and the cell-free filtrate (0.9 ml) was added to counting vials to determine the amount of vitamin released.

Measurement of Release of Vitamin B6 from Cells—Cells which had been filtered and washed were boiled with small amounts of water, resuspended in water to a known concentration (about 2 mg dry weight/ml), and stored for not longer than 5 hours at 0-4°C.

To determine the forms of the vitamin that accumulated, the filtered and washed cells were boiled with small amounts of water (approximately 0.2 ml/mg of cells) for 10 min. Individual vitamin forms in the extract or the medium were then separated by successive column chromatography on acid (Whatman P11) and basic (Whatman DE52) cellulose (15).

Measurement of Release of Vitamin B6 from Cells—Cells which had been allowed to accumulate labeled vitamin for various times ("loaded" cells) as described in the previous section were filtered, washed with water, resuspended in various buffer solutions at pH 4.5, and incubated at 30°C with shaking. At various times, aliquots were removed and filtered and the cell-free filtrate (0.9 ml) was added to counting vials to determine the amount of vitamin released.

RESULTS

Demonstration and General Characteristics of Pyridoxine Transport

[¹⁴C]Pyridoxine was rapidly accumulated by resting cells in Salts M + 1% Glc medium (Fig. 1A). Assuming that fresh cells contain 2.1 ml of free intracellular water/g of dry solids (21), peak intracellular concentrations of pyridoxine were from 50- to 3000-fold higher than those supplied in the medium, depending on the initial substrate concentration. A marked "overshoot" in transported vitamin was seen with higher substrate concentrations. Separate trials showed that uptake was proportional to cell concentration over the range tested (0.05 to 5.0 mg dry weight/ml) except when more than 50% of the vitamin in the medium had been transported.

Washed cells stored for 5 hours at 0-4°C in water or growth medium (lacking pyridoxine) retained their full uptake capacity for at least 5 hours; at 20°C under these same conditions, or in Salts M (without glucose) at 4°C, cells lost 40% of their uptake capacity in this same period.
incubation period. Energy uncouplers and inhibitors (30 mM azide, 1 mM iodoacetate, or 1 mM dinitrophenol), effectively abolished the concentration of [3H]pyridoxine by the cells in Salts M + 1% Glc, p-Chloromercuribenzoate (0.1 mM), which blocks carrier mediated transport of sugars in Escherichia coli (22-24) and yeast (25), decreased initial net uptake rates by 50% and steady state levels by 15%.

Cells grown under conditions where vitamin B6 is not required, i.e. in the absence of both thiamin and pyridoxine, exhibited uptake parameters (preincubation effects, rate and levels of pyridoxine uptake, and overshoot) essentially identical with those of cells grown in the presence of both thiamin (260 ng/ml) and pyridoxine (10 ng/ml). The uptake system(s) therefore appear to be constitutive in these cells.

Kinetics of Pyridoxine Uptake in Salts M + 1% Glc

At concentrations of [14C]pyridoxine from 0 to 5 μM, pyridoxine uptake exhibited typical Michaelis-Menten kinetics. The apparent K_m of 0.36 μM did not vary significantly during incubation times ranging from 30 s to 15 min, but decreased to 0.12 μM by 60 min (Table II). Above 5 μM external pyridoxine, Lineweaver-Burk plots departed from linearity, possibly due to contributions from a lower affinity uptake system or to diffusion. V_max values were markedly influenced by preincubation and incubation times as net uptake and not inflow was being measured. The maximal uptake rate obtained after a 1-min incubation with [14C]pyridoxine was 99 pmol-min⁻¹·mg⁻¹ cells, equivalent to an increase in concentration of 47.1 μM min⁻¹ in intracellular vitamin.

Structural Requirements for Binding to Pyridoxine Transport System

The effects of various analogs of pyridoxine and of thiamin on the uptake of [14C]pyridoxine are shown in Table III. K_i values were calculated assuming a K_m value for pyridoxine of 0.36 μM (Table II) and competitive inhibition. Such K_i values must reflect some component of the transport system, but do not necessarily indicate transport. Binding was almost eliminated by substitution at positions 1 or 6 of pyridoxine (cf. 1-methylpyridoxine, 6-methylpyridoxal). However, potent antagonists of pyridoxine uptake resulted from appropriate variations at positions 2, 3, 4, or 5 of pyridoxine (cf. 2'-methylpyridoxine, 3-NH₂-3-deoxypyridoxine, 4-deoxy- pyridoxine, 5'-deoxypyridoxine, 5'-deoxypropyridoxal, and isopyridoxamine). 5'-Deoxypyridoxine was the most effective inhibitor found; toxopyrimidine also is a potent antagonist of pyridoxine uptake.

In each of these compounds the new substituent is rather similar in size to that replaced. In the case of 2-alkyl substituents, the methyl group found in pyridoxine itself permits optimum binding, but analogs supplying an ethyl or propyl group (2'-methylpyridoxine, 2'-ethylpyridoxine) bind better than that containing only the smaller hydrogen atom (2-norpypyridoxine); a progressive decrease in binding accompanies increasing size beyond that of the methyl group. Strongly ionic groups (e.g. pyridoxamine versus pyridoxine or pyridoxal; isopyridoxamine versus pyridoxine, etc.) impede binding under these conditions even when similar in size to the substituent replaced, whereas weakly basic aromatic amino groups (e.g. in 5-NH₂-3-deoxypyridoxine or in toxopyrimidine) do not. The relative contributions of ionic charge versus increased size to the failure of pyridoxine-5'-P or 5-pyridoxic acid to bind effectively is not known; both factors undoubtedly play a role.

The apparent affinity of the uptake system for pyridoxamine is considerably lower than that for pyridoxine or pyridoxal, a surprising observation, considering their similar growth-promoting activities. As shown subsequently, this anomaly is apparently explained by the existence of a second uptake system with higher affinity for pyridoxamine.

Effect of pH on Vitamin B6 Uptake and Analog Binding

Pyridoxine uptake exhibits two distinct pH optima in both phosphate-citrate (Fig. 2) and 25 mM potassium phosphate buffers. At the concentrations tested, the low pH system (pH
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Table III

Structural specificity for binding to pyridoxine uptake system as revealed by inhibition of pyridoxine uptake

Cells (1 mg/ml) were incubated at 30° for 30 min in Salts M + 1% Glc, pH 4.5, before the addition of [14C]pyridoxine (0.5 μM) and analog (5 or 50 μM). [14C]Pyridoxine uptake was measured after 1 and 5 min and Ki values were calculated assuming competitive inhibition. Competitive inhibition was verified by more extensive data for those entries shown in italics.

| Compound and source | Side chain at position x replaced by y | K<sub>i</sub> | Compound and source | Side chain at position x replaced by y | K<sub>i</sub> |
|---------------------|--------------------------------------|------------|---------------------|--------------------------------------|------------|
| PL<sup>b</sup>      | CHO                                  | 0.35       | 4'-methoxy-5-de-(CH<sub>2</sub>OH)PN | CH<sub>2</sub>OH<sub>3</sub> | 20         |
| PM<sup>a</sup>      | CHO                                  | 1.1        | 6-MePL              | CHO                                  | 50         |
| 4de(CH<sub>2</sub>OH).PN | H                                   | 11         | 4-de-(CH<sub>2</sub>OH)-6-(CH<sub>2</sub>OH)PN | H                | 26         |
| 4norPN              | CHO                                  | 17         | 3-deoxoPN           | H                                   | 2.9        |
| 4′-deoxyPN<sup>b</sup> | CH<sub>3</sub>                | 1.1        | 3-NH<sub>2</sub>-3-deoxyPN | NH<sub>2</sub>       | 1.1        |
| PL-oxime            | CHO                                  | 23         | 3-NH<sub>2</sub>-3-deoxyPN | CHO<sub>3</sub>       | 5.2        |
| 5′-deoxyPN          | CH<sub>3</sub>                | 0.20       | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |
| 5-isop               | CH<sub>3</sub>                | 7.7        | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |
| 5-Pyridoxoic acid   | CHO                                  | 20         | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |
| 5-isop              | CHO                                  | 52         | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |
| 5′-deoxyPL           | CHO                                  | 60         | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |
| 5′-pyridoxo-lactone  | CHO                                  | 45         | 3-NH<sub>2</sub>-3-deoxyPN | OCH<sub>3</sub>       | 17         |

a The compounds have been abbreviated as derivatives of pyridoxine (PN), pyridoxal (PL), or pyridoxamine (PM) according to published recommendations (27). The structure of each is obtained by reference to columns x and y and the adjoining formula for pyridoxine (I). References to synthetic procedures for these compounds have been summarized by Korytnyk and Ikawa (28) and by Florentiev et al. (29). Test samples of compounds not available commercially were obtained from representatives of the cited laboratories or synthesized here. We are especially indebted to Drs. K. Packer for certain of these samples. The following compounds also were tested but had no significant affinity (K<sub>i</sub> > 100 μM) for the uptake system: pyridoxal<sup>z</sup>-, pyridoxamine<sup>z</sup>-, pyridoxine<sup>z</sup>-, 4-pyridoxic acid, 4-pyridoxolactone, 2-methyl-3-hydroxypropyridine-4,5-dicarboxylic acid, 3-hydroxy-4-formylpyridine, thiamine<sup>z</sup>-, 4-methyl-5-(2-hydroxyethyl)thiazole, 1-methylpyridoxal, 2-methyl-3-amino-4,5,6-trihydroxymethylpyridine.

b These compounds were obtained commercially. Toxopyrimidine is 2-methyl-4-amino-5-hydroxymethylpyridimine.

3.5 to 4.0) is most effective for pyridoxal, whereas the high pH system (pH 5.5 to 6.5) is most effective for pyridoxamine. The effects of pH on the affinity of these and related compounds for the uptake systems are shown in Table IV. Results at pH 4.5, which lies between the two optima for pyridoxine uptake but is close to the pH optimum for growth, are also included. Each of the compounds tested showed slightly lower affinity for the transport system in citrate-phosphate buffer at pH 4.5 (Table IV) than in Salts M + 1% Glc solution at this same pH (cf. Table III). Both binding and transport of pyridoxal were maximal at pH 3.5 and practically nonexistent at pH 6, while binding and transport of pyridoxamine were maximal at pH 6. Pyridoxine was bound well and was transported at both pH values. The 5′-deoxy analog of pyridoxine, pyridoxal, and pyridoxamine resemble their parent compounds with respect to pH optima, but, in each case, showed greater affinity as judged by K<sub>i</sub> values. The K<sub>i</sub> values for 5′-deoxopyridoxal were considerably higher than the K<sub>i</sub> values, an effect most obvious at pH 8. This compound, unlike pyridoxal, also exhibits substantial uptake at pH 6. This uptake may reflect nonspecific binding of this compound (through interaction of its free formyl group with amino groups of the cell); if so, the K<sub>i</sub> values most closely reflect the true affinities for the transport system. This effect was not noted with pyridoxal, probably because (unlike 5′-deoxopyridoxal) very little of the free aldehyde form is present in the pH range studied (30). All analogs inhibited [14C]pyridoxine uptake competitively and had lower affinities at pH 7, as judged by K<sub>i</sub> values, than at pH 6. Pyridoxal-P, pyridoxine-P, and pyridoxamine-P had no affinity (K<sub>i</sub> > 100 μM) at any pH tested.

An overshoot in transported [14C]pyridoxine similar to that already noted at pH 4.5 (Fig. 1A) also was observed at pH 3.5 and 6.0.

Effect of Other Ions on Pyridoxine Uptake

Certain buffer effects led us to examine the effect of various ions on transport, even though no attempts were made to obtain ion-deficient media or cells. Since the rate of transport
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**Effect of pH on Vitamin B6 Uptake**

Cells (1 mg/ml) were preincubated at 30° for 30 min in 12.5 mM KH_{2}PO_{4}, 12.5 mM K_{2} citrate-HCl buffer (indicated pH values) plus 1% glucose before the addition of \(^{[14}C\)pyridoxine (PN) (0.5 \(\mu\)M), \(^{[3}H\)pyridoxal (PL) (0.30 \(\mu\)M), or \(^{[3}H\)pyridoxamine (PM) (0.47 \(\mu\)M). Uptake of labeled vitamin was measured after 5 min.

**TABLE IV**

| Compound tested and parameter measured | \(K_{m}\) (\(\mu\)M) | \(V_{max}\) (\(\mu\)M/min) |
|----------------------------------------|---------------------|---------------------|
| Pyridoxine                            |                     |                     |
| \(K_{m}\)                              | 0.46                | 0.70                |
| \(V_{max}\)                            | 101                 | 62.4                |
| Pyridoxal                              |                     |                     |
| \(K_{m}\)                              | 1.74                |                     |
| \(K_{f}\)                              | 1.20                | 3.48                |
| \(V_{max}\)                            | 85                  |                     |
| Pyridoxamine                           |                     |                     |
| \(K_{m}\)                              | 20.6                | 2.2                 |
| \(K_{f}\)                              | 13.6                | 2.8                 |
| \(V_{max}\)                            | 28.6                |                     |
| 5'-Deoxypyridoxine                     |                     |                     |
| \(K_{m}\)                              | 0.25                | 0.22                |
| \(K_{f}\)                              | 0.23                | 0.20                |
| 5'-Deoxypyridoxal                      |                     |                     |
| \(K_{m}\)                              | 1.50                |                     |
| \(K_{f}\)                              | 0.30                | 0.31                |
| 5'-Deoxypyridoxamine                   |                     |                     |
| \(K_{m}\)                              | 7.3                 | 3.8                 |
| \(K_{f}\)                              | 8.0                 |                     |

**Effect of Temperature on Vitamin B6 Uptake**

Pyridoxine uptake showed an unexpected relationship to temperature (Fig. 3). Between 1 and 40°, typical results for a transport system were seen: transport first increased from negligible values to an initial rate optimum near 35° and a steady state optimum of 30° (the optimum growth temperature), then decreased as the temperature was raised to 40°. Above 40°, however, a rapid rise in pyridoxine uptake occurred with an apparent optimum at 50°, before inactivation occurred near 60°. The \(Q_{10}\) value between 20 and 30° and between 40 and 50° was 3.3 to 3.4, indicating an active process in each case. A phase transition in membrane lipids, occurring just above 30° (Fig. 4) may be related to the initial decrease in uptake rate.

Some properties of the 50° system were examined briefly with results as follows: (a) the dual temperature optimum shown in Fig. 3 for uptake at pH 4.5 was also present at pH 3.5 and pH 6.0; (b) the affinities for pyridoxine, pyridoxamine, pyridoxal, and 5'-deoxypyridoxine at 50° were about one-half those shown in Table III for these same substrates at 30°; (c) uptake at 50° was markedly dependent upon the presence of glucose during both preincubation and uptake, stimulation by

**Fig. 2. Effect of pH on vitamin B6 uptake.** Cells (1 mg/ml) were preincubated at 30° for 30 min in 12.5 mM KH_{2}PO_{4}, 12.5 mM K_{2} citrate-HCl buffer (indicated pH values) plus 1% glucose before the addition of \(^{[14}C\)pyridoxine (PN) (0.5 \(\mu\)M), \(^{[3}H\)pyridoxal (PL) (0.30 \(\mu\)M), or \(^{[3}H\)pyridoxamine (PM) (0.47 \(\mu\)M). Uptake of labeled vitamin was measured after 5 min.

**Fig. 3. Effect of temperature on uptake of \(^{[14}C\)pyridoxine.** Cells (1 mg/ml) in Salts M + 1% Glc, pH 4.5, were incubated for 30 min at 30° and for 5 min at the indicated temperatures before the addition of \(^{[14}C\)pyridoxine (0.60 \(\mu\)M). Uptake was measured after 1, 5, and 20 min, as indicated.
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Fig. 4. Effect of temperature on the rotational correlation time ($\tau_c$) of the spin label 2N14 in Saccharomyces carlsbergensis membranes. A cell pellet (0.05 ml) was mixed with 2N14 (2,2,5-trimethyl-5-dodecanoylloxazolidine-1-N-oxyl; 0.2 mM (31)) and scans were performed at the specified temperatures on a Varian V4500 X-band EPR spectrometer equipped with a temperature control to maintain temperature to better than +0.5°. Cells were grown on 5% glucose so very little mitochondrial membrane would have been present (32).

glucose varied from about 18- to 100-fold, depending upon the conditions; (d) preincubation of cells at 50° for 30 min without glucose virtually destroyed their uptake capacity; (e) only a very slight overshoot in transported pyridoxine levels was seen at 50° even with pyridoxine levels as high as 2.2 $\mu$M. This behavior contrasts markedly with the pronounced overshoot observed at 30° at only 0.8 $\mu$M substrate (Fig. 1A).

Nature of Overshoot Phenomenon

The level of accumulated vitamin should represent a dynamic equilibrium determined by the inflow and exit rates at any given time. The overshoot in net uptake observed in Fig. 1 could be a result of a change in this equilibrium or, more trivially, could be caused by counterflow of external labeled vitamin with endogenous unlabeled vitamin. The latter possibility can be eliminated as little or no overshoot was observed when low levels of [14C]pyridoxine were supplied in the uptake medium (Fig. 1A), when cells were not preincubated in the uptake medium (Fig. 1B) or when cells were grown on high levels of unlabeled pyridoxine HCl (100 mM). In addition, if cells were equilibrated with high levels of pyridoxine (250 $\mu$M) or its analogs in the presence of azide (30 mM) and iodoacetate (1 mM), and resuspended in buffer containing [14C]pyridoxine (0.5 $\mu$M) plus metabolic inhibitors, no overshoot in intracellular labeled vitamin was observed, only an equilibration with that in the medium.

If the intracellular level of pyridoxine represents a dynamic equilibrium, small amounts of labeled vitamin should equilibrate with larger amounts of unlabeled intracellular vitamin. Data of Fig. 5 show this to be so. The initial rate of uptake of this equilibrating vitamin represents the actual inflow rate and, although rapid, appears to be unaffected by prior incubation with 0.2 or 2 $\mu$M unlabeled vitamin for periods up to 60 min, even when overshoot is in progress. Thus, modification of the inflow mechanism does not appear to be responsible for the overshoot phenomenon.

Exchange of intracellular [14C]pyridoxine with excess external unlabeled pyridoxine similarly should have an initial rate equivalent to the outflow rate at that time, as reuptake of exchanged [14C]pyridoxine should be virtually eliminated. Such rates, too, are rapid; however, this rate is significantly higher in cells containing high levels of intracellular vitamin (Table V), i.e. during and after the overshoot period, suggesting that activation of the exit rate is one key to the overshoot phenomenon. When unlabeled pyridoxine was replaced in these exchange experiments by azide (25 mM) plus iodoacetate (1 mM), negligible rates of outflow occurred.

When cells which had undergone overshoot and had achieved a steady state level of intracellular vitamin (e.g. those of Curve 3, Fig. 1B) were washed exhaustively and resuspended in uptake mixtures containing the same level of [14C]pyridoxine, only slight uptake sufficient to replace the small amount of vitamin lost by washing was observed; no re-overshoot occurred showing that accumulation of intracellular vitamin (or a product formed only in the presence of such high levels) was responsible for the activation of the exit mechanism.

To examine this conclusion further, the effects of permitting cells to accumulate [14C]pyridoxine from comparatively low external concentrations (0.04 or 0.2 $\mu$M) for various times on their subsequent behavior toward a high external concentration (1.6 $\mu$M) of the labeled vitamin were determined (Fig. 6). Incubation with 0.04 $\mu$M vitamin did not result in overshoot and did not affect the rate of inflow when 1.56 $\mu$M vitamin was added. In fact, addition of 1.56 $\mu$M vitamin after 60 min resulted in slightly greater uptake than at 1 or 15 min. Cells incubated with 0.2 $\mu$M [14C]pyridoxine before addition of...
maximum intracellular levels of 30 and 70 ELM, respectively, not essential for the transport of vitamin B6.

falling to about 30% of maximum in 60 min. We conclude that doxine or 5'-deoxy[3H]pyridoxal. These vitamin analogs were vitamin.

accumulation of intracellular vitamin, a vitamin derivative, or transported almost as rapidly as pyridoxine itself, reaching those in Fig. I A were conducted with 1 mM 5'.deoxy['H]pyri-

some other product formed in response to the high levels of 

after 2.5 to 5.0 min. They, too, exhibit overshoot, their levels after 2.5 to 5.0 min. They, too, exhibit overshoot, their levels in their ability to accumulate high levels of the additional vitamin to 1.6 pM and uptake (solid lines) was again followed with time. At 1, 15, or 60 min (as indicated by arrows) sufficient 

FIG. 6. Effect of intracellular vitamin B6 concentration on uptake and overshoot. Cells (1 mg/ml) were incubated at 30° for 30 min in Salts M + 1% Glc, pH 4.5, before the addition of either 0.04 (A), or 0.2 (B) μM [14C]pyridoxine (PN). Net uptake (broken line) was followed with time. At 1, 15, or 60 min (as indicated by arrows) sufficient [14C]pyridoxine was added to bring the concentration of [14C]pyridoxine to 1.6 μM and uptake (solid lines) was again followed with time.

[14C]pyridoxine to 1.6 μM showed a progressive decay with time in their ability to accumulate high levels of the additional vitamin. This decay is consistent with activation of outflow by accumulation of intracellular vitamin, a vitamin derivative, or some other product formed in response to the high levels of vitamin.

To determine whether phosphorylated forms of vitamin B6 were the products promoting overshoot, experiments similar to those in Fig. 1A were conducted with 1 mM 5'-deoxy[14H]pyri-
doxine or 5'-deoxy[14H]pyridoxal. These vitamin analogs were transported almost as rapidly as pyridoxine itself, reaching maximum intracellular levels of 30 and 70 μM, respectively, after 2.5 to 5.0 min. They, too, exhibit overshoot, their levels falling to about 30% of maximum in 60 min. We conclude that phosphorylation-dephosphorylation at least in the position 5' is not essential for the transport of vitamin B6.

Effect of Intracellular Concentration of Pyridoxine on its Exit Rate—To eliminate effects of metabolism of pyridoxine and to reduce reuptake effects, efflux was measured over a short time span (2 to 8 min) and with cells that had been loaded with different external levels of [14C]pyridoxine for only 5 min. The labeled vitamin in such cells was essentially all pyridoxine (cf. Table I). The results (Fig. 7) show that very little net efflux occurs at concentrations of intracellular pyridoxine less than about 20 μM. At higher concentrations, activation of the exit mechanism appears to occur and net efflux becomes a linear function of the intracellular vitamin concentration. This linear relationship is consistent with loss by simple diffusion, but since exit is inhibited by triethanolamine acetate, which also inhibits uptake, exit through a system which was not saturated in the range of intracellular concentrations tested seems more likely.

At high concentrations of intracellular [14C]pyridoxine, the rate of loss of vitamin approximated first order kinetics with rate constants for exit of 0.103 min⁻¹ in Salts M + 1% Glc and 0.027 min⁻¹ in triethanolamine acetate (Table VI). After 15 to 30 min, net outflow ceased in each case and a net influx was observed which was most marked in the presence of glucose. Although metabolism of intracellular pyridoxine to other vitamin forms which were less easily released coupled with reuptake of released pyridoxine could explain this effect, it was noteworthy that the changeover from net outflow to net inflow occurred when the intracellular vitamin concentration fell to about 50 μM and thus may represent repression or deactivation of the exit mechanism. Initial exit rates were increased in the presence of glucose (Table VI) and by addition of unlabeled analogs. It is unlikely that this effect was due simply to inhibition of reuptake of released labeled vitamin as 5'-deoxypyri-
doxine, the most effective analog inhibitor of pyridoxine uptake, did not cause as large an increase in exit rate as did pyridoxine and pyridoxal (Table VI). It is possible that excess external vitamin activated the exit mechanism but more prob-
able that the vitamin was transported into the cell before this activation occurred. Exit rates were increased at 50° and were accelerated in the absence of glucose and in the presence of azide (Table VI).

Interconversion and Efflux of Pyridoxine Metabolites—It was shown in Table I that pyridoxine was the major form of intracellular vitamin during the first 30 min of uptake with [14C]pyridoxine and was also the form of the vitamin released during the overshoot period; after 120 min much of the intracellular vitamin had been phosphorylated and all forms of the vitamin were present. Separate tests showed that outflow of labeled vitamin from cells loaded with [14C]pyridoxine for 120 min was affected by buffer salts, glucose, and unlabeled pyridoxine in the same general way as in cells loaded for only 5 min; however, the initial rapid outflow (that during the first 15 min) was considerably lower, accounting for only about 15% of the intracellular labeled vitamin, i.e. about the amount of the nonphosphorylated forms of the vitamin present at this time (cf. Table I). A much slower outflow followed (data not shown). To examine the specificity of this slow release, exchange experiments were carried out in which cells loaded for 120 min with [14C]pyridoxine were washed, then suspended in Salts M + 1% Glc containing unlabeled pyridoxine or related comp-
ounds for 150 min before filtering and analyzing the filtrate for released forms of 14C-labeled vitamin B6. The results

Table V

| Effect of Intracellular Vitamin B6 |
|-----------------------------------|
| Exchange of extracellular unlabeled pyridoxine with intracellular [1H]pyridoxine at different stages of overshoot |
| Cells (1 mg/ml) were incubated at 30° for 30 min in Salts M + 1% Glc, pH 4.5, before the addition of either 0.04 (A), or 0.2 (B) μM [14C]pyridoxine (PN). Net uptake (broken line) was followed with time. At 1, 15, or 60 min (as indicated by arrows) sufficient [14C]pyridoxine was added to bring the concentration of [14C]pyridoxine to 1.6 μM and uptake (solid lines) was again followed with time. |
Effect of intracellular \[^{14}C\]pyridoxine concentrations on exit rate. Cells (1 mg/ml) were preincubated at 30° for 30 min in Salts M + 1% Glc, pH 4.5, before the addition of various concentrations (0.024 to 2.13 \(\mu\)M) of \[^{14}C\]pyridoxine. After 5 min, the cells were washed with water and an aliquot counted to determine total intracellular vitamin. The remaining cells were resuspended (1 mg/ml) in the indicated media and release of label was followed with time.

**Table VI**

| Buffer, pH 4.5 | Glucose | Temperature °C | Exit rate, \(k\) min\(^{-1}\) | Additions to medium |
|---------------|---------|----------------|-----------------------------|---------------------|
|               | %       |                |                             | None               |
| Salts M       | 0       | 30             | 0.045                      | 0.069* 0.070\(^9\) 0.054 —<\(c\) |
|               | 1       | 30             | 0.103 0.192\(^{10}\) 0.171\(^{11}\) 0.146 <\(c\) |
| Triethanolamine acetate (50 mM) | 0       | 30             | 0.019 0.023 0.029\(^{12}\) 0.020 —<\(c\) |
| Salts M       | 0       | 50             | 0.178 0.184 0.170 0.155 >0.8\(^{13}\) |
|               | 1       | 60             | 0.140 0.141 0.162 0.130 —<\(c\) |

\(^{*}\) The abbreviations used are: PN, pyridoxine; PL, pyridoxal.

\(^{+}\) In these cases the exit rate was significantly different (\(P < 0.01\)) from that obtained in the absence of unlabeled analogs.

\(^{<}\) Not determined.

(Table VII) showed that the total net efflux of all vitamin forms was increased almost 100% by excess unlabeled pyridoxine or pyridoxal and 40% by excess pyridoxamine or 5'-deoxy-pyridoxine; essentially all of this increase resulted from increased release of \[^{14}C\]pyridoxine and, to a lesser extent, of \[^{14}C\]pyridoxamine. One effect of these analogs would be to inhibit reuptake of released \[^{14}C\]pyridoxine, but since 5'-deoxy-pyridoxine is the most effective analog inhibitor of uptake (cf. Table III) and did not cause as large a release of \[^{14}C\]pyridoxine as did pyridoxal, the major effects of the analogs must have been on the intracellular metabolism of the vitamin. At sufficiently high levels, all of these analogs would prevent reporphorylation by pyridoxal kinase of free \[^{14}C\]labeled vitamin B6 (1) and would thus permit increased release of labeled vitamin arising by hydrolysis of the intracellular phosphorlated compounds. However, very little pyridoxine-P is present intracellularly, and it is noteworthy that unlabeled pyridoxal increased outflow of \[^{14}C\]pyridoxine but not that of \[^{14}C\]pyridoxal. Although the exit system thus appears relatively specific for pyridoxine, this apparent specificity could be a result of the conversion of free pyridoxal to pyridoxine by the pyridoxine dehydrogenase of yeast (33, 34) prior to release. The latter explanation is favored by the fact that release of \[^{14}C\]pyridoxamine also is enhanced by addition of each of the unlabeled compounds. Relatively large amounts of \[^{14}C\]pyridoxal-P were released by the cells and, as \[^{14}C\]pyridoxamine-P was not released, it was unlikely that this was due to loss in cell viability. The amounts released were not increased by addition.
of the unlabeled vitamins. The values reported for 4-pyridoxic acid 5'-phosphate (and related compounds) may represent pyridoxal-P oxidized during the chromatographic procedures (35-37).

**DISCUSSION**

Pyridoxine uptake by nonproliferating cells of Saccharomyces carlsbergensis possessed characteristics of a carrier-mediated, active process (38-40). The vitamin was taken up and retained against a concentration gradient and the transport process was dependent on energy, pH, temperature, and the ionic environment, and also displayed structural specificity and saturation kinetics. Uptake of pyridoxal, pyridoxamine, 5'-deoxypyridoxal, and 5'-deoxypyridoxal also displayed the properties of an active process in those characters measured. The $K_m$ of $3.6 \times 10^{-7}$ M for uptake of pyridoxine at pH 4.5 is in the range reported for active transport systems acting on biotin (41) and thiamine (42) in yeast.

Uptake of pyridoxine exhibits twin pH optima of 3.5 and 6.0. These systems have different structural specificities for transport, pyridoxal being transported primarily by the pH 3.5 system and pyridoxamine by the pH 6 system, whereas pyridoxine is transported rather effectively by both systems. These observations may explain the fact that pyridoxamine frequently appears somewhat less active than pyridoxine or pyridoxal in supporting yeast growth: most such assays are conducted near pH 4.5. Although neither uptake system showed an absolute requirement for ions under the conditions tested (no effort to obtain K+-deficient yeast was made) uptake at pH 3.5 was stimulated 4-fold by low concentrations of K+ and to a lesser extent by higher concentrations of Li+ and Na+. Uptake at pH 6 was stimulated slightly by K+.

An overshoot in transported pyridoxine levels was observed and was apparently due to an acceleration of net outflow that occurs when internal vitamin concentrations increase sufficiently. Although much of the pyridoxine had been metabolized, mainly to pyridoxal-P and pyridoxamine-P, by the time steady state levels of vitamin B6 had been reached, intracellular pyridoxine was still maintained at concentrations considerably above those in the external medium.

The study of the exit mechanism was complicated by metabolism and reuptake of pyridoxine. No saturation of the exit system was detected within the range of intracellular pyridoxine concentrations tested. Other properties of the exit system, however, were indicative of a carrier-mediated system. Exit was accelerated by exchange with external pyridoxine or its analogs and was inhibited by azide plus iodoacetate, and by diethanolamine acetate, which also inhibited uptake. Unexpectedly, exit was also stimulated by glucose. This effect could not be studied under conditions of complete glucose starvation as cells could be preloaded with [14C]pyridoxine only in the presence of glucose; the degree of stimulation by energy sources may therefore be more marked than noted.

The level of vitamin in the cell depends on a balance between inflow and outflow and the overshoot effect represents a change in this equilibrium. Dreyfuss and Pardee (43) concluded that overshoot in sulfate transport in Salmonella typhimurium was caused by repression of inflow by an unstable high energy metabolite, 3'-phosphoadenosine 5'-phosphosulfate, while Becker and Lichtstein (41) concluded that biotin inflow in Saccharomyces cerevisiae was repressed by intracellular biotin. In both of these cases, exit was by diffusion. In the case of pyridoxine, studied here, there were no apparent differences in the initial equilibration rates of pyridoxine before, during, and after the overshoot period, and apparently, therefore, no repression of inflow. Exit rates, however, measured by exchange experiments, were accelerated during and after the overshoot period and appeared to be responsible for this phenomenon. Pyridoxine or a stable derivative of this vitamin appeared responsible for this activation in exit rate and the effect was time-dependent. Since 5'-deoxypyridoxine and 5'-deoxypyridoxal both exhibited a similar overshoot in transport, 5'-phosphorylation of the vitamin apparently is not required either for this activation or for transport. β-Galactosidase exit in E. coli is accelerated in the absence of an energy source by a lowering of the exit $K_m$ (22, 44), and high intracellular levels of pyridoxine may act similarly by lowering the exit $K_m$ for pyridoxine in S. carlsbergensis. The facts (a) that the cooperative effect on exit rate evoked by intracellular pyridoxamine was manifested at about the same concentration that re-overshoot was inhibited, (b) that the apparent $K_m$ for inflow of pyridoxine decreases as the intracellular pyridoxine concentration increases (Table II), (c) that the time dependency between inhibition of overshoot by intracellular vitamin and the observed decrease in apparent inflow $K_m$ are similar, and (d) that 5'-deoxypyridoxine also exhibits the overshoot phenomenon, all support the view that these changes are a function of the transport system itself rather than a consequence of intracellular metabolism of the vitamin.

Overshoot might be considered a protective mechanism by which cells prevent the accumulation of excessive vitamin (41) or, more likely, a means of keeping intracellular vitamin concentration relatively constant despite large variations in the external environment (43). In the latter connection it is interesting that the intracellular level of vitamin B6 in cells grown without addition of this vitamin was about 53 ± 10 µM (10), a concentration in close agreement with the steady state levels found after uptake with a wide range of [14C]pyridoxine concentrations (Fig. 1A).

Pyridoxal-P does not effectively replace pyridoxal as a growth factor for yeast (10-12) or several bacteria (12), and because of this it is sometimes assumed that vitamin B6 phosphates do not in general cross cell membranes. However, pyridoxamine-P is an essential growth factor for some bacteria (8), and pyridoxal-P has been shown to cross the red blood cell membrane without hydrolysis (3). Similarly, many bacteria excrete vitamin B6 into their growth medium, mainly in the form of pyridoxal-P and sometimes pyridoxamine-P (46-48). Evidence has also been presented for the transport of pyridoxal-P or pyridoxine-P across placental membranes (16, 49) and possibly the blood-brain barrier in mammals (16, 50, 51).

We report herein that yeast cells preloaded with pyridoxine convert much of it to pyridoxal-P within 120 min, and excrete one-half of this amount into the medium within 150 min. This release was not affected by exchange with excess nonphosphorylated forms of the vitamin. The phenomenon is of interest.
since pyridoxal-P is almost inactive in replacing pyridoxine as a growth factor for this yeast (12) and has little or no affinity for the transport system studied here.

The most unusual feature of the pyridoxine uptake system is its behavior at high temperatures. Up to 40°C, the effect of temperature is typical of an active process with a maximal uptake rate at 30–35°C. However, the second sharp, energy-dependent optimum at 50°C with its greatly increased initial uptake rate was unexpected. The activation at high temperatures is even more marked than indicated by Fig. 3, as some loss in cell viability occurs at this temperature. Overshoot was barely detectable, and one possibility is that the exit rate of the transport system has not been studied extensively but it resembles that of 5'-deoxypyridoxine.

It seems clear from the dual pH optima, and the different structural requirements for uptake at these two optima, that at least two transport systems operate in providing vitamin B6 to yeast cells. The fact that both systems exhibit such unusual temperature relationships indicates that they share certain elements in common.

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