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Pyridoxal 5′-Phosphate Related Changes in Retention of 1,25-Dihydroxy Vitamin D–Receptor Ligands in Rat Intestinal Mucosa Cell Nuclei

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After feeding rats a vitamin B-6-deficient diet, we observed a decrease in pyridoxal 5′-phosphate concentrations in intestinal mucosa cells to 32 and 48% of control in cytoplasm and cell nuclei, respectively. Correlation analysis suggested that there were two pyridoxal 5′-phosphate pools in the nuclei: a "mobile" pool (equivalent to about 5% the concentration of the cytoplasmic pyridoxal 5′-phosphate), and a "stable" pool, which was independent of cytoplasmic fluctuations of pyridoxal 5′-phosphate (about 9 pmol pyridoxal 5′-phosphate/mg DNA). Reduction in pyridoxal 5′-phosphate content in the cells of vitamin B-6-deficient animals was accompanied by a substantial increase in 1,25-dihydroxyvitamin D–receptor ligand concentration in the cell nuclei (76.6 ± 19.7 vs 762 ± 291 fmol/mg DNA, mean ± SEM). The degree of 1,25-dihydroxyvitamin D accumulation in the nuclei appeared to be an exponential function of the "mobile" nuclear pyridoxal 5′-phosphate concentration. Semilogarithmic transformation of the data yielded a straight line, representing an inverse correlation between the cytoplasm-related nuclear pool of pyridoxal 5′-phosphate and the logarithm of the 1,25-dihydroxyvitamin D concentration in the nuclei ($r = -0.95$). These data suggest that pyridoxal 5′-phosphate may be related to 1,25-dihydroxyvitamin D retention in the nuclei, possibly through interaction of the pyridoxal 5′-phosphate with the vitamin D receptor protein in the nuclei.

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INTRODUCTION

Pyridoxal 5′-phosphate (PLP), the major active form of vitamin B-6, is a cofactor for many enzymes, mainly those involved in intermediary metabolism of amino acids [1, 2]. The mechanism of action of PLP consists of reacting with the free amino groups of a large variety of compounds, including amino acids, to produce a Schiff base. By binding to a lysyl residue near or at the active site, PLP can also inhibit several enzymes [3–8].

Since the mid 1970s, data have been published which suggest that PLP may also be a physiological modulator of steroid hormone receptor action [9–21]. Thus, in several *in vitro* experiments, PLP inhibited the association of steroid hormone–receptor complexes to ATP–Sepharose or to phosphocellulose [9] or to DNA–cellulose [9–11]. Supraphysiological millimolar concentrations of PLP effectively and specifically extracted vitamin D receptor [12], androgen receptor [11, 13], glucocorticoid receptor [14] or estrogen receptor [15] from chromatin or cell nuclei of the targeted tissues. Exogenously added PLP inhibited translocation of activated glucocorticoid [10], androgen [11] and estrogen [15] hormone–receptor complexes to nuclei in cell-free systems or in the intact uterus, as in the case of estrogen receptor [15]. Unlike the DNA-binding and aggregation properties, the hormone-binding abilities of the steroid receptor molecule remained unaffected by PLP action [9, 11, 12, 17, 18].

At the same time, it was documented that the interaction of PLP with steroid hormone receptors appeared

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to be the result of a Schiff base formation [9, 10, 15–18]. To date, there have been only a few papers which have indicated the possibility that PLP can affect *in vivo* steroid hormone receptor mechanisms of action. In experiments carried out with rats fed a vitamin B-6-deficient diet, a nuclear fraction of estrogen receptor in the uterus was increased compared with tissue from rats fed a vitamin B-6-adequate diet, while the total number of estrogen receptors per gram of tissue remained unaffected [19, 20]. Similar results were obtained in the case of glucocorticoid receptors in liver [16] and of vitamin D receptors in intestine [21] of rats. In the present study, a substantial increase in accumulation of vitamin D (1,25-dihydroxyvitamin D)–receptor ligand in the intestinal mucosa nuclei of vitamin B-6-deficient rats was confirmed. Furthermore, we present data which indicate that the amount of 1,25-dihydroxyvitamin D found in the nuclear fraction was correlated with the PLP concentrations within both the nuclei and the cytoplasm.

EXPERIMENTAL

Animals

The experiments were performed on male Wistar outbred rats. The animals were housed and cared for in accordance with the "NIH Guide for the Care and Use of Laboratory Animals". After weaning at 4 weeks of age, the animals were pair-fed for 5 weeks with one of the two experimental diets (Table 1): the vitamin B-6-deficient diet (−B-6 group), or the diet supplemented with 7 mg of pyridoxine HCl/kg (+B-6, control pair-fed group). Before excision of the intestines, rats were fasted for 24 h and then anesthetized by intraperitoneal injection of a mixture of combelen (2.5 mg/kg body wt) and ketamine (90 mg/kg body wt).

Chemicals

1,25-Dihydroxy-23,24(*n*)-[^{3}H]vitamin D_3 was purchased from Amersham (Amersham, UK; sp. act. 6.66×10^{12} Bq/mmol, and >95% purity, as determined by HPLC analysis). Nonradioactive 1,25-dihydroxyvitamin D_3 was a generous gift from Dr Michael F. Holick (Boston University School of Medicine, Boston, MA). Vitamin-free casein and corn starch were obtained from Teklad Test Diets (Harlan Sprague–Dawley, Madison, WI). All vitamins used for the diets were from Sigma (Sigma, St Louis, MO). Other reagents were of analytical grade.

Preparation of cell nuclei from rat intestinal mucosa

Following onset of anesthesia, approx. 1 ml of blood was taken from the dorsal artery of a rat to determine PLP concentration and alkaline phosphatase activity in serum. Immediately after this, a container of 154 mmol/l NaCl at 37°C was connected to the artery through the same needle, and perfusion of intestine together with kidneys and liver was conducted. Intestinal mucosa cell nuclei were prepared following the method of Bloor *et al.* [22] with some modifications. The small intestines, 20 cm of length distal to the pylorus, were removed, rinsed with ice-cold 154 mmol/l NaCl + 2.5 mmol/l dithiothreitol (DTT), pH 7.4, and then filled with the same solution. The ends were clamped and the intestinal "sacs" incubated for 3 min at 37°C in 154 mmol/l NaCl. Then the intestines were rinsed again with ice-cold 154 mmol/l NaCl + 2.5 mmol/l DTT, pH 7.4, blotted to remove excess buffer, and slit longitudinally with scissors. The intestines were placed on a glass Petri dish placed on ice with the serosal side down and the mucosa was scraped from the serosa with the use of glass microscope slides. The scrapings were homogenized, 10% w/v, in ice-cold homogenization buffer [10 mmol/l sodium barbital, 0.32 mol/l sucrose, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 2.5 mmol/l DTT, 0.2 mmol/l phenylmethylsulfonyl fluoride (PMSF), pH 7.4] using 20 up-and-down strokes of a loose-fitting Dounce homogenizer. Aliquots (1 ml) of the homogenates were frozen for subsequent 1,25-dihydroxyvitamin D–receptor investigation as well as for PLP and DNA determinations. The remaining homogenate was centrifuged for 10 min at 0°C and 1250*g* (2000 rpm, $r_{av} = 7$ cm) in a swinging bucket rotor of the K_{26D} centrifuge (Janetzki, Germany), followed by one wash with the homogenization buffer. The crude nuclear pellet (about 0.3 ml/rat) was suspended by vortexing in 3.5 ml of SCD-buffer (2.4 mol/l sucrose, 1 mmol/l $CaCl_2$, 2.5 mmol/l DTT, pH 7.5) and centrifuged for 1 h

Table 1. Composition of the experimental diets

Ingredients	+ B-6 diet (g/kg diet)	− B-6 diet (g/kg diet)
Casein[a]	309	—
Vitamin-free casein[b]	—	286.8
Cornstarch[b]	150	154.7
Sucrose	400	417.5
Sunflower oil	100	100
Salt mix[c]	40	40
Vitamin mix[d]	1	—
Vitamin mix without pyridoxine[d]	—	1

[a]Casein Hammarsten Biochemical, BDH Chemicals Ltd, Poole, England.

[b]Teklad Test Diets, Madison, WI.

[c]Concentration: (g/kg salt mix); calcium citrate, 308.30; potassium phosphate dibasic, 218.8; potassium chloride, 124.70; calcium phosphate monobasic, 112.80; sodium chloride, 77.10; calcium carbonate, 68.60; magnesium sulfate, 38.30; magnesium carbonate, 35.20; ferric citrate, 13.70; ammonium alum, 0.99; sodium fluoride, 0.50; manganese sulfate, 0.20; cupric sulfate, 0.08 (all from Polskie Odczyniki Chemiczne, Gliwice, Poland).

[d]Final concentrations: (in mg/kg diet); choline chloride, 1653.4; ascorbic acid, 1016.6; *p*-aminobenzoic acid, 110.13; inositol, 110.13; nicotinic acid, 99.12; α-tocopherol acid succinate, 80.0; calcium pantothenate, 66.08; menadione, 49.56; riboflavin, 22.026; thiamine, 22.0; pyridoxine HCl, 22.0 (in vitamin B-6-adequate diet only, none added in vitamin B-6-deficient diet); retinal palmitate, 11.24 mg; folic acid, 1.98: (in μg/kg diet); biotin, 441; cholecalciferol, 55.2; vitamin B-12, 29.7; (all from Sigma, St Louis, MO).

at 0–5°C and 50,000 rpm (220,000 *g*, r_{av} = 9.1 cm) in an SW-60 rotor of a Beckman L8-80 ultracentrifuge (Beckman, Palo Alto, CA). After centrifugation, the supernatant was carefully removed along with the brownish layer of cellular debris floating on the top. The white pellet of purified nuclei was resuspended in the homogenization buffer and frozen in liquid nitrogen for further investigations. Efficiency of the nuclei preparations was calculated on the basis of DNA recovery.

Extraction of 1,25-dihydroxyvitamin D–receptors from whole cell homogenates and purified nuclear fractions of intestinal mucosa.

To 0.25 ml of mucosa homogenate or nuclei suspension in the homogenization buffer, 1.75 ml of ice-cold BEDK-0.3 buffer was added (final concentrations of the buffer components in the extraction mixtures were 10 mmol/l sodium barbital, 1.5 mmol/l EDTA, 5 mmol/l DTT and 0.3 mol/l KCl, pH 7.4) and homogenized with 7–10 strokes of a glass–glass Potter homogenizer. Of this suspension, 0.2 ml aliquots were frozen at −30°C for subsequent PLP estimations.

Immediately following removal of this aliquot, 0.2 ml of the BEDK-0.3 buffer plus 100 mmol/l Na_2MoO_4, pH 7.4, and 20 µl of 2 mmol/l PMSF in ethanol was added (to give final concentrations of 10 mmol/l Na_2MoO_4 and 0.2 mmol/l PMSF), followed by vigorous mixing. It was essential not to add the molybdate and PMSF before taking samples for PLP monitoring due to strong interference of these reagents in the PLP determination [23]. Previously it had been determined that a 30 min delay in addition of molybdate (a 1,25-dihydroxyvitamin D–receptor stabilizer [24]) and a protease inhibitor (PMSF) had no influence on the receptor–ligand binding ability (data not shown). The final extraction mixtures were centrifuged for 25 min at 0–5°C and 200,000 *g* (55,000 rpm, r_{av} = 6.25 cm) in an 80 Ti rotor of a Beckman L8-80 ultracentrifuge. The supernatants (crude 1,25-dihydroxyvitamin D–receptor extracts) were subsequently used for evaluation of unoccupied 1,25-dihydroxyvitamin D–receptor binding sites as well as for estimation of the receptor ligands. The pellets were used for DNA estimation.

Evaluation of unoccupied 1,25-dihydroxyvitamin D–receptor binding sites

Samples (0.2 ml) of 1,25-dihydroxyvitamin D–receptor extracts from whole cells or purified nuclei were incubated for 2 h in an ice-water bath with 10 µl of [^{3}H]1,25-dihydroxyvitamin D_3 in ethanol (sp. act. 6.66 × 10^{12} Bq/mmol; final concentration of 1 nmol/l) with (nonspecific binding) or without (total binding) a 200-fold excess of unlabeled 1,25-dihydroxyvitamin D_3 in 10 µl ethanol. At the end of the incubation, 0.1 ml of dextran-coated charcoal (DCC) slurry was added for 20 min to separate bound from free ligand. The samples were then centrifuged at 2500 rpm for 10 min at 0–5°C in a K_{26D} centifuge. Radioactivity of the supernatants was determined in a Beckman LS-7500 counter using scintillation fluid consisting of 5 g PPO and 0.1 g POPOP dissolved in 0.3 l of Triton X-100 plus 0.7 l of toluene. Specific binding of the 1,25-dihydroxyvitamin D–receptor was calculated by subtracting nonspecific binding from total binding. The amount of unoccupied 1,25-dihydroxyvitamin D–receptor binding sites was determined by calculation, including the specific activity of the tracer and the dilution which occurred during the extraction procedure.

Determination of 1,25-dihydroxyvitamin D and related compounds in the receptor extracts

The extracts from whole cells or purified nuclei were diluted to a volume of 1 ml with 154 mmol/l NaCl. To each set of estimations, two 1 ml samples of the standard serum, and two 1 ml samples of the standard serum which had been preincubated for 1 h with 6 × 10^5 Bq of [^{3}H]1,25-dihydroxyvitamin D_3, were added in order to monitor reliability of the assay and the tracer recovery, respectively. Preparation of 1,25-dihydroxyvitamin D extracts was performed according to the method of Reinhardt *et al.* [25]. Generally, the samples and the standard sera were extracted with an equal volume of acetonitrile. The extract was alkalized and purified by passing through a Sep-Pak C_{18} followed by silica Sep-Pak minicolumns (Waters, Milford, MA). Final eluates, containing 1,25-dihydroxyvitamin D_2, 1,25-dihydroxyvitamin D_3 and related compounds, were dried under a stream of nitrogen and redissolved in ethanol for the assay. The 1,25-dihydroxyvitamin D–receptor ligands were estimated in a nonequilibrium competitive binding assay using calf thymus receptor preparation, as described by Grądzka *et al.* [26].

Other determinations

PLP was determined by an enzymatic stimulation micro-method of Andon and Reynolds [23] with the use of tyrosine apodecarboxylase. Alkaline phosphatase activity (EC 3.1.3.1) in sera was measured with *p*-nitrophenylphosphate as a substrate following the method of Walter and Schutt [27]. The same enzyme activity in mucosa preparations was measured as described by Weisser [28], except that the final concentration of *p*-nitrophenylphosphate was 2.3 mmol/l. Lactate dehydrogenase activity (EC 1.1.1.27) was assayed by the method of Wróblewski and LaDue [29], NADPH–cytochrome *c* reductase activity (EC 1.6.2.4) was quantified by the procedure of Masters *et al.* [30], succinate:2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride reductase activity was measured by the method of Morré [31]. DNA was determined by the diphenylamine method of Burton [32] using calf thymus DNA as a standard. Protein was estimated by the method of Bradford [33] with human gamma-globulin as a standard.

Table 2. Body weights, serum PLP concentrations and serum alkaline phosphatase activities in rats fed a vitamin B-6-deficient (−B-6) or vitamin B-6-supplemented (+B-6) diet

Diet	Body weight (g)	Serum pyridoxal 5′-phosphate (nmol/l)	Serum alkaline phosphatase activity (U/l)
+B-6	163 ± 4[a]	81.0, 102.2	70.8 ± 9.7
	$n = 6$	$n = 2$	$n = 6$
−B-6	148 ± 5	4.0, 4.6	197.9 ± 61.0[b]
	$n = 6$	$n = 2$	$n = 6$

[a]Mean ± SEM.
[b]$P < 0.05$ between +B-6 and −B-6 groups.

Statistics

Results were expressed as the mean ± SEM. The statistical significance of differences between means were determined by Student's t-test [34] at $P = 0.05$. Pearson's linear correlation was calculated for related observations.

RESULTS

After 5 weeks of feeding the experimental diets, PLP concentrations in sera of randomly chosen rats were 4.0 and 4.6 nmol/l in the −B-6 group, vs 81.0 and 102.2 nmol/l in the pair-fed control group (Table 2). At the beginning of the 3rd week of feeding the diets, animals deprived of vitamin B-6 had lower body weights and increased alkaline phosphatase activities in serum compared to the pair-fed controls.

The yield of purified nuclei from the intestinal mucosa of both groups was 9.4 ± 2.2% (mean ± SEM, $n = 22$). Phase-contrast micrography of the nuclear suspension in the homogenization buffer showed no apparent contamination with plasma membranes or unbroken cells. Nucleoli were also easily visible inside the nuclei. Purity of the preparation was additionally examined by comparison of several marker enzyme activities in the mucosa homogenate and cell nuclei preparation (Table 3). Specific activities of the enzymes typical for cytosol, microvilli, and microsomes were decreased in the purified nuclei compared to the homogenate. The succinic:2-(*p*-iodophenyl-)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride reductase activity remained at nearly the same level in both homogenate and nuclei. This indicates a possibility of some contamination of the nuclei preparation with mitochondrial membranes. However, this should not interfere with the estimation of 1,25-dihydroxyvitamin D in the nuclear compartment because it has been shown that there was no specific accumulation of the vitamin D–receptor ligand in intestinal mucosa cell mitochondria [22]. The protein/DNA ratio in purified nuclei was 4.0 which was similar to the value of 3.1 reported earlier by Bloor *et al.* [22] for the same preparation.

PLP concentrations in individual nuclei preparations were closely correlated with PLP concentrations in adequate vitamin B-6 cytoplasm specimens [Fig. 1(a and b)]. The cytoplasm-dependent PLP pool in the nuclei was equivalent to about 5% of the amount of PLP in the cytoplasm, as calculated from the curve in Fig. 1(a). It should be noted, however, that the extrapolated plot of the relationship was shifted upwards and intersected the Y-axis at 9 pmol of nuclear PLP/mg DNA [Fig. 1(a)] or 2.3 pmol/mg of nuclear protein [Fig. 1(b)]. Thus, in addition to the PLP pool

Table 3. Marker enzyme activities of the rat intestinal mucosa cell homogenate and nuclei preparations

Enzyme (subcellular location)	Specific activity (nmol product/mg protein.min^{-1})	
	Homogenate	Nuclei
Lactate dehydrogenase (cytosol)	1,408	176
Alkaline phosphatase (microvilli)	112,479	17,053
Succinic:2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride reductase (mitochondria)	402	492
NADP–cytochrome *c* reductase (microsomes)	18.9	10.0
Protein/DNA ratio (mg/mg)	18.3	4.0

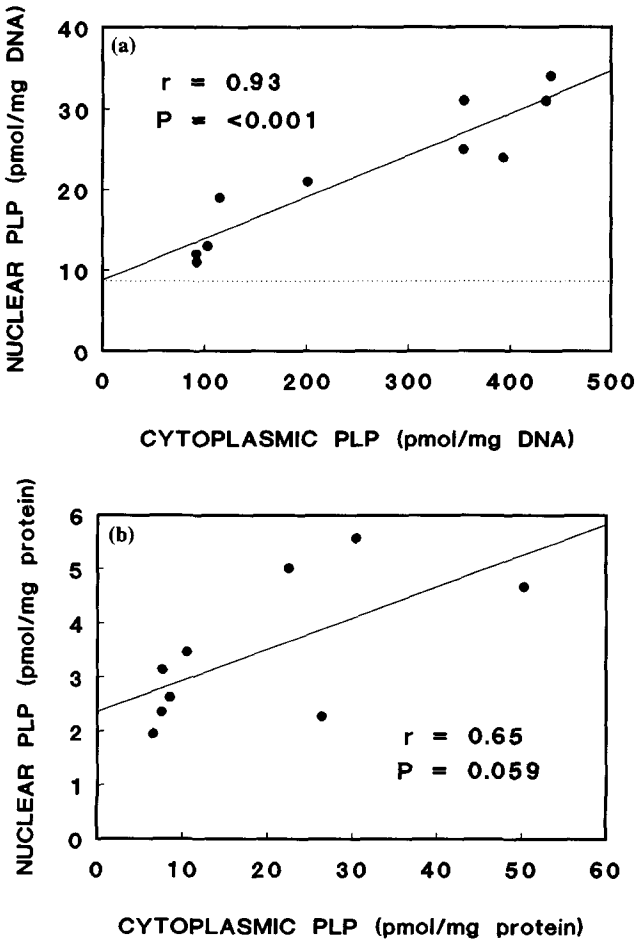

Fig. 1. Correlation between PLP concentration in cytoplasm and nuclei of rat intestinal mucosa cells. PLP was estimated in mucosa homogenates and purified nuclei in BEDK-0.3 buffer (see Experimental). PLP concentrations in cytoplasm were calculated by subtracting values found in purified nuclei from the values in homogenates, and expressed as (a) pmol/mg DNA, or (b) pmol/mg protein in cytoplasm or nuclei. Protein content in the cytoplasm was determined by subtracting the content measured in the nuclei from that found in the homogenates.

inside the nuclei which equilibrates with the cytoplasm, there appeared to be a stable pool of PLP which was independent of cytoplasmic fluctuations.

Quantities of unoccupied 1,25-dihydroxyvitamin D–receptors measured in extracts from total mucosa and purified nuclei are presented in Table 4. There was some tendency to increase the amount of the unoccupied receptor binding sites during vitamin B-6 deficiency in both cell and nuclei extracts in comparison to the mean values from the control group, although none of the differences were statistically significant ($P > 0.1$).

The content of occupied 1,25-dihydroxyvitamin D–receptor binding sites in the individual extracts was evaluated indirectly through estimation of the receptor ligands. This approach was derived from the fact that the method using mersalyl acid, which is commonly used to estimate occupied 1,25-dihydroxyvitamin D–receptors [35], did not have sufficient sensitivity for the small amounts of the receptor extracts available in these experiments.

In the intestine of rats fed a vitamin D_3-supplemented diet, the main compound bound to the 1,25-dihydroxyvitamin D–receptor is 1,25-dihydroxyvitamin D_3, which comprised over 80% of all vitamin D_3 derivatives in the cell [36, 37]. The remaining compounds of related affinity to the receptor were early oxidation products of 1,25-dihydroxyvitamin D_3, including 1,24,25-trihydroxyvitamin D_3, 1,25-dihydroxy-24-oxo-vitamin D_3, 1,23,25-trihydroxy-24-oxo-vitamin D_3, 1,25,26-trihydroxy-23-oxo-vitamin D_3 and 1,25-dihydroxyvitamin D_3-26,23-lactone. Because of a low dissociation constant of the rat 1,25-dihydroxyvitamin D–receptor ($K_d = 10^{-11}$–10^{-10} mol/l; [26], practically all the ligands present in the salt extracts of tissue should remain bound to the receptor. In the present experiments, the vitamin D_3 derivatives after organic solvent extraction and purification (see Experimental) were estimated to react with the calf 1,25-dihydroxyvitamin D–receptor, which has nearly the same specificity towards the compounds considered [38] as does the rat receptor [26].

On the basis of these observations, we assumed that the level of 1,25-dihydroxyvitamin D–receptor ligands (mainly 1,25-dihydroxyvitamin D_3) can reasonably reflect occupancy of the receptor in the tissue preparations. As shown in Table 4, the level of the 1,25-dihydroxyvitamin D–receptor ligands measured in the nuclear extracts was tenfold higher in the −B-6 group than in the pair-fed control group. The method we used was unsuccessful in estimating the 1,25-dihydroxyvitamin D–receptor ligands in the whole cell extracts, possibly due to the presence of hydroxylases and oxidases which degraded the vitamin D_3 derivatives to forms which did not react with the receptor [37].

Figure 2(a) shows the 1,25-dihydroxyvitamin D content in the nuclei as an exponential function of the nuclear PLP concentration. This can be confirmed by a semilogarithmic transformation of this relationship, which gives a straight line [Fig. 2(b)] with an inverse correlation ($r = 0.95$) between the logarithm of 1,25-dihydroxyvitamin D and PLP concentrations in the nuclei. The 1,25-dihydroxyvitamin D level rose dramatically when PLP concentrations approached an asymptotic value of about 10 pmol/mg DNA [Fig. 2(a)] which is close to the value established earlier for the cytoplasm-independent PLP pool in the nuclei [Fig. 1(a)]. Thus, it seems that only the cytoplasm-dependent PLP pool is engaged in the regulation of nuclear 1,25-dihydroxyvitamin D content. At concentrations above 40 pmol PLP/mg DNA, 1,25-dihydroxyvitamin D was undetectable in the nuclei.

DISCUSSION

Although several laboratories have documented the influence of PLP on steroid receptor action *in vitro* [9–18], the question still remains of whether PLP plays an essential role in the modulation of the steroid hormone action *in vivo* or if it is only a useful chemical reagent for receptor characterization. Feeding animals with a vitamin B-6-deficient diet results in a gradual decrease of the PLP level in blood and tissues. Thus, a few investigators have used the vitamin B-6 deficiency model to observe changes in steroid receptor behavior. According to the results from *in vitro* experiments in which PLP prevented the steroid receptors from binding to DNA [9–11], chromatin [12, 13] or isolated nuclei [12, 14], experiments by DiSorbo *et al.* [16] demonstrated that the efficiency of translocation of [^{3}H]triamcinolone acetonide (a glucocorticoid–receptor ligand) to the liver cell nuclei was higher in vitamin B-6-deficient rats compared to that in control animals. Subsequently, Bowden *et al.* [19] showed that in the uteri of vitamin B-6-deficient rats, nuclear retention of [^{3}H]estradiol was substantially increased. Also, the estrogen receptor concentration in the uterine cell nuclei of the vitamin B-6-deficient rats was greater than in the control uteri, as reported by Bunce and Vessal [20], although the total number of estrogen receptors per gram of tissue remained unaffected.

Recently, Sergeev and Spirichev [21] investigated the effect of vitamin B-6 deficiency on the 1,25-dihydroxyvitamin D–receptor properties in rat intestinal mucosa cells. They reported an increase in the content of occupied 1,25-dihydroxyvitamin D–receptors in both chromatin fraction (by 1.8 times) and cytosol (by 1.3 times) of cells derived from vitamin B-6-deficient rats compared to control, while unoccupied 1,25-dihydroxyvitamin D–receptors were at the same level in both groups. In addition, binding of the *in vivo* occupied 1,25-dihydroxyvitamin D–receptors with DNA–cellulose increased by 42% in vitamin B-6 deficiency.

The present work was designed to extend our knowledge concerning the *in vivo* distribution of 1,25-dihydroxyvitamin D receptors inside rat intestinal mucosa cells as a function of the vitamin B-6 status of the animals. In our experiments, the following conditions were established to improve correction of the measurements of PLP concentrations and its effects within the intestinal cells. Plasma and erythrocytes, both known to contain high concentrations of PLP [39], were removed from the system by perfusion of the intestines. Moreover, in all solutions, Tris buffer was replaced by barbital. Tris, commonly used in the vitamin D–receptor methodology, can react with PLP to form a Schiff base [40], thus making accurate estimations of the PLP amount and actions difficult. For example, it was shown that inhibition of progesterone receptor binding to ATP–Sepharose caused by PLP can be reversed after addition of Tris [9]. Also, the activity of tyrosine aminotransferase, a PLP-requiring enzyme, was diminished considerably in the presence of Tris buffer [41].

As a consequence of the vitamin B-6 depletion in our experiments, the PLP level in the serum of the rats decreased to about 5% of control values. At the same time, concentrations of PLP in the cytoplasm and nuclei fraction of the intestinal mucosa cells decreased only to about 30 and 50% of control, respectively. There appears to be an adaptive mechanism which enables the retention of substantial amounts of PLP in the intestinal mucosa, even during a severe vitamin B-6 deficiency. Considering this phenomenon, one should remember that in the intestinal wall, PLP can be synthesized from less biologically active B-6-vitamers such as pyridoxine phosphate or pyridoxal [42].

Concentrations of PLP per mg protein were considerably lower in the nuclear fraction than in the cytoplasm [Fig. 1(b)]. At the same time, a correlation

Table 4. 1,25-dihydroxyvitamin D receptors and their ligands in whole mucosa cell extracts and cell nuclei extracts after consumption of a vitamin B-6-adequate (+B-6) or -deficient (−B-6) diet

Diet	Extract	Unoccupied $1,25(OH)_2$ vitamin D receptors (fmol/mg DNA)	Unoccupied $1,25(OH)_2$ vitamin D receptors (fmol/mg protein)	$1,25(OH)_2D$–receptor ligands, $1,25(OH)_2D_3$ and related compounds[a] (fmol/mg DNA)	$1,25(OH)_2D$–receptor ligands, $1,25(OH)_2D_3$ and related compounds[a] (fmol/mg protein)
+B-6	Whole cell	2379 ± 1108[b]	124 ± 30	ND[c]	ND
		$n = 6$	$n = 6$		
	Nuclei	66.7 ± 20.2	9.7 ± 3.6	76.6 ± 19.7	14.7 ± 5.3
		$n = 6$	$n = 6$	$n = 6$	$n = 6$
−B-6	Whole cell	3232 ± 250	162 ± 9	ND	ND
		$n = 5$	$n = 5$		
	Nuclei	83.7 ± 9.8	16.4 ± 3.0	762 ± 291[d]	144 ± 56[d]
		$n = 6$	$n = 6$	$n = 6$	$n = 6$

[a]"Related compounds" refers to early degradation products of 1,25-dihydroxyvitamin D_3 which still retain high affinity for the 1,25-dihydroxyvitamin D receptor (see Results section for further details).

[b]Mean ± SEM.

[c]ND, not determined.

[d]$P < 0.05$ between +B-6 and −B-6 groups.

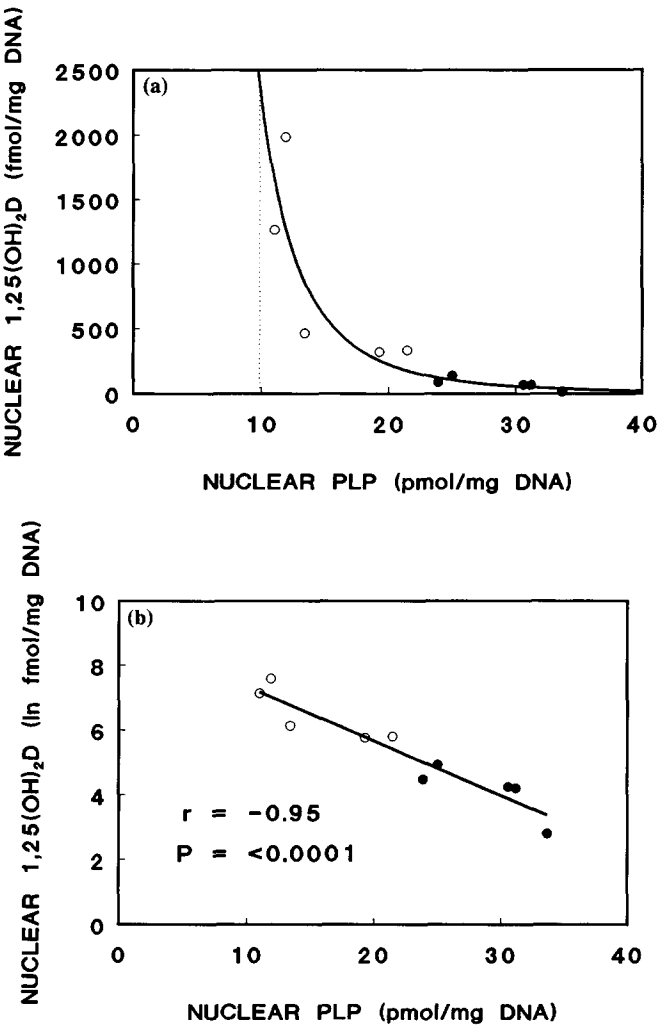

Fig. 2. Relationship between 1,25-dihydroxyvitamin D and PLP concentrations in rat intestinal mucosa cell nuclei. Both 1,25-dihydroxyvitamin D and PLP were estimated in purified nuclei fractions as described in Experimental. ○, Vitamin B-6-deficient; ●, vitamin B-6-adequate rats. (a) Decrease in PLP concentrations (up to about 10 pmol/mg DNA), caused by vitamin B-6 deficiency resulted in the rapid, nonlinear increase in 1,25-dihydroxyvitamin D accumulation in the nuclei. (b) Semilogarithmic transformation of the plot from Fig. 2(a) produced a straight line with correlation coefficient $r = -0.95$.

($r = 0.65$) existed between PLP amounts in both compartments [Fig. 1(b)]. These observations suggest that differential equilibrium of PLP across the nuclear membrane occurs. Assuming that the major amount of cellular PLP (because of its facility to react with free amino groups) is bound to proteins, one can imagine that only selective protein PLP-carriers could cross the cytoplasm/nucleus barrier, the remaining PLP being engaged in other processes in the cell. This proposed transport of protein-bound PLP across the nuclear membrane may be analogous to the transport of albumin-bound PLP across the hepatic plasma membrane [43], although the mechanisms for movement of proteins across nuclear membranes (and thus of any protein-bound PLP) is different from that of the movement of proteins across cell membranes [44]. Cytoplasm-related or "mobile" nuclear PLP constitutes about 5% of cytoplasmic PLP, as calculated from Fig. 1(a).

On the other hand, extrapolation of the correlation from Fig. 1(a and b) indicates the existence of an amount (about 9 pmol/mg DNA or 2.3 pmol/mg protein) of nuclear PLP which is independent of cytoplasmic PLP fluctuations. The presence of this "stable" PLP pool may explain why the PLP content in the nuclei after vitamin B-6 depletion diminished only to 50% and not 30% of control, as occurred in the cytoplasm.

The mean amount of 1,25-dihydroxyvitamin D (reflecting the amount of occupied 1,25-dihydroxyvitamin D–receptors) found in the nuclear fraction from intestinal mucosa cells of vitamin B-6-deficient rats was tenfold higher than in controls (Table 4). A similar trend was observed by Sergeev and Spirichev [21] in the chromatin fraction from intestinal rat mucosa although the content of the occupied 1,25-dihydroxyvitamin D–receptors in chromatin derived from vitamin B-6 depleted animals was only 1.8-fold greater compared to controls. The lesser size of the difference reported by the latter authors probably arose from the fact that the protein content of chromatin (including 1,25-dihydroxyvitamin D–receptor) had been partially lost after using 0.3 mol/l KCl directly in the buffer for the mucosa homogenization [21]. In our protocol, the high salt content buffer was used only after the native nuclei were purified, so that the resulting extract contained both unbound and bound nuclear 1,25-dihydroxyvitamin D–receptors. Additionally, the Tris buffer used by Sergeev and Spirichev [21] could interfere with the PLP effects.

Further analysis of our experimental data showed that the 1,25-dihydroxyvitamin D concentration in the nuclei was an exponential function of the cytoplasm-related nuclear PLP amount [Fig. 2(a)], as was confirmed by semilogarithmic transformation of this relationship [Fig. 2(b)]. This transformation produced a straight line with an inverse correlation between the "mobile" nuclear PLP content and the logarithm of 1,25-dihydroxyvitamin D concentration in the nuclei ($r = -0.95$). To our knowledge, this is the first demonstration of an *in vivo* relationship between PLP and the steroid–receptor ligand retention in the nucleus. Following our former assumption, the amount of 1,25-dihydroxyvitamin D reflects the amount of the occupied vitamin D receptor in the nuclei. Thus, PLP efficiently prevented the occupied vitamin D receptors from entering the nucleus, although inhibition of binding of the receptors to DNA in the nucleus by this compound could not be excluded by our results.

This regulation could occur through interaction of PLP with the receptor protein involving Schiff base formation, as proposed earlier [9], but an indirect

influence on the receptor through other PLP-modified proteins is also possible. Both processes could lead to changes in the vitamin D receptor conformation and result in loss of its ability to cross the nuclear membrane and/or bring about the dissociation of the ligand from the receptor. In effect, PLP would enter the nucleus instead of 1,25-dihydroxyvitamin D. Similar functions describe extraction and solubilization processes, but the conclusion that PLP acts on 1,25-dihydroxyvitamin retention in the nuclei rather unspecifically as a solvent would not necessarily be true. It will be interesting to investigate the effects of PLP-dependent, 1,25-dihydroxyvitamin D fluctuations in the nuclei on the expression of specific vitamin D-dependent genes. The specificity, if any, in the final effect of 1,25-dihydroxyvitamin D decrease or increase is unknown. Another question to pursue is whether the PLP–1,25-dihydroxyvitamin D relationship can be extended to other compounds related to the thyroid and steroid hormone superfamily.

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