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Transmural Absorption of Pyridoxine·HCl *in Vitro* in the Rat Jejunum[1] (41207)

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Abstract. Transmural absorption of [^{3}H]pyridoxine·HCl ([^{3}H]PN) was studied in Sprague–Dawley rats *in vitro* utilizing everted sacs and perfused (serosal lumen) everted segments of jejunum. The predominant form of vitamin B_6 to appear in the serosal fluid was [^{3}H]PN. Phosphorylated vitamin ([^{3}H]B_6PO_4) was limited primarily to the jejunal wall (44.6 ± 3.4% of total tissue ^{3}H) with only minimal amounts detectable in the serosal fluid (7.0 ± 1.5% of total serosal ^{3}H). The appearance of absorbed vitamin in the serosal perfusate, expressed on a per micromolar [^{3}H]PN basis, was significantly increased when the mucosal PN concentration was increased to 1 m*M*. Likewise, serosal appearance of vitamin was increased for 2 μM [^{3}H]PN when 1 m*M* 4-deoxypyridoxine was added to the mucosal solution. When a high phosphate buffer (80 m*M*) was substituted for the Krebs' bicarbonate buffer (1.1 m*M* phosphate) in the serosal perfusing solution, tissue content of [^{3}H]B_6PO_4 was increased a significant 49.7%. In contrast, substitution of phosphate buffer in the mucosal solution had no significant effect on tissue [^{3}H]B_6PO_4. Conclusions are (i) PN is the principal form of absorbed vitamin B_6 to appear on the serosal side of everted rat jejunum *in vitro;* (ii) intracellular metabolism leads to a compartmentalization of [^{3}H]PN after its uptake by mucosa; (iii) this metabolism is associated with a significant decrease in the serosal appearance of absorbed vitamin; and (iv) *in vitro* increases in tissue [^{3}H]B_6PO_4 by phosphate inhibition of phosphatase hydrolysis are dependent upon the high phosphate concentrations being present on the serosal, not the mucosal, side of the jejunum.

Previous studies both *in vitro* and *in vivo* indicate that pyridoxine·HCl (PN)[3] is taken up in the rat jejunum by passive diffusion and then undergoes substantial phosphorylation intramurally (1–6), presumably as a result of the cytoplasmic enzyme, pyridoxal kinase (5). The phosphorylated vitamin so formed ([^{3}H]B_6PO_4) also undergoes dephosphorylation by a process which is inhibited by high phosphate concentrations and is compatible with a phosphatase reaction (3). An understanding of the significance of this phosphorylation and dephosphorylation is incomplete. Short-term absorption studies for PN *in vivo* in isolated loops of rat jejunum have indicated that there is a delay in the serosal exit of absorbed, radiolabeled vitamin which correlates with the presence of phosphorylation (4). This delay, as well as the fact that [^{3}H]B_6PO_4 is the predominate form of radiolabeled vitamin in tissues during *in vivo* absorption, suggests that there may be compartmentalization of the vitamin after mucosal uptake (4).

The purpose of the present studies was to evaluate *in vitro* [^{3}H]PN absorption further by assessing transmural—and thus indirectly—serosal net flux of absorbed vitamin. The specific aims were (i) to measure directly the serosal efflux of vitamin during absorption, (ii) to evaluate further the apparent compartmentalization of absorbed vitamin by studying the distribution of ^{3}H-labeled vitamin B_6 compounds in medium, tissue, and serosal fluid of everted sacs during *in vitro* incubation, and (iii) to de-

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[3] Abbreviations used: PN, pyridoxine; PL–PM, pyridoxal–pyridoxamine; [^{3}H]B_6PO_4, phosphorylated vitamin B_6 derived from absorbed pyridoxine.

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termine the relative effects of high phosphate concentrations on the mucosal and serosal sides of the bowel wall.

Materials and Methods. Male Sprague–Dawley rats (Holtzman Company, Madison, Wisc.) weighing 100–200 g were maintained on a commercial stock diet consisting of 4.5 ppm vitamin B_6 (Purina Rat Chow, Cherokee Feed and Supply Co., Atlanta, Ga.) without fasting and were permitted free access to water. The animals were killed by cervical dislocation; the proximal jejunum was removed and everted over a glass rod. At this point the everted tissue was then processed according to one of two experimental models to be described. Incubation media consisted of either a Krebs' bicarbonate buffer or an 80 m*M* phosphate buffer to which [^{3}H]pyridoxine·HCl (1.1–1.5 Ci/mmole; 94–96% radiopurity: Amersham/Searle, Arlington Heights, Ill.) had been added. Media were gassed with either 95% O_2–5% CO_2 (pH 7.4) or 100% O_2 (pH 7.4) and were maintained at 37° in a water bath. All chemicals were standard laboratory reagents.

In vitro perfused everted segments. After removal and eversion of jejunum, glass tubing was inserted into each end of a 5- to 6-cm segment and was held in place with silk ligatures. The serosal lumen so formed was then perfused with buffer by means of a peristaltic pump connected in series with both the everted segment and a serosal reservoir containing 30 ml of buffer solution maintained at 37° and gassed with either 95% O_2–5% CO_2 or 100% O_2. Flow through the segment was approximately 8 ml/min. Following a 5-min preincubation at 37° in a pyridoxine-free buffer, the segment was transferred to an incubation medium containing 2 μM PN in a total volume of 25 ml. Serosal perfusion was continued; incubation continued for 30 min with 100-μl aliquots being taken from the serosal reservoir as needed. At the end of the incubation, the segment was removed, rinsed, weighed, and then processed for determination of either dry tissue weight or tissue [^{3}H]B_6PO_4 by paper chromatography. Dry tissue weight was determined in preweighed vials after heating overnight at 80°. For chromatography wet tissue weights were determined; segments were homogenized and washed with 5% TCA; and supernatants were processed as previously described (2).

In vitro everted sac model. Everted sacs (5 cm) were prepared by filling the serosal lumen of the everted jejunum with Krebs' bicarbonate buffer and closing the ends with silk ligatures as previously described (2). After a 5-min preincubation in pyridoxine-free buffer, the sacs were incubated for 30 min in medium consisting of 2 μM PN in Krebs' bicarbonate buffer. At the end of the incubation, the sacs were removed and rinsed, and serosal fluids were flushed into centrifuge tubes using 2.0 ml of 0.9% NaCl. The flushed solutions were centrifuged to remove any possible solid debris, and equal volumes of 10% TCA were added to the decanted supernatants. Aliquots of supernatants from the tissue and serosal fluid as well as incubation medium were then processed by paper chromatography.

Chromatography. The separation of phosphorylated forms of vitamin B_6 from the nonphosphorylated forms utilized techniques as previously described (1, 2). Briefly, the supernatants following homogenization and washing of tissue with 5% TCA were combined and extracted with peroxide-free ethyl ether. Aliquots of the resulting extracts were streaked on strips of Whatman 3MM paper and were run overnight at 4° in the dark utilizing *t*-butanol:acetone:water:diethylamine (8:7:4:1) as a solvent. The strips were then dried, cut into 1-cm strips, and counted in a scintillation spectrometer. This chromatographic method isolates all phosphorylated forms of the vitamin into a single group at the origin ([^{3}H]B_6PO_4) and results in some overlap in the migrations of pyridoxal and pyridoxamine (PL–PM) (2). No attempts were made in these studies to resolve either [^{3}H]B_6PO_4 or [^{3}H]PL–PM into their respective components. Such resolution would have added little to the specific information sought or to the conclusions drawn.

Statistics. Data were expressed as mean ± SEM. Statistical significance was determined by Student's *t* test (7).

Results. *Distribution of ^3H.* To study the distribution of ^{3}H-labeled B_6 compounds across the intestinal wall during *in vitro* incubation in [^{3}H]PN, everted sacs with Krebs' bicarbonate buffer in the serosal lumina were incubated for 30 min in medium containing 2 μM [^{3}H]PN in Krebs' bicarbonate buffer. Figure 1 demonstrates the resulting distribution of ^{3}H-labeled vit B_6 compounds in medium jejunal walls, and serosal fluids. [^{3}H]B_6PO_4 was limited primarily to the tissue compartment where it constituted 44.6 ± 3.4% of total tissue ^{3}H. It was present in negligible amounts in the medium and represented only 7.0 ± 1.5% of total label in serosal fluid. The primary constituent of the serosal fluid was [^{3}H]PN, which accounted for 80.3 ± 2.7% of total serosal fluid label. Significant amounts of [^{3}H]PL−PM were also present, representing 12.3 ± 0.6% of tissue label and 13.7 ± 1.4% of serosal fluid label.

Serosal appearance of [^3H]vit B_6. To determine the serosal appearance of ^{3}H-vit B_6 from rat jejunum *in vitro,* the perfused everted segment model was used. Aliquots (100 μl) of buffer from the serosal reservoir were taken at 10-min intervals for 30 min, placed in scintillation fluid, and counted.

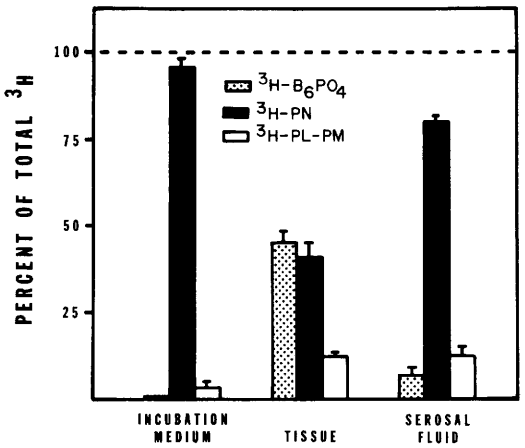

FIG. 1. Distribution of [^{3}H]vitamin B_6 after incubation of jejunal everted sacs for 30 min in 2 μM PN in Krebs' bicarbonate buffer. Data are expressed as the means ± SEM for 11 determinations for tissue and 3 determinations each for incubation medium and serosal fluid. Percentages total 100% each for medium, tissue, and serosal fluid.

The mucosal incubation medium contained 2 μM PN, 1 mM PN, or 2 μM PN with 1 mM 4-deoxypyridoxine (4-DP). The results are illustrated in Fig. 2. A compares serosal appearance for 2 μM PN and 1 mM PN. Serosal [^{3}H]vit B_6, expressed as nanomoles per micromolar PN per 100 mg dry tissue, was significantly higher for 1 mM PN than for 2 μM PN. B compares 2 μM PN with and without 1 mM 4-DP; serosal appearance was significantly greater in the presence of 4-DP than in its absence.

Differential effect of high phosphate concentrations in mucosal and serosal fluids. To determine if the previously described inhibitory effect of phosphate on the phosphatase hydrolysis of tissue [^{3}H]-B_6PO_4 could be localized to either the mu-

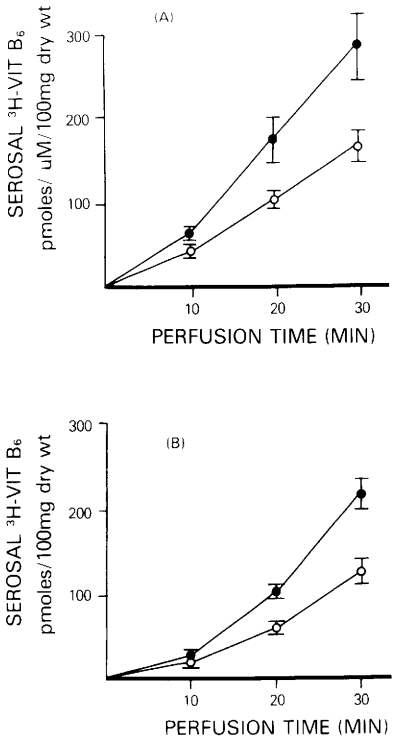

FIG. 2. Appearance of [^{3}H]vitamin B_6 in serosal perfusates during *in vitro* incubation of segments of rat jejunum in medium containing [^{3}H]PN. A compares 2 μM (open circles) and 1 mM (closed circles) [^{3}H]PN. B compares 2 μM (open circles) with 2 μM PN plus 1 mM 4-deoxypyridoxine (closed circles). Each circle represents the mean ± SEM for 10 (A) or 6 (B) determinations.

cosal or the serosal side of the everted sac, the effect of high phosphate buffer in either the medium or the serosal fluid only was studied (3). Initial experiments in everted sacs, while continuing to show an increase in tissue [^{3}H]B_6PO_4 when 80 m*M* phosphate buffer was used on both serosal and mucosal sides, failed to demonstrate similar increases when 80 m*M* phosphate was used on either the mucosal or the serosal side alone. To circumvent the possibility that the very small amount of buffer present in the serosal lumen of everted sacs might not be sufficient to maintain high phosphate concentrations on the serosal side during prolonged incubation in the presence of a high serosal-mucosal gradient, the *in vitro* perfused everted segment model was used. Control studies utilized Krebs' bicarbonate buffer in both medium and serosal fluid. Experimental perfusions substituted 80 m*M* phosphate buffer for either the medium or the serosal fluid. Table I depicts the results obtained. When phosphate buffer was used in the incubation medium (mucosal side), tissue [^{3}H]B_6PO_4 was not statistically different from that of controls. When high phosphate buffer was used in the serosal perfusing solution, however, tissue [^{3}H]-B_6PO_4 was significantly increased 49.7% over that of controls.

Discussion. Jejunal uptake of low concentrations of PN is associated with intestinal phosphorylation of a significant portion of the absorbed vitamin (1–4, 6). The data in Fig. 1 demonstrate that phosphorylated vitamin so formed is limited largely to the tissue compartment. The comparable amounts of [^{3}H]PN and [^{3}H]B_6PO_4 in tissue and the much lower relative amounts of [^{3}H]B_6PO_4 in serosal fluid suggest that the [^{3}H]B_6PO_4 formed during vitamin uptake exits poorly from the jejunal wall. Such results have been observed by others (8) and are qualitatively similar to the failure of [^{3}H]PLP to exit from red blood cells incubated in [^{3}H]PN (9). The failure of this apparent gradient to affect the transfer of intact [^{3}H]B_6PO_4 from tissue to serosal fluid could be the result of intracellular binding, low permeability of epithelial basolateral membranes, or interference by the underlying muscle layers with diffusion into the serosal fluid. The last possibility seems unlikely, however, since the tendency for phosphorylated vitamin to remain within the tissue compartment has also been observed *in vivo* where diffusion through muscle layers is not a factor (4). The relative importance of the first two possibilities is speculative and will require further study for resolution. Whatever the exact cause of the compartmentalization of phosphorylated vitamin in tissue, it is quite clear that the predominant form of the vitamin exiting into the serosal fluid is still PN. Such results suggest that intestinal absorption of PN is best characterized by a net mucosal-to-serosal flux of unchanged PN.

In light of the limitation of [^{3}H]B_6PO_4 primarily to the tissue compartment, what is the effect of phosporylation on transmural vitamin flux? Previous studies from this laboratory, utilizing the *in vivo*-isolated loop model have demonstrated that transmural absorption of 2 μM [^{3}H]PN lags behind mucosal uptake and that transmural absorption can be significantly increased by experimental conditions which minimize the relative effect of phosphorylation (4).

TABLE I. LOCALIZATION OF EFFECT OF HIGH PHOSPHATE CONCENTRATION ON JEJUNAL [^{3}H]B_6PO_4

Experiment	No. rats	Phosphate concentration		Tissue [^{3}H]B_6PO_4[a]	P[b]
		Medium (m*M*)	Perfusate (m*M*)		
Control	5	1.1	1.1	0.775 ± 0.057[c]	—
Mucosal PO_4	7	80	1.1	0.861 ± 0.083	NS[d]
Serosal PO_4	7	1.1	80	1.16 ± 0.10	<0.01

[a] Units are in nmole/g wet tissue and represent total tissue content after a 30-min incubation.
[b] Statistical analysis compares each high phosphate result with the control result.
[c] Mean ± SEM.
[d] NS = not statistically significant.

The present data confirm these previous studies by utilizing a model which permits direct measurement of the serosal appearance of vitamin. Similar to the *in vivo* data, both 1 m*M* pyridoxine (far in excess of saturating concentrations for the kinase) and 1 m*M* 4-deoxypyridoxine (which acts as a competitive inhibitor for the kinase) were associated with greater serosal appearance of absorbed vitamin compared to control experimental conditions (2 μM PN only) (4, 5). In contrast to these data, Buss *et al.*, utilizing the vascularly perfused rat small intestine *in vitro,* have reported a linear relationship between luminal pyridoxine doses and the appearance of pyridoxine in the vascular perfusate over a 10,000-fold range of PN concentrations (6).

The reason for the different results from this laboratory and from that of Buss *et al.* is not known with certainty. Differences in experimental design must always be considered possible factors. However, it is of note that the lowest dose of intraluminal PN used by Buss, *et al.* was 0.2 μmole. Though that amount of PN was low compared to the daily nutritional requirements for a rat, the 1.0-ml volume in which it was dissolved should have yielded an initial intraluminal PN concentration of 20 μM (6, 10). Both *in vitro* and *in vivo* studies from this laboratory suggest that the phosphorylating system in rat jejunum is already near saturation at 20 μM (3, 4). Therefore, the demonstration of increases in serosal appearance at high PN concentrations both *in vivo* and *in vitro* from this laboratory and the lack of such an observation by Buss *et al.* may possibly relate to differences in the lowest PN concentrations studied.

The observation that high-phosphate concentrations increase the tissue content of $[^3H]B_6PO_4$ only if it is on the serosal side of the jejunum is of interest. Previous studies have demonstrated that this increased tissue $[^3H]B_6PO_4$ is related to inhibition of a phosphatase reaction (3). Why the high phosphate concentration must be on the serosal side is not known. Several possibilities, however, warrant mention. First, permeability differences between mucosal and serosal sides might permit differing concentrations of phosphate at the site of phosphatase activity. Since alkaline phosphatase is localized primarily to the brush border membrane in the small intestine, however, it seems unlikely that serosal phosphate would be a more effective inhibitor than mucosal phosphate (11). Second, alkaline phosphatase has been demonstrated histochemically on the basolateral membrane of absorptive cells (12). If it is this phosphatase that is hydrolyzing $[^3H]$-B_6PO_4, then the effect of serosal phosphate concentrations would appear more reasonable. However, such phosphatase activity must be quite low compared to mucosal activity, and the present data really do not give any specific information about the localization of enzyme activity beyond the necessity for inhibiting concentrations of phosphate to be on the serosal side. Finally, the increase in $[^3H]B_6PO_4$ might possibly represent an increase in the muscular layers of the intestine rather than the mucosal layer. Though the present studies did not specifically measure the differential localization of $[^3H]B_6PO_4$, such a possibility seems unlikely since previous studies both in this laboratory and elsewhere have demonstrated that phosphorylated vitamin is primarily in the mucosal layer, not in the deeper muscular layers (3, 6).

Thus, the need for serosal high phosphate concentrations to inhibit phosphatase hydrolysis of $[^3H]B_6PO_4$ has been demonstrated; the cellular basis for this requirement, however, is not known at present and needs further evaluation. Whatever the explanation, one conclusion based upon the data available seems reasonable. It is unlikely that variations in intraluminal phosphate concentrations (i.e., dietery intake) under normal *in vivo* conditions has a significant effect on intracellular hydrolysis of phosphorylated B_6.

In summary, transmural absorption of PN by everted sacs in the rat jejunum *in vitro* consists principally of a net flux of PN from medium to serosal fluid. Concurrent with this net flux is a phosphorylation of significant quantities of absorbed PN which leads to compartmentalization; this in turn at sufficiently low concentrations leads to significant delay in the net transmural flux of the absorbed vitamin. The ability to in-

hibit the phosphatase hydrolysis of phosphorylated vitamin appears to depend upon sufficiently high concentrations of phosphate on the serosal side of the bowel segment and appears to be unrelated to mucosal concentrations.

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