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Pyridoxal 5′-Phosphate Disappearance from Perfused Rat Jejunal Segments: Correlation with Perfusate Alkaline Phosphatase and Water Absorption[1,2] (41733)

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Abstract. The relative effects of perfusate alkaline phosphatase activity and net water absorption on 2 μM pyridoxal 5′-phosphate (PLP) luminal disappearance from rat jejunum were studied in a single-pass, *in vivo* perfused intestinal segment model. Perfusate consisted of unlabeled PLP in buffer (pH = 7.4). Net water flux was monitored by inclusion of [^{3}H]polyethylene glycol. PLP was measured by the [^{14}C]tyrosine apodecarboxylase assay. Single and multiple regression analysis of results during perfusion of 2 μM PLP in Krebs bicarbonate buffer demonstrated no correlation between perfusate alkaline phosphatase activity and net water absorption and significant correlations between PLP luminal disappearance and both perfusate alkaline phosphatase activity and net water absorption. Correlation for the latter was improved when disappearance results were corrected for variations in perfusate alkaline phosphatase activity. When perfusate buffers were selected to yield divergent rates of net water absorption, the one associated with greater net water absorption was also associated with greater PLP disappearance. That this could not be explained by changes in perfusate alkaline phosphatase activity was demonstrated both by assessment of the rate of decay of PLP added *in vitro* to exited perfusate incubated at 37°C and by measurement of alkaline phosphatase activity under conditions defined by the buffers using a modified spectrophotometric assay. Conclusions were: (1) *In vivo* PLP luminal disappearance correlates significantly with both perfusate alkaline phosphatase activity and net water absorption; (2) these two factors appear to act as independent variables; and (3) future studies on PLP intestinal absorption will need to take both of these variables into account in the interpretation of results.

Vitamin B_6 exists in both nonphosphorylated (pyridoxine, pyridoxal, and pyridoxamine) and phosphorylated (pyridoxine 5′-phosphate, pyridoxal 5′-phosphate, and pyridoxamine 5′-phosphate) forms. Pyridoxine·HCl is the one used in medicinal preparations and in food supplementation and has been the one most extensively evaluated to date in intestinal absorption studies (1–6). The phosphorylated forms are present in food, however, and are known to be absorbed from the gastrointestinal tract (7, 8). Therefore, an adequate understanding of the intestinal absorption of vitamin B_6 requires evaluation not only of pyridoxine but also of the phosphorylated forms of the vitamin.

Studies to date indicate that pyridoxal 5′-phosphate (PLP)[4] disappearance from the intestinal lumen is due at least in part to alkaline phosphatase hydrolysis. The relative contributions of mucosal brush border-bound and intraluminal (perfusate) alkaline phosphatase to this hydrolysis are not known. Evidence of intraluminal hydrolysis, however, has been demonstrated in rat intestine using both *in vitro* vascular perfusion (9) and *in vivo* luminal perfusion (10) techniques. In the *in vivo* model, a significant correlation was found between alkaline phosphatase activity in the exiting perfusate and PLP luminal disappearance when a mannitol-containing buffer that prevented net water absorption was used (11).

The effect of changes in net water flux on vitamin B_6 uptake has been evaluated only to a limited extent. Serebro *et al.* demonstrated

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[4] Abbreviations used: PLP, pyridoxal 5′-phosphate; PEG, polyethylene glycol.

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a significant linear correlation between net water and pyridoxine uptake rates from *in vivo* intestinal loops in rats (2). Nelson *et al.* noted in man that *in vivo* luminal disappearance of total vitamin B_6 from a synthetic solution containing pyridoxine, pyridoxamine, and PLP was enhanced in the presence of increased uptake of water (12). No studies, however, to this author's knowledge, have specifically dealt with correlations between net water absorption and PLP luminal disappearance or the relative importance of perfusate alkaline phosphatase activity and net water absorption as factors in PLP disappearance. The present studies were carried out to evaluate those correlations.

Materials and Methods. *Experimental procedure—in vivo perfusion.* All studies were performed in male Sprague–Dawley rats weighing 75–150 g and maintained on a stock commercial diet. The animals were fasted the night prior to use but were permitted free access to water. They were anesthetized with intraperitoneal pentobarbital (55 mg/kg). The proximal jejunum was identified through a midline abdominal incision, and a 5-cm segment was prepared with inflow and outflow catheters secured by silk ligatures as previously described (10). The segment was perfused with buffer containing 2 μM unlabeled PLP (Sigma Chemical Company, St. Louis, Mo.) and [^{3}H]polyethylene glycol (PEG) (New England Nuclear Company, Boston, Mass.; 1.6 mCi/g), utilizing a constant-flow pump set at a nominal flow rate of 0.123 ml/min. The [^{3}H]PEG was used as a nonabsorbable marker to calculate net water flux.

Three different buffers were used: (i) Krebs bicarbonate buffer, containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.6 mM KCl, 1.1 mM KH_2PO_4, and 1.1 mM $MgSO_4 \cdot 7H_2O$; (ii) Krebs bicarbonate with glucose buffer, made by decreasing the NaCl to 90 mM and adding 56 mM dextrose; and (iii) a mannitol bicarbonate buffer, made from the Krebs buffer by substituting mannitol (230 mM) and $KHCO_3$ (25 mM) for the NaCl and the $NaHCO_3$. All three buffers were gassed with 95% O_2–5% CO_2 (pH = 7.4). Osmolarities by freezing point depression were 274, 277, and 290 mOsm/liter, respectively. The PLP in the buffer was added from a stock solution which was monitored daily spectrophotometrically for actual PLP concentrations with 0.1 N NaOH at 388 mμ (10).

Segments were perfused at a constant rate of 0.123 ml/min for a period of 30 min, after which 50-μl aliquots were collected from the tip of the outflow catheter for [^{3}H]PEG, PLP, and alkaline phosphatase activity (10). Samples for measurement of [^{3}H]PEG were placed in scintillation fluid and counted in a liquid scintillation spectrometer (Model LS 9000, Beckman Instruments, Inc., Fullerton, Calif.). Aliquots for PLP determinations were placed in 2 ml of 5% trichloroacetic acid (TCA) and centrifuged; supernatants were then stored at −20°C until assayed (10). Aliquots were also collected from the perfusion syringes at the beginning and end of each day's experiments and were used as standards. In one group of rats, exiting perfusate was collected between 15 and 30 min of perfusion in a convenient vessel for more extensive evaluation of alkaline phosphatase activity and *in vitro* PLP decay.

Assay of alkaline phosphatase activity. Activity in the exiting perfusates was measured in two ways. First, activity in 50-μl aliquots was measured for all rats by a commercially available assay (Reagent SET, Bio-Dynamics/bmc, Indianapolis, Ind.) utilizing standard assay conditions (25°C, 10 mM p-nitrophenylphosphate as substrate, 1 M diethanolamine buffer, pH = 9.8). Generation of p-nitrophenol was measured spectrophotometrically, and enzyme activity was expressed as mU/ml.

Second, the *in vitro* conditions for assaying alkaline phosphatase activity were modified to approximate more closely actual *in vivo* perfusion conditions by adding a 100-μl aliquot of perfusate to an assay medium consisting of 1 mM p-nitrophenol phosphate in buffer corresponding to that used in the perfusion. Activity was measured spectrophotometrically at 405 mμ by generation of p-nitrophenol; and enzyme activity was expressed in arbitrary units per milliliter, since standard conditions were not in use. Validation studies demonstrated that this latter system yielded values for alkaline phosphatase activity in proportion to the amount of enzyme present. Generation of p-nitrophenol was linear with respect to time. Further validation studies demonstrated that the presence of known inhibitors of intestinal alkaline phosphatase significantly reduced measured activity. In Krebs

bicarbonate buffer, in Krebs bicarbonate buffer containing 10 m*M* L-phenylalanine (a stereospecific inhibitor of intestinal alkaline phosphatase), and in an 80 m*M* phosphate buffer, measured activities of a constant quantity of enzyme were 152 ± 9, 61.4 ± 4.4 (59.6% inhibition), and 8.3 ± 3.0 (94.5% inhibition) arbitrary units/ml, respectively. These degrees of inhibition were similar to those previously published for *in vitro* PLP decay in collected perfusates (10).

In vitro PLP decay. In vitro PLP decay was measured as previously described (10). After determination of perfusate alkaline phosphatase activity under standard conditions, samples of exited perfusates were diluted in corresponding buffers to a calculated final activity of 50 mU/ml. Aliquots (1.0 ml) were transferred to test tubes in a water bath at 37°C and *in vitro* PLP decay was measured as previously described (10). In brief, PLP was added to yield a final concentration of 2 μM, and a 50-μl aliquot was obtained and designated as the 0-time sample. Each tube was then allowed to incubate for 5 min, after which another 50-μl aliquot of incubating perfusate was taken. All aliquots upon collection were immediately added to 2 ml of 5% TCA and centrifuged; supernatants were stored at −20°C until assayed.

Assay of PLP. Frozen samples for PLP determination were thawed on the day of assay and were extracted with water-saturated, peroxide-free ethyl ether. PLP was measured by the tyrosine apodecarboxylase assay as described by Hamfelt (13) and later modified by Stone *et al.* (14). Use of this assay for *in vivo* perfusion studies and *in vitro* PLP decay studies has been described elsewhere (10, 11).

Calculations and statistics. Water uptake and PLP disappearance were calculated by standard formulas for single-pass constant perfusion models (10). PLP disappearance was corrected for changes in intraluminal PLP concentration by dividing actual disappearance by the geometric mean concentration of PLP in the segment lumen and expressing the results on a per-micromolar PLP basis (15). PLP decay was expressed in terms of percentage decay from 0-time levels. All results were recorded as mean ± SEM. Single and multiple linear regression analyses by the least-square method were used to evaluate correlation (16). Comparison of means for statistical significance utilized the Student's *t* test for unpaired data (16).

Results. *Relative effects of perfusate alkaline phosphatase activity and net water absorption.* To evaluate correlations for perfusate alkaline phosphatase activity, net water absorption, and PLP luminal disappearance, lumens of rat jejunum were perfused with 2 μM PLP and [^{3}H]PEG in Krebs bicarbonate buffer. PLP disappearance, net water uptake, and alkaline phosphatase activity in exiting perfusate were determined for each rat. The results of these perfusions are depicted in Figs. 1–4 and Table I. Figure 1 demonstrates the absence of any significant correlation between net water absorption and perfusate alkaline phosphatase activity. Figure 2 demonstrates a statistically significant correlation between perfusate alkaline phosphatase activity and PLP luminal disppearance ($Y = 44.4 + 0.614X$; $r = 0.734$; $P < 0.001$). Figure 3 demonstrates a relatively low but still statistically significant correlation between net water uptake and PLP disappearance ($Y = 58.4 + 4.7X$; $r = 0.374$; $P < 0.025$). To convert the data in Fig. 3 to a constant alkaline phosphatase activity and

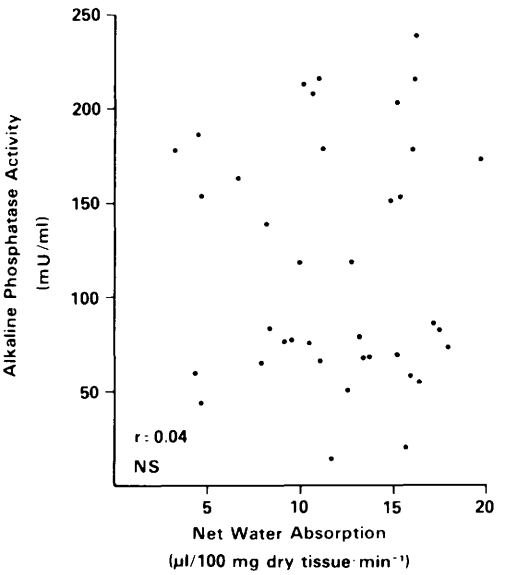

FIG. 1. Correlation between net water absorption and exiting perfusate alkaline phosphatase activity during *in vivo* perfusion of jejunal segments with 2 μM PLP in Krebs bicarbonate buffer.

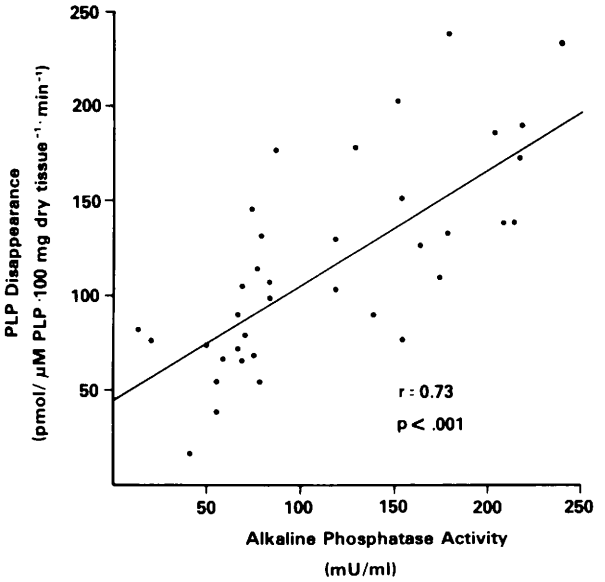

FIG. 2. Correlation between perfusate alkaline phosphatase activity and PLP luminal disappearance during *in vivo* perfusion of jejunal segments with 2 μM PLP in Krebs bicarbonate buffer.

thus to eliminate variability caused by differences in alkaline phosphatase, the difference between actual alkaline phosphatase activity and 100 mU/ml was determined for each rat and multiplied by the slope for the regression in Fig. 2. The resulting answer was then either added to or subtracted from the actual PLP luminal disappearance as appropriate to determine a "corrected" PLP value. When this "corrected" value was used, the correlation with water absorption (Fig. 4) was increased compared to that seen in Fig. 3 ($Y = 43.6 + 4.91X$; $r = 0.53$; $P < 0.001$).

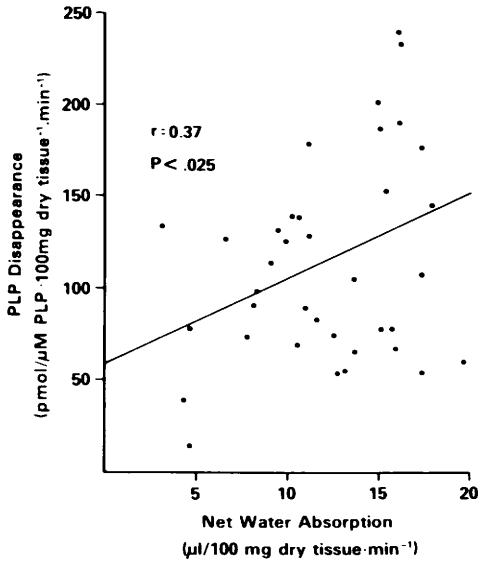

FIG. 3. Correlation between net water absorption and PLP luminal disappearance during *in vivo* perfusion of jejunal segments with 2 μM PLP in Krebs bicarbonate buffer.

When the results were submitted to multiple regression analysis, the formula $Y = -6.92 + 0.603X_1 + 4.36X_2$ was obtained in which X_1 was the perfusate alkaline phosphatase activity and X_2 was net water absorption (Table I). This multiple regression was statistically significant with an F value of 33 ($P < 0.01$). Both X_1 and X_2 contributed significantly to the regression (F values of 51.4 and 11.9 for X_1 after X_2 removed and X_2 after X_1 removed, respectively). The calculation of the standard partial regression coefficients, as a measure of the relative importance of the two independent variables in the regression, yielded values of 0.720 and 0.346 for X_1 and X_2, respectively. The fraction of the variance of PLP disappearance not attributable to either perfusate alkaline phosphatase activity or water absorption was 0.36.

PLP luminal disappearance—comparison in different buffers. To maximize differences in net water absorption, 2 μM PLP in either

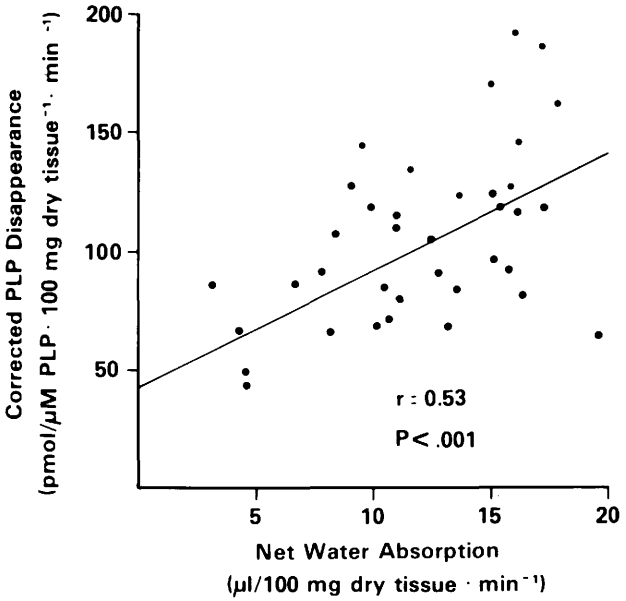

FIG. 4. Correlation between water absorption and "corrected" PLP luminal disappearance (see Methods) during *in vivo* perfusion of jejunal segments with 2 μM PLP in Krebs bicarbonate buffer.

mannitol bicarbonate buffer or Krebs bicarbonate with glucose buffer was perfused through the lumens of *in vivo* jejunal segments. Table II demonstrates the results for eight rats in each group. Both PLP disappearance and net water absorption were significantly greater when segments were perfused with Krebs bicarbonate with glucose buffer than when perfused with mannitol bicarbonate buffer. In contrast, perfusate alkaline phosphatase activity, as measured under standard assay conditions, was greater with mannitol buffer than with the glucose-containing buffer, though this difference did not achieve statistical significance at the $P < 0.05$ level.

Assessment of the effect of buffer changes on alkaline phosphatase activity. Since the ionic strengths, substrate concentrations, and pHs of the perfusates were different from those of the commercial assay used for measuring alkaline phosphatase activity, two separate studies were carried out to determine the effect of buffer changes on alkaline phosphatase activity.

First, alkaline phosphatase activity was determined using the modified spectrophotometric assay with 1 mM p-nitrophenol phosphate in perfusion buffer at pH = 7.4 (see Methods). Enzyme activities in exiting perfusates containing the two types of buffers are

TABLE I. REGRESSION ANALYSIS OF PLP DISAPPEARANCE[a]

	$Y = -6.98 + 0.603X_1 + 4.36X_2$			
Analysis	X_1[b]	X_2[c]	F	P
Multiple regression	+	+	32.6	<0.01
X_1 with X_2 removed	+		51.4	<0.01
X_2 with X_1 removed		+	11.9	<0.01
Standard partial regression coefficient	0.720	0.346		

[a] Rat jejunal segments perfused with 2 μM PLP in Krebs bicarbonate buffer (N = 37).
[b] X_1 = perfusate alkaline phosphatase activity.
[c] X_2 = net water absorption.

TABLE II. COMPARISON BETWEEN TWO PERFUSATE BUFFERS

Parameters (units)	Mannitol[a,b]	NaCl–Glu[a,b]	*P*
PLP luminal disappearance (pmole/μM PLP · 100 mg dry tissue^{-1} · min^{-1})	102 ± 9	151 ± 16	<0.025
Net water absorption (μl/100 mg dry tissue · min^{-1})	−9.83 ± 2.32	22.5 ± 1.9	<0.001
Perfusate alkaline phosphatase			
Activity			
(a) Standard assay (mU/ml)	171 ± 22	108 ± 23	NS[c]
(b) Modified assay (arbitrary units/ml)[d]	168 ± 21	112 ± 22	NS
5 min *in vitro* PLP decay			
(a) Media alkaline phosphatase activity after dilution (mU/ml)	53.4 ± 1.8	48.4 ± 2.5	NS
(b) PLP decay (%)	13.1 ± 2.6	10.4 ± 2.9	NS

[a] Mannitol = mannitol bicarbonate buffer; NaCl–Glu = Krebs bicarbonate with glucose buffer.
[b] Data expressed as mean ± SEM.
[c] NS = not statistically significant at $P < 0.05$ level.
[d] See text for details of assay and rationale for "arbitrary units."

demonstrated in Table II and Fig. 5. Table II demonstrates the activities for each buffer as measured both at standard assay conditions and at the modified, more "physiologic" conditions. When these values were used to calculate a ratio of modified:standard activities

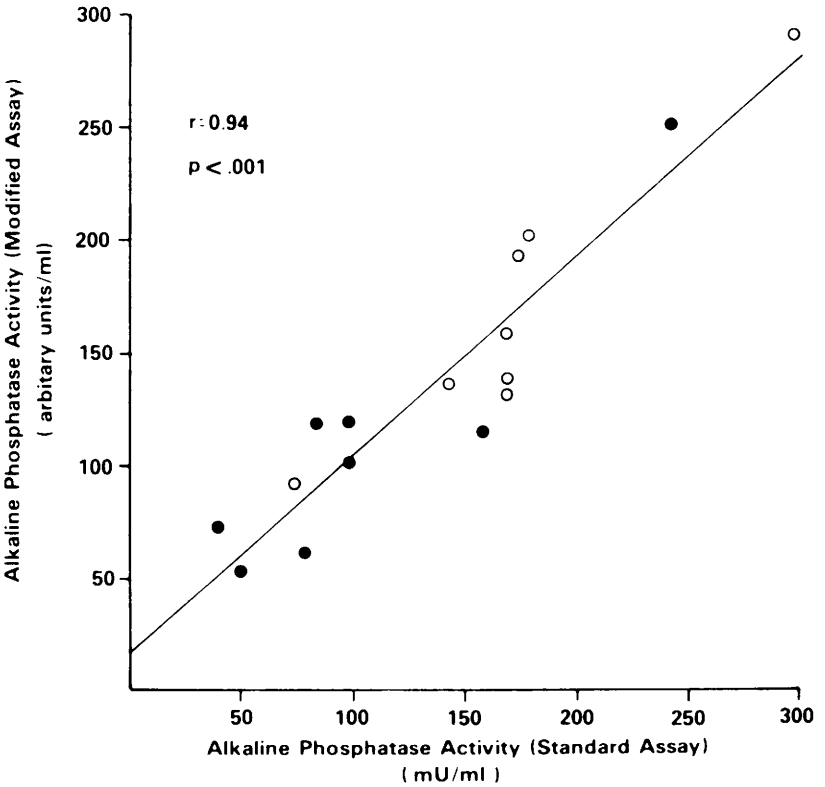

FIG. 5. Alkaline phosphatase activity in exiting perfusates as measured by the standard assay (abscissa) and the modified assay (ordinate). Open circles represent results obtained from perfusates containing mannitol bicarbonate buffer; closed circles, results obtained from perfusates containing Krebs bicarbonate with glucose buffer.

for each buffer, the ratios were not statistically different. Figure 5 demonstrates that individual enzyme activities for the two buffers defined a single regression line when standard assay activity on the *X*-axis was plotted against modified assay activity on the *Y*-axis. The *Y*-intercept of the regression was not statistically significantly different from an intercept passing through the origin (16).

Second, aliquots of perfusates were diluted with corresponding buffer to a calculated final alkaline phosphatase activity of 50 mU/ml as described under Methods, and then 5-min PLP decay studies were performed. As illustrated in Table II, enzyme activity after dilution was slightly greater in the mannitol buffer, but the difference was not statistically significant. Likewise, the percentage PLP decay was slightly greater in the mannitol bicarbonate buffer, but the difference was not statistically significant. Thus, PLP decay rates were similar in the two buffers when alkaline phosphatase activities as measured by the standard assay were also similar, indicating no differential effects of the buffer on enzyme activity.

Discussion. That PLP disappearance correlates with alkaline phosphatase activity in the exiting perfusate in this model now seems secure. Earlier studies had demonstrated the presence of alkaline phosphatase activity in the perfusate as it exited from the jejunal segment and had demonstrated that PLP disappearance both from the lumen of jejunal segments *in vivo* and from exited perfusates incubated *in vitro* correlated with measured alkaline phosphatase activity (11). In the *in vivo* portion of those studies, perfusate consisted of a mannitol-containing, sodium-free buffer which prevented net water absorption. The present studies, using a Krebs buffer which was associated with net water absorption, showed a similar strong correlation between perfusate alkaline phosphatase activity and PLP luminal disappearance (Fig. 2).

The present studies, in addition, demonstrate that PLP disappearance is also correlated with net water absorption. This was done in two ways. First, single and multiple regression analyses on results from perfusions with 2 μM PLP in Krebs bicarbonate buffer demonstrated (a) a significant correlation between net water absorption and PLP disappearance by single regression analysis which was improved when correction was made for variability in perfusate alkaline phosphatase activity, and (b) a significant contribution of water absorption in the multiple regression analysis. No significant correlation between perfusate alkaline phosphatase activity and net water absorption was found. Thus, both perfusate alkaline phosphatase activity and net water absorption appear to act as independent variables, with both significantly correlating with PLP disappearance but with the former being the more important of the two (higher standard partial regression coefficient). It would appear that in future *in vivo* PLP studies both of these factors—perfusate alkaline phosphatase activity and net water absorption—will need to be taken into consideration in the interpretation of results.

The second way of demonstrating a correlation between net water absorption and PLP disappearance was by varying buffer conditions to yield widely divergent rates of net water absorption. When this was done, significantly increased mean PLP disappearance was associated with the buffer yielding the greater mean net water absorption (Table II). Thus, as with the regression studies, there appeared to be an association between net water absorption and PLP disappearance.

This second means of demonstrating an association between the two parameters illustrated well how interpretation of results required consideration of both alkaline phosphatase activity and net water absorption. Before one could postulate that mean differences in net water absorption and PLP disappearance were associated with each other, it was necessary to establish that the difference in PLP disappearance rates could not be explained by differences in perfusate alkaline phosphatase activity. The standard assay and the PLP decay studies were used, and the modified *p*-nitrophenol phosphate assay was developed to assess such a possibility. Alkaline phosphatase activity as determined by the standard, commercially available assay was actually higher in the buffer yielding the lower mean PLP disappearance rate, though the difference was not statistically significant ($0.10 > P > 0.05$). When alkaline phosphatase was measured under conditions which more closely approximated actual perfusion con-

ditions (PLP decay and modified *P*-nitrophenol phosphate assay), similar results for enzyme activity were obtained. Thus, higher PLP disappearance from the buffer associated with greater water absorption (Krebs bicarbonate with glucose buffer) could not be explained by the presence of more enzyme or by buffer-related alterations in enzyme activity.

Alkaline phosphatase activity may vary with changing incubation conditions (substrate concentration, pH, ionic strengths, etc.) (17, 18). Standard assays for alkaline phosphatase activity, however, are normally performed under well-defined, rigid incubation conditions; thus, the results obtained will reflect assay, not necessarily biological and/or experimental conditions. Such a situation should not be a problem as long as the samples being measured are always from the same source (i.e., serum) or as long as only one experimental condition is used (i.e., Figs. 1–4, Table I). However, if buffer conditions change in experimental studies, as in Table II, the measurement of alkaline phosphatase under standard conditions may not necessarily reflect actual activity under experimental conditions. For example, L-phenylalanine, a stereospecific inhibitor of intestinal alkaline phosphatase, inhibits both luminal disappearance of PLP in this *in vivo* model and PLP decay in *in vitro* studies (10, 11). Alkaline phosphatase activities measured in exiting perfusate by standard assay techniques, however, are similar for perfusates with and without L-phenylalanine (11). Thus, standard assay conditions do not reflect actual enzyme activity under those particular experimental conditions.

In the evaluation of alkaline phosphatase activity under experimental conditions in the present studies, PLP decay was probably more "physiologic" in that it duplicated not only pH and buffer compositions but also substrate and substrate concentration. The multiple steps required, however, made the method rather cumbersome for routine monitoring. The modified spectrophotometric assay, on the other hand, had the advantage of simplicity, ease of handling multiple samples, and maintenance of experimental pH and ionic concentrations during the assay. The main theoretical disadvantage compared to PLP decay was the inability to lower the substrate concentration below 1 m*M* and still maintain adequate sensitivity. In spite of this, the validation results with the modified assay when phosphatase inhibitors (phosphate and L-phenylalanine) were included were similar to previous PLP decay studies. The modified phosphatase assay, therefore, represented a compromise which was a simple, rapid, apparently reliable method for measuring and comparing alkaline phosphatase activities under near actual experimental conditions. This method should be applicable to future studies of PLP absorption.

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