J. Inher. Metab. Dis. 13 (1990) 891–896

Increased Plasma Pyridoxal-5′-phosphate Levels before and after Pyridoxine Loading in Carriers of Perinatal/Infantile Hypophosphatasia

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Summary: We measured plasma levels of pyridoxal-5′-phosphate (PLP), a cofactor form of vitamin B6 and apparent natural substrate for alkaline phosphatase (ALP), in carriers and in non-carriers of the severe perinatal and infantile forms of hypophosphatasia, both before and after an oral load of pyridoxine (i.e. $\frac{1}{3}$ mg/kg body weight). The assignment of carrier status was determined by serum ALP activity, level of serum inorganic phosphate, and if necessary urinary phosphoethanolamine excretion. Plasma PLP levels were significantly increased in the carriers both before and especially after B6 loading.

Hypophosphatasia is a metabolic bone disease which is characterized biochemically by decreased activity of the liver/bone/kidney (tissue non-specific) isoenzyme of alkaline phosphatase (Whyte, 1989a). Severe perinatal (lethal) and infantile forms and milder juvenile and adult forms have been identified based on the age at clinical presentation (Fraser, 1957; Whyte, 1989a). Perinatal and infantile hypophosphatasia are considered to be autosomal recessive conditions (McKusick 24150), whereas the inheritance pattern for the mild forms is less clear (Moore, 1990; Whyte, 1989a).

In all clinical forms of hypophosphatasia, there is endogenous accumulation of three phosphocompounds: phosphoethanolamine (PEA), inorganic pyrophosphate (PPi), and pyridoxal-5′-phosphate (PLP) (Whyte, 1989b). PLP is a cofactor form of vitamin B6 (Schideler, 1983), and acts as a coenzyme for a large number of apoenzymes which regulate the catabolism of amino acids, glycogen, and short chain fatty acids. The metabolism of PLP appears to be regulated by the liver/bone/kidney form of

MS received 8.11.89 Accepted 30.3.90

ALP, since markedly increased plasma levels of PLP have been documented in patients with all clinical forms of hypophosphatasia (Whyte *et al.*, 1988; Whyte, 1989a). Recent studies indicate that the liver/bone/kidney form of ALP acts as an ectoenzyme to control extracellular levels of PLP (Fedde *et al.*, 1988; Whyte *et al.*, 1988; Whyte, 1989b).

An increased incidence of all clinical forms of hypophosphatasia has been documented in the Mennonite community of Manitoba with an estimated carrier frequency of approximately 4% (MacPherson *et al.*, 1972; Chodirker *et al.*, 1990). We have found recently that as in subjects with the childhood and adult forms of hypophosphatasia, relative hyperphosphataemia also occurs in the carriers of severe hypophosphatasia (Chodirker *et al.*, 1990). We have developed accurate methods of carrier assignment for our high-risk population using combined information from assays of serum ALP activity, serum inorganic phosphate (Pi) levels, and urinary PEA excretion (Chodirker *et al.*, 1990). Such carrier assignment methods allowed us to test our hypothesis (Coburn and Whyte, 1988; Whyte, 1989b, 1989c) that aberrant extracellular metabolism of PLP in hypophosphatasia would also be reflected by elevated plasma PLP levels in carriers for severe hypophosphatasia, especially after they received an oral challenge with vitamin B6.

SUBJECTS AND METHODS

As part of a larger study on the genetics of hypophosphatasia, 58 individuals were found to be carriers for severe hypophosphatasia, and 82 individuals were found to be non-carriers. Carrier assignment was based on serum ALP, serum Pi levels and urinary PEA excretion as previously described (Chodirker *et al.*, 1990). Carrier status was inconclusive for 12 individuals based on these biochemical tests.

Following informed consent, dietary and drug histories were recorded. Ages and weights of all participants were documented. Plasma samples were then obtained for basal measurements of PLP. Participants were subsequently given vitamin B6 as $\frac{1}{3}$ mg of pyridoxine hydrochloride (ICN Canada) /kg body weight daily by mouth in the morning for six consecutive days. On the seventh morning, another blood sample was obtained. Participants were instructed to fast overnight prior to venepuncture for both the pre-load and post-load tests.

Plasma was separated by centrifugation of the heparinized blood samples within 4 h of collection and stored at $-20°C$. The anticoagulant used was sodium heparin. Plasma samples were shipped on dry ice to the laboratory of one of the investigators (S.P.C.) generally within one month of collection. PLP and other forms of vitamin B6 (e.g., pyridoxal, pyridoxamine and pyridoxine) were measured by high pressure liquid chromatography as previously described (Coburn and Mahuren, 1983) without knowledge of the assigned carrier status. Although only the plasma levels of PLP were compared between carriers and non-carriers, the pattern of the other compounds containing vitamin B6 was examined to determine if individuals were receiving exogenous B6 supplementation.

Individuals taking other drugs or exogenous vitamins or who had abnormal plasma pyridoxal and pyridoxamine levels were excluded from further study. Similarly, only

those whose carrier status was certain based on the routine tests were included. Accordingly, in this study we analysed pre-load plasma samples from 64 non-carriers and from 45 carriers. Post-loading plasma was available from 31 non-carriers and 22 carriers. The Student t test or χ^2 analysis was used to compare the data from the two groups.

RESULTS

Seven individuals (6 non-carriers and 1 carrier) stated that they were taking vitamin pills which contained or possibly contained vitamin B6. Two of these non-carriers also had elevated plasma pyridoxal and pyridoxamine levels. Two other non-carriers who did not volunteer a history of vitamin intake had elevated plasma pyridoxal and pyridoxamine levels suggesting exogenous B6 supplementation. One other non-carrier had very low plasma pyridoxal and pyridoxamine levels and was assumed to be vitamin B6 deficient. These 10 individuals were therefore excluded from further analysis. No study participant admitted to taking drugs known to alter vitamin B6 metabolism except for one carrier who was taking an oral contraceptive. She was excluded from the study as well. The decision to exclude these 11 individuals was made independently of knowledge of their carrier status.

For the pre-load study there were 36 male and 28 female non-carriers compared to 20 male and 25 female carriers. For the post-load study there were 15 male and 16 female non-carriers versus 13 male and 9 female carriers. In both instances, the differences in sex distribution were not significant ($p = 0.22$ and 0.44 respectively). The age and weight distributions of the study participants are shown in Table 1. No significant differences were seen except that female carriers in the post-load study were somewhat heavier than female non-carriers.

Table 2 summarizes the plasma PLP levels observed before and after pyridoxine loading. Plasma PLP levels were significantly elevated in carriers both before and especially after the oral load. The oral load was well tolerated with no adverse symptoms reported.

The distributions of pre- and post-load PLP in carriers and non-carriers are shown in the figures. As shown in Figure 1, 63 out of 64 non-carriers had a pre-load PLP

Table 1 Age and sex distribution

Test	*Participants*	*Non-carrier Mean (± SD)*	*Carrier Mean (± SD)*	*p*
Age (y)	Pre	39.3 (± 15.8)	37.0 (± 13.6)	0.4
	Post	36.7 (± 13.1)	34.4 (± 14.3)	0.5
Weight (kg)	Pre (males)	81.1 (± 10.3)	80.4 (± 13.5)	0.9
	Pre (females)	64.5 (± 14.8)	70.2 (± 14.6)	0.2
Weight (kg)	Post (males)	81.7 (± 9.5)	81.2 (± 14.6)	0.9
	Post (females)	63.6 (± 11.5)	75.7 (± 15.7)	0.04

Pre and Post = Participants for whom pre-load or post-load results were available respectively

Table 2 Plasma pyridoxal-5′-phosphate levels before and after B6 loading

Test	*Non-carrier*			*Carrier*			
	Mean (± SD)	*Range*	*n*	*Mean (± SD)*	*Range*	*n*	*p*
Pre-load PLP (nmol/L)	45.1 (± 30.1)	4–207	64	250.7 (± 221.1)	31–1182	45	<0.0001
Post-load PLP (nmol/L)	299.4 (± 162.9)	81–980	31	1313.5 (± 808.9)	104–3077	22	<0.0001

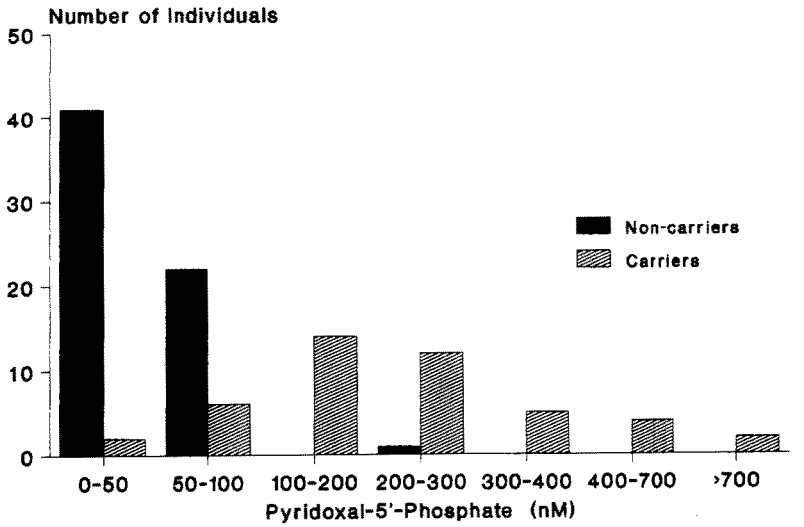

Figure 1 Distribution of pyridoxal-5′-phosphate (PLP) levels in carriers and non-carriers before an oral load of vitamin B6. Note the irregular scale used on the horizontal axis.

level of 100 nmol/L or less; only 8 out of 45 carriers had such a level. Similarly, 30 out of 31 non-carriers had post-load levels under 600 nmol/L compared to only 4 out of 22 carriers.

DISCUSSION

In 1985, plasma levels of PLP were discovered to be increased in 14 subjects who represented all of the various clinical forms of hypophosphatasia (Whyte, 1989a). In 1989, elevated plasma PLP levels were reported in all of the 33 probands who had been tested to date (Whyte, 1989a). That same year subjects with the various clinical forms of hypophosphatasia, including those who are mildly affected, were found to be particularly well distinguished by their plasma PLP levels following an oral load of pyridoxine (as used herein) (Whyte, 1989b). Accordingly, we suggested that pyridoxine challenge might be a good way to identify carriers of hypophosphatasia (Whyte, 1989c). This is now the first documentation of elevated plasma PLP in carriers for the perinatal/infantile form of hypophosphatasia.

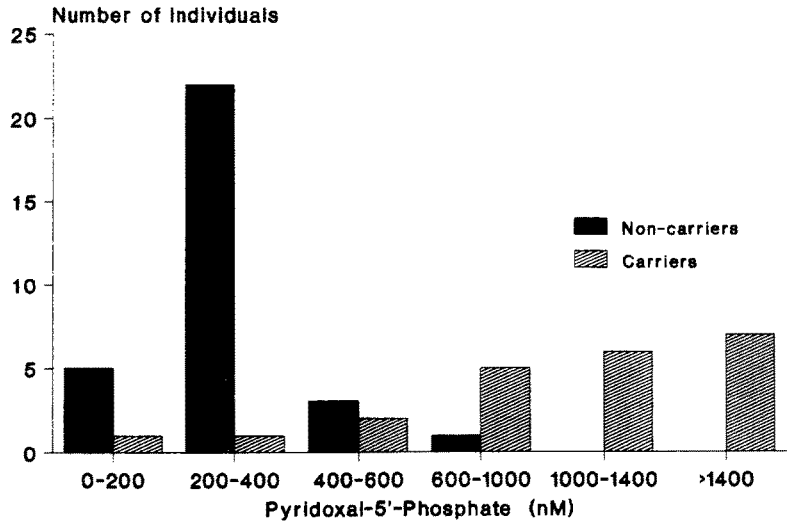

Figure 2 Distribution of pyridoxal-5'-phosphate (PLP) levels in carriers and non-carriers after an oral load of vitamin B6. Note the irregular scale used on the horizontal axis.

We have previously reported that 6% of individuals in our Mennonite study population could not have their carrier status definitively assigned based on our routine biochemical testing (assay of ALP, Pi and PEA) (Chodirker *et al.*, 1990). This original group consisted of 19 adult obligate carriers and 32 adult controls. If first degree relatives of the obligate carriers are studied as well, 8% (12 individuals) could not be classified by the routine methods. We found measurements of plasma PLP levels before or after oral loading with vitamin B6 (pyridoxine) to be a useful adjunct for carrier detection of hypophosphatasia in our high-risk Mennonite population and possibly in other populations as well. For example, of the 12 individuals for whom routine biochemical tests (i.e. ALP, Pi or PEA) gave inconclusive results for carrier assignment and were therefore not included in this study, 2 in fact were obligate carriers, i.e. parents of an affected infant. One had a highly elevated post-load PLP (1176 nmol/L) with a pre-load PLP of 85 nmol/L while the other had an elevated pre-load PLP (164 nmol/L) with a post-load level of 308 nmol/L. If a screening program designed to detect hypophosphatasia carriers is to be established in our community, it is crucial that a reliable back-up diagnostic test be available when these routine investigations are equivocal. Our results confirm that assay of plasma PLP levels, both before and/or after oral vitamin B6 loading, represents such a useful ancillary test. We would therefore recommend that in individuals for whom carrier status is uncertain after routine testing, the PLP level be measured both before and after an oral load of vitamin B6. This certainly applies in this population of Manitoba Mennonites: whether or not it would be true in other populations remains to be seen.

At present the genetic relationship between the milder juvenile and adult forms of hypophosphatasia is being studied in our high-risk population. Segregation analysis

may prove to be a helpful technique in delineating patterns of inheritance. Plasma PLP levels can now be used as an additional marker for such studies, and such an analysis of the data collected from these kindreds is underway.

ACKNOWLEDGEMENT

This project was generously supported by the Children's Hospital of Winnipeg Research Foundation, by the Manitoba Health Research Council, by grant no. 85-CRCR-1-1554 from the USDA Competitive Grant Program, and the Shriners Hospitals for Crippled Children. The secretarial assistance of Josie Diato and the technical assistance of Wayne E. Schaltenbrand, J. Dennis Mahuren and Cathy Mallory are gratefully acknowledged.

REFERENCES

Chodirker, B. N., Evans, J. A., Seargeant, L. E., Cheang, M. S. and Greenberg, C. R. Hypophosphatemia in infantile hypophosphatasia: Implications for carrier diagnosis and screening. *Am. J. Hum. Genet.* 46 (1990) 280–285

Coburn, S. P. and Mahuren, J. D. A versatile cation-exchange procedure for measuring the seven major forms of vitamin B6 in biological samples. *Anal. Biochem.* 129 (1983) 310–317

Coburn, S. P. and Whyte, M. P. Role of phosphatases in the regulation of vitamin B6 metabolism in hypophosphatasia and other disorders. In Leklem, J. E. and Reynolds, R. D. (eds.), *Clinical and Physiological Applications of Vitamin B6*, Liss, New York, 1988, pp. 65–93

Fedde, K. N., Lane, C. C. and Whyte, M. P. Alkaline phosphatase is an ectoenzyme which degrades natural substrates at micromolar concentration and physiologic pH in human osteosarcoma (SAOS-2) cells. *Arch. Biochem. Biophys.* 264 (1988) 400–409

Fraser, D. Hypophosphatasia. *Am. J. Med.* 22 (1957) 730–746

MacPherson, R. I., Kroeker, M. and Houston, C. S. Hypophosphatasia. *J. Can. Assoc. Radiol.* 23 (1972) 16–26

Moore, C. A., Ward, J. C., Rivas, M. L., Magill, H. L. and Whyte, M. P. Infantile hypophosphatasia: Autosomal recessive transmission to two related sibships. *Am. J. Med. Genet.* 36 (1990) 15–22

Rasmussen, K. Phosphorylethanolamine and hypophosphatasia. *Danish Med. Bull.* 15 (suppl. II) (1968) 1–112

Schideler, C. D. Vitamin B6: An overview. *Am J. Med. Technol.* 49 (1983) 17–22

Whyte, M. P. Hypophosphatasia. In Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D. (eds.), *The Metabolic Basis of Inherited Disease, 6th edn.*, McGraw-Hill, New York, 1989a, pp. 2843–2856

Whyte, M. P. Alkaline phosphatase: Physiologic role explored in hypophosphatasia. In Peck, W. A. (ed.), *Bone and Mineral Research, 6th edn.*, Elsevier Science Publishers, Amsterdam, 1989b, pp. 175–218

Whyte, M. P. Pediatric forms of hypophosphatasia. In Kleerekoper, M. and Krane, S. M. (eds.), *Clinical Disorders of Bone and Mineral Metabolism*, Mary Ann Liebert, New York, 1989c, pp. 383–391

Whyte, M. P., Mahuren, J. D., Fedde, K. N., Cole, F. S. and Coburn, S. P. Perinatal hypophosphatasia: Tissue levels of vitamin B6 are unremarkable despite markedly increased circulating concentrations of pyridoxal-5'-phosphate (evidence for an ectoenzyme role for tissue non-specific alkaline phosphatase). *J. Clin. Invest.* 81 (1988) 1234–1239