A carrier-mediated mechanism for pyridoxine uptake by human intestinal epithelial Caco-2 cells: regulation by a PKA-mediated pathway

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Said, Hamid M., Alvaro Ortiz, and Thomas Y. Ma. A carrier-mediated mechanism for pyridoxine uptake by human intestinal epithelial Caco-2 cells: regulation by a PKA-mediated pathway. Am J Physiol Cell Physiol 285: C1219–C1225, 2003.—Vitamin B₆ is essential for cellular functions and growth due to its involvement in important metabolic reactions. Humans and other mammals cannot synthesize vitamin B₆ and thus must obtain this micronutrient from exogenous sources via intestinal absorption. The intestine, therefore, plays a central role in maintaining and regulating normal vitamin B₆ homeostasis. Due to the water-soluble nature of vitamin B₆ and the demonstration that transport of other water-soluble vitamins in intestinal epithelial cells involves specialized carrier-mediated mechanisms, we hypothesized that transport of vitamin B₆ in these cells is also carrier mediated in nature. To test this hypothesis, we examined pyridoxine transport in a model system for human enterocytes, the human-derived intestinal epithelial Caco-2 cells. The results showed pyridoxine uptake to be (1) linear with time for up to 10 min of incubation and to occur with minimal metabolic alteration in the transported substrate, (2) temperature and energy dependent but Na⁺ independent, (3) pH dependent with higher uptake at acidic compared with alkaline pHs, (4) saturable as a function of concentration (at buffer pH 5.5 but not 7.4) with an apparent Michaelis-Menten constant (Kₘ) of 11.99 ± 1.41 μM and a maximal velocity (Vₘₐₓ) of 67.63 ± 3.87 pmol·mg protein⁻¹·min⁻¹, (5) inhibited by pyridoxine structural analogs (at buffer pH 5.5 but not 7.4) but not by unrelated compounds, and (6) inhibited in a competitive manner by amiloride with an apparent inhibitor constant (Kᵢ) of 0.39 mM. We also examined the possible regulation of pyridoxine uptake by specific intracellular regulatory pathways. The results showed that whereas modulators of PKC, Ca²⁺/calmodulin (CaM), and nitric oxide (NO)-mediated pathways had no effect on pyridoxine uptake, modulators of PKA-mediated pathway were found to cause significant reduction in pyridoxine uptake. This reduction was mediated via a significant inhibition in the Vₘₐₓ, but not the apparent Kₘ, of the pyridoxine uptake process. These results demonstrate, for the first time, the involvement of a specialized carrier-mediated mechanism for pyridoxine uptake by intestinal epithelial cells. This system is pH dependent and amiloride sensitive and appears to be under the regulation of an intracellular PKA-mediated pathway.

VITAMIN B₆ REPRESENTS A GROUP of structurally related compounds (pyridoxine, pyridoxal, and pyridoxamine) that exist in the diet in different proportions in phosphorylated and unphosphorylated forms. Vitamin B₆ acts as a cofactor in a number of metabolic reactions that include amino acid metabolism, biosynthesis and degradation of sphingolipid, and carbohydrate metabolism. Deficiency of vitamin B₆ leads to a variety of clinical abnormalities and occurs in patients with a variety of conditions including alcoholism and diabetes and in patients on long-term use of isoniazid and other hydrazines (see Ref. 13 for review). Low suboptimal levels of vitamin B₆ have also been reported in patients with vitamin B₆-dependent seizures, an autosomal-recessive disorder believed to be due to an abnormality in pyridoxine transport into cells (5).

Humans and other mammals cannot synthesize vitamin B₆ and thus obtain the vitamin from exogenous sources via absorption in the intestine. The intestine, therefore, plays a central role in maintaining and regulating normal vitamin B₆ body homeostasis. Absorption of dietary vitamin B₆ occurs after hydrolysis of its phosphorylated forms in the intestinal lumen (7, 15, 16). Previous studies have reported that no net trans-epithelial transport or intracellular accumulation of vitamin B₆ occurs in the intestine (25). In addition, studies using intestinal preparations, such as everted sacs, vascularly perfused intestinal segments, and brush-border membrane vesicles, have failed to show saturation in the vitamin B₆ uptake process (7, 14, 31). Thus the uptake process of vitamin B₆ has been suggested to occur via simple diffusion. However, given the water-soluble nature of vitamin B₆ forms and the demonstration of involvement of specialized and regulated carrier-mediated mechanisms for uptake of other water-soluble vitamins in intestinal epithelial cells (transport events that were earlier thought to occur via simple diffusion), we sought in this investigation to reexamine the issue of intestinal vitamin B₆ uptake by using optimal physiological conditions that allow the uncovering of any carrier-mediated mechanism. We used a well-established model system for human enterocytes, the human-derived intestinal epithelial Caco-2 cells, and pyridoxine as a vitamin B₆ substrate.

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in our investigations. Our results indicate that vitamin B₆ uptake by human intestinal epithelial Caco-2 cells occurs via a specialized, carrier-mediated mechanism. This mechanism is pH dependent and amiloride sensitive and appears to be under the regulation of an intracellular protein kinase A (PKA)-mediated pathway.

MATERIALS AND METHODS

[3H]pyridoxine (custom-made; sp act 20 Ci/mmol, radiochemical purity >97%) was obtained from American Radiolabeled Chemical (St. Louis, MO). The radiochemical purity of [3H]pyridoxine was verified in our laboratory by thin-layer chromatography (TLC) procedure by using silica-precoated thin-layer plates and a solvent system of t-butanol, acetone, water, and diethylamine (8:7:4:1 vol/vol ratio) (14). The same chromatography procedure was used to determine the degree of metabolism in the transported pyridoxine into Caco-2 cells. All chemicals and reagents used in these studies were of analytical grade and were obtained from commercial sources. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). DMEM, trypsin, fetal bovine serum, and other cell culture reagents were obtained from Life Technologies (Grand Island, NY).

Caco-2 cells (passages 17 to 27) were grown as we described previously (19, 20). Uptake studies were performed on Caco-2 monolayers 3–5 days after confluence. Uptake of pyridoxine was examined in cells incubated in Krebs-Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO₄, 0.85 CaCl₂, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 5.5, unless otherwise stated) at 37°C. Labeled and unlabeled pyridoxine was added to the incubation medium at the onset of the uptake experiment. In certain experiments, cells were pretreated with the compound under study for a specific period of time before the addition of pyridoxine and the start of uptake experiments. Unless otherwise specified, uptake was examined over a period of 3 min, i.e., initial rate, and the reaction was terminated by the addition of 2 ml of ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer, digested with 1 ml of 1 N NaOH, neutralized with HCl, and counted for radioactivity. The protein content of cell digests was measured in parallel wells by using a Bio-Rad kit (Bio-Rad, Richmond, VA).

Uptake data presented in this paper are expressed as means ± SE of multiple separate uptake determinations and are expressed in terms of pico- or femtomoles per milligram of protein per unit time. Statistical differences were analyzed by Student’s t-test and ANOVA, with statistical significance being set at 0.05 (P < 0.05). Kinetic parameters of the saturable pyridoxine uptake process [i.e., the apparent Michaelis-Menten constant (Kₘ) and maximal velocity (Vₘₐₓ)] were calculated by using a computerized model of the Michaelis-Menten equation as described by Wilkinson (29).

RESULTS

General characteristics of pyridoxine uptake by Caco-2 cells. Figure 1 depicts the results of uptake of 0.1 and 10 μM pyridoxine by Caco-2 cells as a function of time. As can be seen, uptake of pyridoxine was linear at both concentrations examined (r = 0.99 for both) and occurred at a rate of 0.13 and 8.07 pmol·mg protein⁻¹·min⁻¹, respectively. Thus 3 min was used as the standard incubation time in all subsequent studies (unless otherwise stated).

In another study, we examined the metabolic form of the transported substrate after incubation of Caco-2 cells with 25 nM [3H]pyridoxine for 3 and 10 min. TLC procedure was used in the investigation (see MATERIALS AND METHODS). The results showed that 95 and 91% of the transported ³H radioactivity was in the form of intact pyridoxine.

We also examined the effect of varying the incubation temperature on the initial rate of pyridoxine (15 nM) uptake by Caco-2 cells. The results showed that decreasing the incubation temperature from 37 to 21 and 4°C caused a significant (P < 0.01) decrease in the initial rate of pyridoxine uptake (117.4 ± 3.2, 53.3 ± 1.5, and 12.4 ± 1.9 fmol·mg protein⁻¹·3 min⁻¹, respectively).
Role of Na⁺ and H⁺ in pyridoxine uptake by Caco-2 cells. In these investigations, we investigated the role of Na⁺ and H⁺ in the incubation medium on the initial rate of uptake of pyridoxine (15 nM) by Caco-2 cells. The role of Na⁺ was examined by testing the effect of Na⁺ removal from the incubation media on pyridoxine uptake (K⁺ was used to replace Na⁺). The results showed that pyridoxine uptake was similar in the presence and absence of Na⁺ (111.4 ± 6.0 and 111.9 ± 10.6 fmol·mg protein⁻¹·3 min⁻¹, respectively). In another study, we examined the effect of pretreatment of Caco-2 cells for 30 min with the Na⁺/K⁺-ATPase inhibitor, ouabain (1 mM), on the initial rate of pyridoxine (15 nM) uptake. The results showed no effect of such pretreatment on the vitamin uptake (110.3 ± 3.9 and 108.5 ± 3.9 fmol·mg protein⁻¹·3 min⁻¹ in the presence and absence of ouabain, respectively).

The role of H⁺ on pyridoxine uptake by Caco-2 cells was examined by investigating the effect of varying incubation buffer pH on pyridoxine uptake. The results showed that gradual lowering of the incubation buffer pH from 8 to 5 was associated with a gradual increase in initial rate of pyridoxine (15 nM) uptake (Fig. 2). Maximum uptake was reached at around buffer pH 5.5, and thus we used this buffer pH in all the studies described in this paper (unless otherwise stated). In another study, we examined the effect of the protein ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) on the uptake of pyridoxine (15 nM) by Caco-2 cells incubated at buffer pH 5.5. The results showed that FCCP (100 and 150 μM) caused significant (P < 0.01) inhibition in the initial rate of pyridoxine uptake (105.8 ± 4.5, 68.9 ± 3.6, and 48.0 ± 4.3 fmol·mg protein⁻¹·3 min⁻¹ for control and in the presence of 100 and 150 μM FCCP, respectively). We also examined the effect of lowering the intracellular pH of Caco-2 cells (i.e., decreasing the transmembrane H⁺ gradient) on the initial rate of pyridoxine uptake by cells incubated at buffer pH 5.5. Cell acidification was performed by a standard procedure as described previously (21, 26). Briefly, confluent Caco-2 monolayers were incubated for 30 min in Krebs-Ringer buffer in which NaCl was replaced with NH₄Cl. This was followed by removal of the buffer, washing the monolayers with KCl-containing Krebs-Ringer buffer (KCl replaced NaCl), and incubating the cells for 3 min in the same KCl-containing Krebs-Ringer buffer in the presence of 15 nM pyridoxine. Results were compared with pyridoxine uptake by cells preincubated for 30 min with a KCl-containing Krebs-Ringer buffer (instead of NH₄Cl-containing Krebs-Ringer buffer), with the rest of the cell handling being the same. A significant (P < 0.01) decrease in the initial rate of pyridoxine uptake was observed in acid-loaded cells compared with control cells (137.3 ± 5.5 and 85.2 ± 5.2 fmol·mg protein⁻¹·3 min⁻¹, respectively).

Evidence for involvement of a carrier-mediated mechanism in pyridoxine uptake by Caco-2 cells. In these studies, we examined the initial rate of pyridoxine uptake by Caco-2 cells incubated in buffer pH 5.5 as a function of a wide range of substrate concentrations (5 nM through 20 μM). The results showed pyridoxine uptake to be linear as a function of concentration in the nanomolar range (5 to 500 nM; Fig. 3A). On the other hand, the initial rate of pyridoxine uptake was found to include a saturable component when examined in the micromolar concentration range (Fig. 3B). Uptake of pyridoxine by the saturable component over the micromolar range was determined by subtracting uptake by the diffusion component (calculated from the slope of the uptake line at 1 mM pyridoxine and the point of origin) from the total uptake at each concentration examined. Kinetic parameters of the saturable component were then determined as described in MATERIALS AND METHODS and found to be 12.0 ± 1.4 μM and 67.6 ± 3.9 fmol·mg protein⁻¹·3 min⁻¹ for the apparent Kₘ and Vₘₐₓ, respectively. In contrast to the saturation observed in the initial rate of pyridoxine uptake as a function of micromolar substrate concentration in cells incubated at buffer pH 5.5, uptake of pyridoxine at buffer pH 7.4 was linear as a function of concentration (Fig. 3C).

In other studies, we examined the effect on the initial rate of [³H]pyridoxine uptake of adding to the incubation medium 50 μM of the unlabeled pyridoxine and the pyridoxine-related compounds pyrdoxal, pyridoxal-5-phosphate, pyridoxamine, and pyridoxic acid. We also examined the effect of the unrelated compounds theophylline, penicillamine, and isoniazid (compounds that have been shown to inhibit the activity of the cytoplasmic pyridoxal kinase, i.e., the enzyme that converts vitamin B₆ compounds from the unphosphorylated to the phosphorylated forms; Ref. 9), as well as that of homocysteine on pyridoxine uptake. The results (Table 1) showed that whereas pyrdoxal and pyridoxal-5-phosphate did not inhibit [³H]pyridoxine uptake, unlabeled pyridoxine, pyridoxamine, 4-deoxyxypyrdoxine, and pyridoxic acid were all found to cause significant (P < 0.01) inhibition in
In another study, we examined possible stimulation of \(^{3}\text{H}\)pyridoxine efflux from Caco-2 cells preloaded with the compound by unlabeled pyridoxine (100 \(\mu\)M). In this study, we first preloaded the cells with \(^{3}\text{H}\)pyridoxine by incubating them with 25 nM \(^{3}\text{H}\)pyridoxine for 10 min and then incubating the cells in Krebs-Ringer buffer pH 5.5 in the presence and absence of 100 \(\mu\)M unlabeled pyridoxine. The results showed that cell content of \(^{3}\text{H}\) radioactivity was significantly \((P < 0.01)\) lower in cells incubated in the presence of unlabeled pyridoxine in the incubation medium compared with those incubated in the absence of unlabeled pyridoxine (cell content of 161.9 \(\pm\) 8.1 and 232.0 \(\pm\) 13.3 fmol \(\cdot\) mg protein \(^{-1}\) \(\cdot\) min \(^{-1}\), respectively).

Effect of membrane transport inhibitors and metabolic poisons on pyridoxine uptake by Caco-2 cells. In these studies, we examined the effect of the membrane transport inhibitors DIDS, probenecid, and amiloride on the initial rate of uptake of pyridoxine (15 nM). The results showed that whereas DIDS and probenecid had no effect on pyridoxine uptake, significant \((P < 0.01)\) inhibition was caused by amiloride (134.0 \(\pm\) 5.7, 135.2 \(\pm\) 15.7, 113.6 \(\pm\) 10.1, and 54.7 \(\pm\) 3.7 fmol \(\cdot\) mg protein \(^{-1}\) \(\cdot\) min \(^{-1}\) for control and in the presence of DIDS, probenecid, and amiloride, respectively). To further characterize the inhibitory effect of amiloride, we examined the effect of different concentrations of the inhibitor on the initial rate of uptake of 0.3 and 3 \(\mu\)M pyridoxine and then applied the data to the Dixon plot. The results (Fig. 4) showed the inhibition to be competitive in nature with a \(K_i\) of 0.39 mM.

In other studies, we examined the effect of pretreating Caco-2 cells (for 30 min) with the metabolic inhibitors azide (5 mM), iodoacetate (1 mM), and dinitrophenol (5 mM) on the initial rate of pyridoxine (15 nM) uptake. The results showed that all of the tested compounds caused significant inhibition in pyridoxine uptake \((110.3 \pm 3.9, 77.4 \pm 4.6 (P < 0.05), 65.1 \pm 10.8 (P < 0.01), \text{and} 17.5 \pm 3.4 (P < 0.01) \text{fmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1} \text{for control and in the presence of azide, iodoacetate, and dinitrophenol, respectively).}

Table 1. Effect of unlabeled pyridoxine and pyridoxine-related and unrelated compounds on the uptake of \(^{3}\text{H}\)pyridoxine by Caco-2 cells

<table>
<thead>
<tr>
<th>Compound (50 (\mu)M)</th>
<th>Pyridoxine Uptake, (\text{fmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1})</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>132.0 (\pm) 4.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>58.2 (\pm) 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>130.0 (\pm) 5.4</td>
<td>NS</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate</td>
<td>131.2 (\pm) 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Pyridoxamination</td>
<td>43.8 (\pm) 1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4-Deoxy-pyridoxine</td>
<td>107.0 (\pm) 4.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pyridoxic acid</td>
<td>92.3 (\pm) 3.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Theophylline</td>
<td>134.3 (\pm) 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>137.0 (\pm) 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Homocystein</td>
<td>153.4 (\pm) 6.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Confluent monolayers of Caco-2 cells were incubated at 37 °C in Krebs-Ringer buffer, pH 5.5, in the presence of 15 nM \(^{3}\text{H}\)pyridoxine and 50 \(\mu\)M of the compound under investigation. Uptake was measured after 3 min of incubation, i.e., initial rate. Data are means \(\pm\) SE of 3–8 separate uptake determinations.

Fig. 3. Pyridoxine uptake by Caco-2 cells as a function of concentration. Confluent monolayers of Caco-2 cells were incubated in Krebs-Ringer buffer at 37 °C in the presence nanomolar (A) and micromolar (B) concentrations of pyridoxine at pH 5.5 and in the presence of micromolar concentration of pyridoxine at buffer pH 7.4 (C). Initial rate of uptake (3 min) by the saturable component (B) was determined as described in RESULTS. Data are means \(\pm\) SE of 3–4 separate uptake determinations. When not shown, error bars are smaller than the symbols.
The possible role of a PKC-mediated pathway in the regulation of pyridoxine uptake by Caco-2 cells was examined by testing the effect of pretreatment of the cells (for 1 h) with modulators of this pathway on the initial rate of uptake of pyridoxine (15 nM). The results showed that neither an activator of PKC (phorbol-12-myristate 13-acetate, PMA; 10 μM) nor inhibitors of the pathway (bisindolylmaleimide I, Bis I, 10 μM; and chelerythrin, 1 μM) significantly affected pyridoxine uptake by these cells (123.1 ± 3.4, 128.3 ± 6.0, and 120.6 ± 3.5 fmol·mg protein⁻¹·min⁻¹ for control and in the presence of PMA, Bis I, and chelerythrin, respectively). We also examined the possible role of CaM-mediated pathway in the regulation of pyridoxine uptake by Caco-2 cells. This was performed by examining the effect of pretreatment of the cells (for 1 h) with inhibitors of this pathway [triﬂuoperazine, TFP, 100 μM; calmidazolium, 50 μM; and N-(4-aminobuty)l-5-chloro-2-naphthalene sulfonamide, W13, 100 μM] on the initial rate of pyridoxine (15 nM) uptake. The results showed that none of the tested compounds caused any significant inhibition in pyridoxine uptake (120.3 ± 0.8, 118.0 ± 2.7, 117.6 ± 0.5, and 119.6 ± 3.1 for control and in the presence of TFP, calmidazolium, and W13, respectively). Similarly, the role of a NO-mediated pathway in the regulation of pyridoxine uptake was examined by investigating the effect of pretreatment of Caco-2 cells (for 6 h) with modulators of the NO-mediated pathway (3-morpholynlysydnoneimine, SIN; and S-nitroso-N-acetyl-penicillamine, SNAP; both were used at 1 mM) on initial rate of pyridoxine (15 nM) uptake. The results showed that neither of these compounds signiﬁcantly affected pyridoxine uptake (127.5 ± 3.0, 132.8 ± 3.0, and 135.0 ± 2.0 fmol·mg protein⁻¹·min⁻¹ for control and after pretreatment with SIN and SNAP, respectively).

In these studies, we examined the possible regulation of the pyridoxine uptake process of Caco-2 cells by specific intracellular regulatory pathways. We focused on the intracellular regulatory pathways that have been shown to exert regulatory effects on transport of other nutrients (including other water-soluble vitamins) in intestinal and other epithelial cells (8, 10, 19, 20, 23, 24). These pathways include PKA, PKC, Ca²⁺/calmodulin (CaM), and nitric oxide (NO)-mediated pathways. The role of the PKA-mediated pathway was investigated by examining the effect of pretreatment of Caco-2 cells (for 1 h) with 1 and 5 mM dibutyryl cAMP (DBcAMP) or with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) on the initial rate of pyridoxine (15 nM) uptake. The results showed significant (P < 0.01) inhibition in pyridoxine uptake by these compounds (125.5 ± 10, 63 ± 10, 40 ± 5, and 46.7 ± 4.6 fmol·mg protein⁻¹·min⁻¹ for control and after pretreatment with 1 and 5 mM DBcAMP and with IBMX, respectively). To determine whether the effect of DBcAMP is mediated through a change in the Vₘₐₓ and/or the apparent Kₘ of the pyridoxine uptake process, we examined the effect of pretreatment with 1 mM DBcAMP on the initial rate of uptake of the vitamin as a function of concentration. We then determined the kinetic parameters of the saturable pyridoxine uptake process and compared the results with that of simultaneously performed (untreated) controls. The results (Fig. 5) showed that pretreatment of Caco-2 cells with DBcAMP led to a significant (P < 0.01) decrease in the Vₘₐₓ of the pyridoxine uptake process with no significant change in its apparent Kₘ (Vₘₐₓ of 36.7 ± 3.4 and 72.4 ± 6.6 fmol·mg protein⁻¹·min⁻¹ and apparent Kₘ of 13.7 ± 2.3 and 12.0 ± 2.1 μM for DBcAMP pretreated and control cells, respectively).

**Fig. 4. Effect of amiloride on pyridoxine uptake by Caco-2 cells.** Confluent monolayers of Caco-2 cells were incubated for 3 min (initial rate) in Krebs-Ringer buffer, pH 5.5, at 37°C in the presence of 0.3 and 3 μM pyridoxine and different concentrations of amiloride. Data, shown in the form of Dixon plot, are means ± SE of 4 separate uptake determinations. When not shown, error bars are smaller than the symbols.

**Fig. 5. Effect of pretreatment of Caco-2 cells with dibutyryl cAMP (DBcAMP) on pyridoxine uptake as a function of concentration.** Confluent monolayers of Caco-2 cells were first pretreated (for 1 h) with 1 mM DBcAMP. Different concentrations of pyridoxine were then added to the incubation medium (Krebs-Ringer buffer, pH 5.5), and uptake was measured after 3 min (initial rate) incubation. Data are means ± SE of 4–8 separate uptake determinations.
DISCUSSION

The aim of these studies was to investigate the mechanism and regulation of vitamin B₆ uptake by human enterocytes by using the human-derived intestinal epithelial Caco-2 cells as a model system and pyridoxine as a vitamin B₆ substrate. These cells were chosen because previous studies have shown that they represent an excellent experimental model system for native human enterocytes because they possess many of the functions and structural features of the native enterocytes, including similar transport mechanisms and regulatory pathways. Our results showed the uptake of pyridoxine by these cells to be appreciable and linear with time for up to 10 min of incubation. Uptake was also found to occur with minimal metabolic alterations in the transported substrate as demonstrated by the studies with TLC. The transport process of pyridoxine was found to be temperature dependent but was Na⁺ independent in nature. The latter conclusion is based on the findings that Na⁺ replacement in the incubation medium did not affect the initial rate of pyridoxine uptake and that pretreatment of the cells with ouabain, the inhibitor of the Na⁺/K⁺-ATPase, had minimal effect on pyridoxine uptake. In contrast, our findings showed that increasing the concentration of H⁺ in the incubation medium (via lowering the incubation buffer pH from 8 to 5) was associated with a marked increase in pyridoxine uptake. This increase was reduced after treatment of cells with the proton ionophore FCCP when the intracellular pH was made more acidic. Both of the latter manipulations led to a decrease in the transmembrane pH gradient. Collectively, these results suggest that the transport of pyridoxine may be occurring via a pyridoxine:H⁺ symport mechanism. Further studies, however, are needed to confirm this suggestion. It is of interest to mention here that the recently cloned pyridoxine transport system of Saccharomyces cerevisiae, i.e., Tpn1, was also found to be acidic pH dependent and proposed to function via a pyridoxine:H⁺ symport (27). The existence of a low pH compartment at the luminal surface of the small intestine, the so-called “intestinal surface acid microclimate” (11, 17), may provide the needed H⁺ for transport of pyridoxine, as has been suggested previously for the intestinal folate uptake, which also occurs by an acidic pH-dependent process (18).

Uptake of pyridoxine by Caco-2 cells was found to be carrier mediated in nature. This conclusion is based on a number of observations. First, the initial rate of pyridoxine uptake at buffer pH 5.5 as a function of concentration was found to include a saturable component (apparent Km of 11.99 ± 1.41 μM and a Vmax of 67.63 ± 3.87 pmol·mg protein⁻¹·3 min⁻¹). Second, significant cis-inhibition was observed in [³H]pyridoxine uptake upon the addition of unlabeled pyridoxine and its structural analogs pyridoxamine, pyridoxic acid, and 4-deoxy-pyridoxine to the incubation medium at buffer pH 5.5. Third, significant trans-stimulation was observed in [³H]pyridoxine efflux from Caco-2 cells preloaded with the [³H]pyridoxine and incubated in the presence of unlabeled pyridoxine in the incubation medium. The carrier-mediated system for pyridoxine uptake by Caco-2 cells was found to be specific in nature because the unrelated compounds theophylline, penicillamine, and homocysteine all failed to affect pyridoxine uptake. It is of interest to mention here that theophylline and penicillamine are known inhibitors of pyridoxal kinase (9), yet they did not affect pyridoxine uptake. This further differentiates between the event of pyridoxine uptake process at the level of cell membrane and interaction with the cytoplasm-metabolizing enzyme pyridoxal kinase. Also interesting is the finding of only one carrier-mediated mechanism for pyridoxine uptake by Caco-2 cells that operates within the low micromolar concentration range. In this regard, pyridoxine appears to be different from uptake of some other water-soluble vitamins (e.g., biotin) in certain cell types in which more than one uptake system have been reported (6, 12). The inability of previous work to demonstrate existence of a carrier-mediated mechanism for pyridoxine uptake by intestinal epithelial cells could be due to the use of unfavorable incubation buffer pH (e.g., pH 7.4 when the carrier system does not function; see RESULTS), the use of a relatively high pharmacological concentration of pyridoxine in uptake studies, and/or the employment of indirect methods to measure intestinal pyridoxine absorption (urinary excretion) (1, 3, 4, 14, 25, 28).

The uptake process of pyridoxine by Caco-2 cells was also found to be energy dependent. This conclusion is based on the observation that pretreatment of cells with metabolic poisons led to a significant inhibition in the initial rate of pyridoxine uptake. Whereas no effect was seen with the membrane transport inhibitors DIDS and probenecid, the uptake process of pyridoxine was found to be sensitive to the inhibitory effect of the diuretic agent amiloride. The amiloride inhibition was found to be competitive in nature with an apparent Ki of 0.39 mM. It is interesting to mention here that amiloride was also found to inhibit pyridoxine uptake by renal epithelial cells (2, 9, 22) and by certain yeast strains (30). The ability of amiloride to inhibit intestinal and renal pyridoxine uptake raises the possibility that this diuretic agent may interfere with normal vitamin B₆ body homeostasis. Further studies are required to test this possibility.

After the delineation of the mechanism of pyridoxine uptake process by Caco-2 cells, we moved to examine the possible regulation of the uptake process by intracellular regulatory mechanisms. We focused on regulatory pathways that have been shown to exert regulatory effects on transport of other nutrients and substrates (including other water-soluble vitamins) in intestinal and other epithelia (8, 10, 19, 20, 23, 24). The results showed that whereas the regulatory pathways mediated by PKC, CaM, and NO did not exert regulatory effect on pyridoxine uptake by Caco-2 cells, the PKA-mediated pathway did appear to have a regulatory role in the vitamin uptake. The latter conclusion is based on the observation that pretreatment of Caco-2 cells with compounds that lead to an increase in intra-
cellular cAMP level, thus activating PKA, cause a significant inhibition in the initial rate of pyridoxine uptake. This inhibition was mediated via a decrease in the activity (and/or the number), but not the affinity, of the pyridoxine uptake process. The latter conclusions were based on the observations that pretreatment of Caco-2 cells with dB cAMP led to a significant decrease in the $V_{\text{max}}$ but not the apparent $K_m$ of the pyridoxine uptake process, respectively. The molecular mechanism by which cAMP-mediated pathway exerts its effect on pyridoxine uptake process is not clear and requires further investigation.

In summary, results of the present studies demonstrate for the first time the involvement of a carrier-mediated mechanism for pyridoxine uptake by intestinal epithelial cells. This system is specific in nature and pH and is amiloride sensitive, and it appears to be under the regulation of an intracellular PKA-mediated pathway.

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