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Research Article

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Markedly Increased Circulating Pyridoxal-5′-Phosphate Levels in Hypophosphatasia

Alkaline Phosphatase Acts in Vitamin B_6 Metabolism

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Abstract

Markedly increased circulating concentrations of pyridoxal-5′-phosphate (PLP) were found in each of 14 patients representing all clinical forms of hypophosphatasia, an inborn error characterized by deficient activity of the tissue nonspecific (bone/liver/kidney) isoenzyme of alkaline phosphatase (AP). The mean PLP concentration in plasma was 1174 nM (range, 214–3839 nM) in the patients and 57±26 nM (mean±SD) in 38 control subjects. In four affected children, urinary excretion of the PLP degradation product, 4-pyridoxic acid, was unremarkable during consumption of normal quantities of dietary vitamin B_6.

Our findings identify increased circulating PLP concentration as a marker for hypophosphatasia and provide further evidence that tissue nonspecific AP acts in vitamin B_6 metabolism. Tissue nonspecific AP appears to function as an ectoenzyme to regulate extracellular but not intracellular levels of PLP substrate. Performing assays of circulating PLP concentration alone to assess vitamin B_6 nutrition may be misleading in disorders associated with altered AP activity.

Introduction

Vitamin B_6 is the term used to describe three nutrients, pyridoxine, pyridoxal, and pyridoxamine, each of which must be converted to pyridoxal-5′-phosphate (PLP)[1] before they act as the coenzyme for a variety of important intracellular reactions (1). Although all cells may be capable of synthesizing PLP from pyridoxal, organ ablation studies in the dog indicate that the majority of circulating PLP is of hepatic origin (2). PLP is synthesized in the liver from pyridoxine, pyridoxal, and pyridoxamine, and then released into the blood where it circulates largely bound to albumin. Albumin-bound PLP does not appear to cross cell membranes directly; instead, the circulating PLP-albumin complex acts as a reservoir for pyridoxal which can enter cells once formed by hydrolysis of small quantities of free circulating PLP (2). Ultimately, intracellular PLP is metabolized largely to 4-pyridoxic acid, which is then excreted in urine (3).

We attempted to corroborate a variety of evidence which indicates that alkaline phosphatase (AP) [orthophosphoric monoester phosphohydrolase (alkaline optimum): EC 3.1.3.1] acts in the metabolism of PLP (2, 4–11) by assaying the major circulating vitamer forms of vitamin B_6 in the circulation and its degradative product, 4-pyridoxic acid, in the urine of patients with hypophosphatasia. Hypophosphatasia is an inborn error characterized biochemically by deficient activity of the tissue nonspecific (bone/liver/kidney) AP isoenzyme (12). We report that patients with hypophosphatasia have markedly elevated circulating PLP concentrations, but normal urinary levels of 4-pyridoxic acid.

Methods

Fourteen patients from 10 families with hypophosphatasia were studied (Table I). 13 subjects are followed by one of us (Dr. Whyte) either at the Metabolic Research Unit, Shriners Hospital for Crippled Children or the Clinical Research Centers, Washington University Medical Center in St. Louis, MO. All clinical forms of hypophosphatasia were represented; four subjects had the mild adult type, five were affected by the childhood form, and two had the severe infantile disease (12). Accordingly, each of these 11 individuals had radiographic evidence of rickets and/or defective bone mineralization (osteomalacia) as demonstrated by iliac crest biopsy. Two hypophosphatasemic adults (M.D. and D.M.) and one boy (R.J.) had no radiographic and/or no biopsy evidence of rickets or osteomalacia, but manifested the dental abnormalities of hypophosphatasia, i.e., odontohypophosphatasia (13). Four of the patients with adult hypophosphatasia were from two kindreds which we have reported in detail (14, 15).

Plasma (heparinized blood) and serum were obtained, after an overnight fast, from the patients with hypophosphatasia, three normal subjects, and two control patients with a different form of osteomalacia—hypophosphatemic bone disease. None of these subjects had an unusual diet or were taking vitamin preparations or pharmaceuticals known to affect vitamin B_6 nutrition or metabolism. Specimens were frozen within 1 h of venipuncture and, within 2 wk, were thawed and promptly assayed. Serum AP activity was measured at the Clinical Chemistry Laboratory, Barnes Hospital, St. Louis, MO at 37°C by a kinetic method using the RA-1000 system (Technicon Instruments, Tarrytown, New York) with 16 mM *p*-nitrophenylphosphate substrate in 0.35 M ampholine buffer (pH 10.35). The seven principal vitamer

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1. *Abbreviations used in this paper:* AP, alkaline phosphatase; HPLC, high performance liquid chromatography; PLP, pyridoxal-5′-phosphate; PPi, inorganic pyrophosphate.

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forms of vitamin B_6 were quantitated, in a blind fashion, by an established cation-exchange high performance liquid chromatography (HPLC) method (16). The sensitivity and coefficient of variation for this method for PLP are 1 ng/injection and 6%, respectively. The tyrosine apodecarboxylase method (17) was then used to confirm the HPLC results in the first four patients and two control subjects studied. In our laboratory, the sensitivity and coefficient of variation of this method are 0.05 ng/reaction flask and 4%, respectively.

To determine what fraction of the increased circulating PLP in the hypophosphatasia patients was protein-bound, plasma samples from two patients and two control subjects were filtered through membranes (Centriflo membrane ultrafilters; Amicon Corp., Danvers, MA) which retain molecules >50,000 mol wt, yet have essentially no retention for molecules <5,000 mol wt. The filtrate and retentate were then assayed for PLP by the HPLC method.

In four subjects, to assess the constancy of the elevated plasma PLP levels, plasma was collected and assayed for the vitamin B_6 vitamers at 4–11 mo after the initial studies.

To explore the mechanism for the markedly increased circulating PLP levels, we assayed urinary excretion of 4-pyridoxic acid in four of the affected children. Each was admitted to the Metabolic Research Unit, Shriners Hospital for Crippled Children where they received normal diets for 4 d which were prepared by the research dieticians and contained the recommended daily allowance of vitamin B_6 (18). Urine collections (24-h) were obtained during the last 2-d period and then assayed for 4-pyridoxic acid and pyridoxamine·2 HCl (16).

Results

Markedly increased plasma PLP levels were found in all subjects with hypophosphatasia (Table I). The average PLP concentration in patients was 1174 nM (range, 214–3839 nM), which was 21-fold greater than the average value for 38 normal adults (mean±SD, 57±26 nM) (16). The two patients with the severe infantile form of hypophosphatasia appeared to have, on average, the greatest plasma PLP concentration (52-fold greater than normal). The lowest plasma PLP concentration (214 nM) was in a child with odontohypophosphatasia. The three normal subjects had unremarkable plasma PLP levels, whereas plasma PLP levels in the two hyperphosphatasemic control patients with hypophosphatemic rickets or osteomalacia were undetectable (<20 nM). Pyridoxine-5′-phosphate was not detected in any patient or control sample. With the exception of S.S., only traces of pyridoxine, pyridoxamine, and pyridoxamine-5′-phosphate were detected in plasma. Plasma concentrations of pyridoxal and 4-pyridoxic acid were mildly elevated in some patients (Table I).

Increased plasma PLP levels were confirmed by the tyrosine apodecarboxylase method in the first four patient samples studied (Table I). The correlation coefficient for the plasma PLP concentrations determined by the cation exchange/HPLC vs. enzymatic methods was +0.99 for these four patient and two control samples.

Filtration studies revealed that, even with plasma PLP concentrations as great as 3053 nM in hypophosphatasia, >90% of the circulating PLP was protein-bound in both patient and control subjects (Table II).

Elevated circulating PLP levels (of similar magnitude) were observed in each of the four patients with hypophosphatasia in whom assays were performed at 4–11 mo after the initial studies (Table I).

Urinary levels of 4-pyridoxic acid were unremarkable (3)

Table I. Circulating Concentrations of Vitamers of Vitamin B_6 in Patients with Hypophosphatasia

Disease form	Subject*	Age/sex	Date of assay	AP‡	Vitamer§ PN‖ (19±33)	PL‖ (23±10)	PM‖ (2±2)	PLP‖ (57±26)	PLP¶	PA‖ (49±19)
		yr	*mo/yr*	*IU/liter*						
Infantile	J.S.	1.5 M	5/84	40 (75–250)	ND	47	<4	2237		96
	J.M.	2 F	10/84	30 (75–250)	ND	162	ND	3701		60
Childhood	B.B.	10 M	3/84	40 (140–340)	<49	98	ND	1424	1351	77
	B.B.	11 M	2/85	37 (140–340)	ND	64	ND	1561		71
	J.B.	7 M	3/84	30 (75–250)	49	47	6	894	1339	65
	J.B.	8 M	2/85	19 (75–250)	ND	39	ND	995		44
	K.M.	11 M	9/84	62 (140–340)	ND	<24	ND	346		19
	K.M.	11 M	1/85	55 (140–340)	ND	93	ND	502		38
	R.J.**	7 M	1/85	63 (75–250)	ND	49	ND	214		27
	S.S.	1.8 F	2/85	50 (75–250)	91	80	64	661		122
Adult	W.K.	55 M	3/84	15 (45–115)	<24	56	ND	926	1011	80
	R.K.	44 M	3/84	25 (45–115)	<20	63	ND	3053	3734	115
	R.K.	45 M	2/85	13 (45–115)	ND	69	ND	3839		158
	A.S.	65 F	5/84	29 (45–125)	ND	88	ND	526		63
	M.D.**	71 F	5/84	15 (45–125)	ND	40	6	365		47
	H.H.	65 F	4/84	19 (45–125)	ND	<98	ND	279		56
	E.S.	50 F	4/84	16 (35–100)	ND	<98	ND	639		107
	D.M.**	45 M	4/84	19 (45–115)	ND	366	ND	1175		132

ND, not detected; PA, 4-pyridoxic acid; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine. * Brackets indicate siblings. ‡ Normal range for age in parentheses. § All concentrations of vitamers of vitamin B_6 are nanomolar. Normal values ($\bar{x}$±SD) are from 38 control subjects (16). ‖ Assayed by cation-exchange/HPLC method (16). ¶ Assayed by tyrosine apodecarboxylase method (17). ** Odontohypophosphatasia.

*Table II. Fractionation of Plasma PLP by Ultrafiltration**

	Subject	Filtrate PLP	Retentate PLP	Percent unbound PLP
Patients	R.K.	244	3,042	8.0
	B.B.	106	1,355	7.8
Controls	M.W.	4.9	52.6	9.2
	M.L.	1.2	28.3	4.2

* PLP concentrations expressed as nanomolar.

in four affected children while they received diets containing recommended daily allowances of vitamin B_6 (Table III).

Discussion

AP is a ubiquitous plasma membrane-bound enzyme whose physiological role remains poorly defined despite intensive investigation (19). Biochemical studies, including amino acid sequence analysis of both partial proteolytic peptide digests and NH_2-terminal regions of AP purified from normal human tissues, suggest that three isoenzymes, each coded by separate genes, occur in man (20). They have been generally referred to as placental, intestinal, and tissue nonspecific (bone/liver/kidney) AP. Posttranslational modifications account for the well-documented physiocochemical differences in the tissue nonspecific AP family; bone, liver, and kidney AP are, therefore, secondary isoenzymes (19).

Study of the inborn error hypophosphatasia has resulted in the confirmation of the biochemical studies which indicate that at least three AP isoenzymes are present in man and in, perhaps, the best insight into the physiological role of AP (12). In the severe (autosomal recessive) infantile form, autopsy studies have revealed profound deficiency of AP activity in bone, liver, and kidney, but normal AP activity in intestine and placenta (fetal trophoblast). In the milder childhood and adult forms, which may be transmitted as an autosomal dominant trait, it has been suggested that the disorder represents clinical expression in heterozygous subjects (14–15). Although autopsy studies of the childhood and adult forms have not been reported, studies of dermal fibroblasts in culture support the hypothesis that all clinical forms of hypophosphatasia are characterized by deficiency of tissue nonspecific AP activity (21).

Although the precise genetic defect(s) in hypophosphatasia is unknown, a fundamental abnormality of this inherited disorder is deficient circulating and tissue activity of the tissue nonspecific AP isoenzyme (12). The clinical nature of hypophosphatasia, a form of rickets or osteomalacia, confirms a variety of evidence that AP functions in bone mineralization (12, 19). The discovery in 1966 that inorganic pyrophosphate (PPi) is an inhibitor of bone mineral deposition and dissolution, and documentation in 1971 that blood and urine levels of PPi are elevated in hypophosphatasia, provided a mechanism for the defective skeletal mineralization in this disorder and suggested that PPi is a natural substrate for AP (12, 19).

Our findings support a variety of evidence that AP acts in the metabolism of vitamin B_6 (2, 4–11). In vitro, PLP has been shown to be hydrolyzed by leukocyte subcellular organelles which are rich in AP activity (6), and an inverse relationship has been reported between the PLP concentration and AP activity in leukocytes from normal subjects compared with patients with Down's syndrome (4). Furthermore, AP purified from rat liver plasma membranes has been shown to hydrolyze PLP (5). In vivo, circulating PLP concentrations have been found to be reduced in patients with a variety of disorders associated with increased circulating AP activity, especially hepatic diseases and malignancy (8–11). Indeed, Anderson and colleagues (11) have demonstrated an inverse relationship between circulating PLP levels and AP activity in patients with either hepatobiliary or bone disease. This evidence would predict that the converse might occur in conditions associated with hypophosphatasemia, i.e., increased circulating PLP concentrations.

We found markedly increased circulating PLP concentrations in each of 14 patients representing all clinical forms of hypophosphatasia. Plasma PLP levels ranged from 3.8 to 67-fold greater than mean value for control subjects. Repeat assay in four patients suggested that the magnitude of the increased levels was consistent for individuals over an extended period of time (several months). The plasma concentration of the other vitamers of vitamin B_6—including the PLP precursors, pyridoxine, pyridoxal, and pyridoxamine; the phosphorylated forms pyridoxine-5′-phosphate and pyridoxamine-5′-phosphate;

*Table III. Urinary Excretion of Vitamers of Vitamin B_6 in the Childhood Form of Hypophosphatasia**

Subject	Date	Age/sex	Weight	Calculated daily intake of vitamin B_6	Urine PA	Urine PM
			kg	*μg*	*μg/d*	*μg/d*
J.B.	2/85	8/M	20.2	1,577	879 (2,198)	4.7 (11.8)
					1045 (2,903)	5.4 (15.0)
B.B.	2/85	11/M	32.8	1,740	716 (1,174)	4.4 (7.2)
					723 (1,225)	3.4 (5.8)
K.M.	1/85	11/M	47.8	2,450	1426 (1,828)	10.6 (13.6)
					1589 (2,091)	6.2 (8.2)
R.J.‡	1/85	7/M	25.5	1,540	533 (1,025)	3.8 (7.3)
					680 (1,063)	5.1 (7.9)

PA, 4-pyridoxic acid; PM, pyridoxamine · 2HCl. * Expressed in parentheses as micrograms per gram creatinine. ‡ Odontohypophosphatasia.

and the excretory product 4-pyridoxic acid—were not remarkably altered. Our ultrafiltration study demonstrated that the elevated circulating PLP levels in hypophosphatasia was an exaggeration of the physiological state, i.e., that the circulating PLP is largely, but not completely protein-bound (2). Lumeng et al. (2) reported that 90% of PLP is protein-bound when the PLP-to-albumin molar ratio is 1:1. Assuming a molecular weight of ~60,000 and a plasma concentration of 4 g/dl for albumin, the plasma PLP concentration could theoretically be as great as 700 μM in man without exceeding this ratio. In our patients with hypophosphatasia, the maximum observed PLP concentration was ~3.8 μM. Therefore, despite markedly elevated plasma PLP concentrations, PLP levels did not approach the binding capacity of albumin. It is of interest that, in most normal subjects, it is difficult to raise circulating PLP concentrations above 1.0 μM even with massive pyridoxine supplementation. Patients with Down's syndrome have been observed to develop somewhat higher plasma PLP levels than those without this disorder in response to pyridoxine supplementation (22). In Down's syndrome, plasma AP is normal, and yet, leukocyte AP is increased (4). Although increased intracellular AP has been shown to decrease the PLP concentration within Down's leukocytes (4), the increased leukocyte AP does not prevent the exaggerated plasma PLP concentrations during administration of pharmacologic doses of pyridoxine. Although leukocyte AP activity may be subnormal in patients with hypophosphatasia (12), together with recent evidence reported by Lumeng and coworkers (23) that PLP is not as vigorously hydrolyzed in blood in vitro as in vivo, these observations suggest that increased circulating PLP concentrations in hypophosphatasia result principally from failure of PLP hydrolysis by tissue nonspecific AP in extravascular sites rather than in the circulation. This conclusion is in keeping with other evidence that AP in the circulation is physiologically less active than in tissues (24).

Although circulating PLP concentrations are markedly increased in hypophosphatasia, vitamin B_6 nutrition does not appear to be markedly deranged. The clinical manifestations of hypophosphatasia are predominantly skeletal (12). The neurotoxicity associated with ingestion of pharmacologic amounts of pyridoxine (25) has not been reported in hypophosphatasia, nor have the clinical hallmarks of vitamin B_6 deficiency, such as dermatitis, stomatitis, peripheral neuritis, depression, or anemia. However, idiopathic seizures and nephrocalcinosis have been described in a few cases of the infantile form of hypophosphatasia (12), and vitamin B_6 deficiency in other subjects has been associated with epilepsy and altered oxalate metabolism (26). Our finding that the degradative product of vitamin B_6, 4-pyridoxic acid, is excreted into the urine in normal quantities in patients with hypophosphatasia who consume the recommended daily allowance of vitamin B_6 is consistent with the aforementioned clinical evidence which suggests that tissue levels of PLP are normal in hypophosphatasia. Furthermore, in 1975, Rodda (27) reported that the growth plates of long bones of vitamin B_6-deficient rats were narrow and reminiscent of starvation in experimental animals. In patients with rickets, including those with hypophosphatasia, the epiphyses are widened. Therefore, this finding is different from what one might expect if AP deficiency in hypophosphatasia lead to intracellular PLP deficiency. Normal urinary levels of 4-pyridoxic acid, together with absence of clinical manifestations of vitamin B_6 deficiency or toxicity, support the hypothesis that tissue levels of PLP are normal in hypophosphatasia. Although additional studies to confirm this hypothesis will be necessary, it appears from our observations that tissue nonspecific AP is acting as an ectoenzyme to regulate extracellular but not intracellular PLP levels.

Finally, our findings which provide further evidence that PLP is a natural substrate for the tissue nonspecific AP isoenzyme and that tissue nonspecific AP directly influences plasma PLP concentrations raise some important clinical considerations. Assays for plasma PLP are available to physicians by commercial laboratories and are used generally to assess vitamin B_6 nutrition. Subnormal circulating PLP levels are often interpreted to reflect vitamin B_6 deficiency (26). Our findings complement the studies which revealed that increased extravascular activity of tissue nonspecific AP is associated with decreased PLP levels in the circulation. Deficiency of serum AP activity in our patients was accompanied by increased plasma PLP levels. Since AP can markedly influence the concentration of PLP in the circulation, measurement of plasma PLP alone may not accurately reflect vitamin B_6 status in situations where AP activity is altered. Such situations include pregnancy where plasma PLP levels fall and AP activity increases (28). Further studies will be necessary to determine whether altered circulating PLP concentrations, resulting from increases or decreases in tissue nonspecific AP activity, are accompanied by changes in cellular PLP concentrations or metabolism.

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