REGULATION OF PYRIDOXAL 5'-PHOSPHATE METABOLISM IN LIVER

Ting-Kai Li, Lawrence Lumeng[1], and Robert L. Veitch[2]

Departments of Medicine and Biochemistry,
Indiana University School of Medicine and
Veterans Administration Hospital
Indianapolis, Indiana 46202

Received September 25,1974

Summary

The pyridoxal 5'-phosphate content of liver *in vivo* and of hepatocytes *in vitro* remains unaltered in the presence of excess unphosphorylated vitamin B_6 precursors. Studies with isolated hepatocytes and subcellular fractions show that while product inhibition of pyridoxine phosphate oxidase does not limit synthesis sufficiently to account for the phenomenon, inhibition of phosphatase activity produces striking increases in pyridoxal 5'-phosphate concentration. Protein-binding protects it against degradation by the phosphatase. The data suggest that protein-binding and the enzymatic hydrolysis of pyridoxal 5'-phosphate, synthesized in excess, act jointly to preserve the constancy of the cellular content of this coenzyme.

Pyridoxal 5'-phosphate (PLP)[3] is quantitatively one of the major vitamin B_6 compounds in mammalian tissues and body fluids (1). Since PLP is highly reactive toward proteins and is, at unphysiologically high concentrations, an inhibitor of numerous enzymes *in vitro* (e.g. 2), mechanisms must exist for regulating its cellular content *in vivo*. It has been suggested that pyridoxine-P oxidase and perhaps phosphatase serve as control points in the pathways of vitamin B_6 metabolism (3). Purified pyridoxine-P oxidase is highly sensitive to product inhibition by PLP (4). On the other hand, various alkaline and acid phosphatases can hydrolyze PLP, but they are not specific for the phosphorylated B_6 compounds (5, 6). This communication evaluates the relative importance of these mechanisms in isolated parenchymal cells and subcellular fractions of rat liver. The role of protein-binding in the regulation of the cellular content of PLP and in the regulation of its degradation by phosphatase is also presented.

---

1 Clinical Investigator, Veterans Administration Career Development Award.
2 Postdoctoral Fellow, U. S. Public Health Service (T01 AM 5173).
3 Abbreviations: Pyridoxal 5'-phosphate, PLP; pyridoxine 5'-phosphate, pyridoxine-P; inorganic phosphate, Pi.

Copyright © 1974 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Materials and Methods

Three groups of 6 male weanling Sprague-Dawley rats were fed ad libitum a basal liquid diet (Bio-Mix 711, pyridoxine-deficient, Bioserv, Inc., Frenchtown, N. J.) to which pyridoxine, 0.5, 5.0 or 50 μg/ml, was added. After 20 days, the rats were sacrificed and brain and liver PLP content measured. Male Sprague-Dawley rats (150-180 g) maintained on a purified rat diet (ICN Pharmaceuticals, Cleveland, Ohio) were employed after a fast of 24-h for the preparation of both hepatic subcellular fractions and isolated hepatocytes. The pyridoxine content of the diet was 22 μg/g. Cytosolic fractions and plasma membranes were prepared by the methods of Hogeboom (7) and of Ray (8), respectively. The method for preparing hepatocytes was essentially that described by Ingebretsen and Wagle (9), except that after perfusion with collagenase (Worthington Biochemical Corp., Freehold, N. J.), the liver was minced and further dispersed with hyaluronidase (Worthington). Viability of the hepatocytes suspended in Krebs-Henseleit medium containing 2.5 g% bovine serum albumin was assessed by trypan-blue exclusion and by the rate of gluconeogenesis from pyruvate. All hepatocyte preparations exhibited dye exclusion in excess of 90% and rates of glucose synthesis exceeded 15 μmoles/h/100 mg protein.

PLP was extracted from brain tissue with metaphosphoric acid (10) and from liver with trichloroacetic acid. Experiments in vitro were performed at 37° C. Reactions which contain hepatocytes and cytosolic fractions were terminated by the addition of trichloroacetic acid and those with plasma membranes alone by heat. PLP was assayed enzymatically with tyrosine apodecarboxylase (11) and protein by the Biuret reaction (12). All chemicals were reagent grade.

Results and Discussion

Animals fed for 20 days the liquid diets supplemented with 0.5, 5.0 or 50 μg pyridoxine/ml exhibited normal growth curves. The mean daily intakes of pyridoxine were 0.025, 0.25 and 2.5 mg, respectively, corresponding to 2-, 20-

TABLE I.

The Effect of Pyridoxine Intake on PLP Content of Rat Liver and Brain

Pyridoxine Intake (mg/day)	Liver PLP (nmoles/g wet weight)	Brain PLP (nmoles/g wet weight)
0.025	38.1±2.0	7.7±0.4
0.25	37.2±4.4	8.1±0.4
2.5	40.5±2.8	8.1±0.4

and 200-times the daily dietary allowance recommended by the National Research Council (13). Despite these large differences in pyridoxine intake, brain and hepatic PLP levels did not vary significantly (Table I). To determine whether the same phenomenon obtains *in vitro*, isolated hepatic parenchymal cells were incubated in a medium containing physiological amounts of Pi (2.6 mM) and varying concentrations of pyridoxine. In all instances, the concentration of PLP did not increase during 45 min of incubation (Fig. 1). Similar results were obtained with pyridoxal as substrate. Thus there exists both *in vivo* and *in vitro* control mechanisms which tightly regulate the cellular content of PLP when vitamin B_6 precursors are in excess.

To ascertain the role of product inhibition of pyridoxine-P oxidase in regulating hepatic PLP content, PLP synthesis by the cytosolic fraction of rat liver was examined (Fig. 2). The addition of pyridoxine and ATP to cytosol resulted in a rapid rise in PLP concentration which reached in 2h a level more than 140% of that found in the rat livers and hepatocytes (40 nmoles/ml homogenate). Furthermore, when 50 μM PLP, an amount which already exceeds that present in liver, was added to the incubation mixture, net PLP synthesis was still apparent and a level 240% of that in liver was attained[4] (Fig. 2).

[4] In these experiments, although the incubation media contained 0.2 M Pi, it is evident that inhibition of the residual phosphatase activity in the cytosol was incomplete, since part of the large excess of PLP added to the incubation was being hydrolyzed. The nonlinear rates of PLP synthesis from pyridoxine can thus be attributed, at least in part, to PLP hydrolysis by the phosphatase.

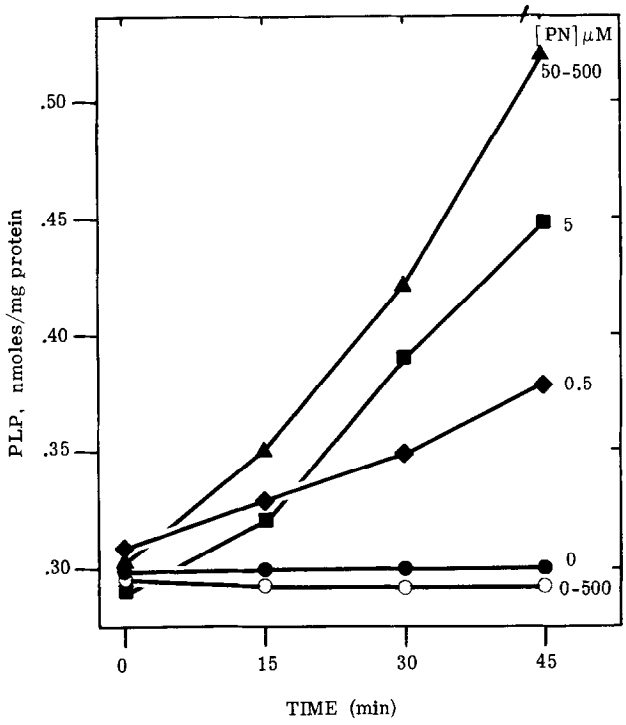

Fig. 1 Effect of [Pi] on the net synthesis of PLP from varying concentrations of pyridoxine (PN) by isolated rat hepatocytes. Hepatocytes (12.2 mg protein/5 ml) were incubated in a medium containing 2.6 mM (o-o) or 80 mM NaPi (closed symbols). The medium (pH 7.4) contained, in addition, KCl, 4.4 mM; $MgSO_4$, 1.2 mM; glucose, 10 mM; PN, 0-500 uM; bovine serum albumin, 2.5 g%; and NaCl in an amount sufficient to bring the osmolarity to 290 mOsM.

Thus, product inhibition of PLP synthesis does not appear to be sufficient to account for the tight regulation of the PLP content that is observed in hepatocytes. The failure of these concentrations of PLP, previously shown to be effective with purified pyridoxine-P oxidase, to inhibit PLP synthesis in cytosol is probably accountable, in part, by the binding of PLP to cytosolic proteins (vide infra), a process which would lower the effective, free concentration of PLP. Snell and Haskell have already pointed out that control of PLP metabolism at the pyridoxine-P oxidase step would be effective only when pyridoxine or pyridoxamine is the substrate (3). Since the studies with isolated hepatocytes showed that the net synthesis of PLP from pyridoxal is also tightly regulated, it is apparent that other control mechanisms must be operative physiologically.

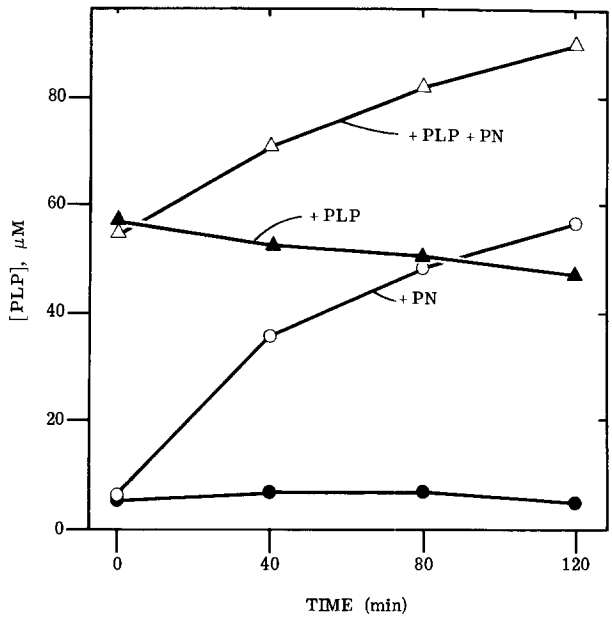

Fig. 2 Synthesis of PLP from pyridoxine (PN) by the cytosolic fraction of rat liver. PN, 2.5 mM and PLP, 50 μM were added where indicated to cytosol, 22.5 mg protein/ml; KPi, 0.2 M (pH 7.0); ATP, 10 mM; and $MgCl_2$, 5 mM.

We have previously reported that the inhibition of the phosphatase activity of erythrocytes leads to striking increases in the cellular content of PLP, synthesized from pyridoxine or pyridoxal (11). The importance of phosphatase activity in the regulation of hepatic cellular PLP content was, therefore, investigated. When isolated hepatocytes were incubated with 0.5 - 500 μM pyridoxine in the presence of 80 mM Pi, time- and dose-dependent net accumulations of PLP were observed (Fig. 1). Similar results were obtained with pyridoxal. In the pathways of PLP synthesis and degradation, high concentrations of Pi appear to affect solely the phosphatase activity. Whereas 80 mM Pi had no measurable effect on PLP synthesis from pyridoxine, catalyzed by pyridoxal kinase and pyridoxine-P oxidase in hepatic cytosol (Fig. 3A), the hydrolysis of PLP by the phosphatase of isolated plasma membranes was almost entirely inhibited (Fig. 3B). Thus hydrolysis of PLP, synthesized in excess, appears to be a physiologically important controlling factor in the regulation of the cellular content of PLP.

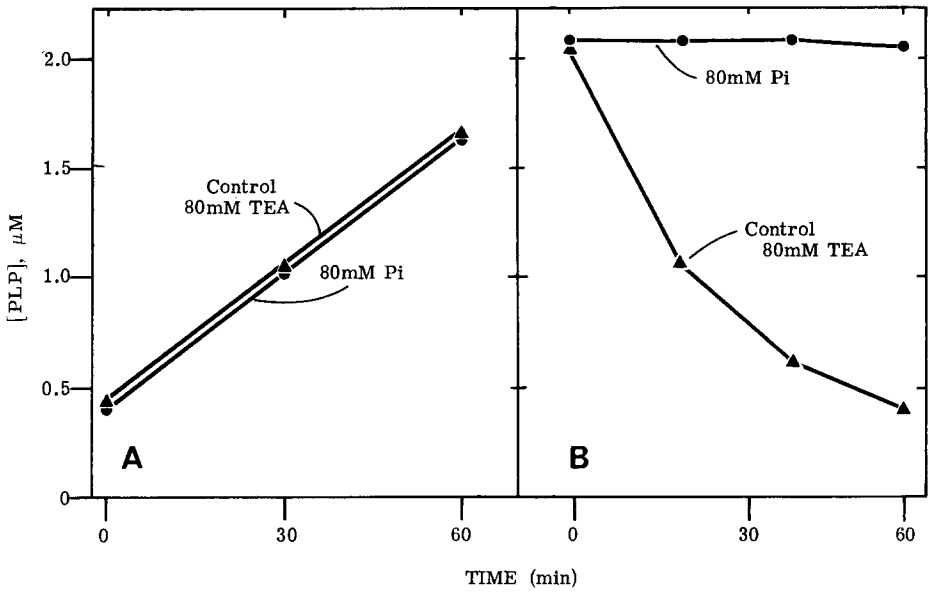

Fig. 3 Effect of [Pi] on PLP-synthesizing and -degradative enzyme activities from liver subcellular fractions. (A) The incubation mixtures contained pyridoxine, 0.5 mM; ATP, 2 mM; $MgCl_2$, 5 mM; cytosol, 2.0 mg protein/2 ml; triethanolamine-HCl (TEA), 80 mM or KPi, 80 mM, pH 8.0. (B) The incubation mixtures contained $MgCl_2$, 5 mM; liver plasma membranes, 35 μg protein/2ml; PLP 2 μM; TEA, 80 mM or KPi, 80 mM, pH 7.4.

The ability of PLP to interact with protein is well known. The extent of this interaction in liver and its role in regulating cellular PLP content were studied (Fig. 4). When hepatic cytosol was dialyzed exhaustively, only 50% of the PLP was lost, indicating that fully one-half of the PLP is firmly bound to proteins. The binding of PLP to cytosolic proteins markedly alters the susceptibility of PLP to degradation by the plasma membrane-associated phosphatase activity. Whereas free PLP was readily hydrolyzed by liver plasma membranes at pH 7.4, PLP in dialyzed cytosol was hardly hydrolyzed at all during the 90 min of incubation. Moreover, the rate of disappearance of the dialyzable portion of PLP or of PLP added to dialyzed cytosol was considerably retarded, suggesting that the PLP that is dialyzable may also bind, but less firmly, to cytosolic proteins.

These data indicate that protein-binding is a significant modifier of PLP hydrolysis by the phosphatase and that these two processes may function jointly

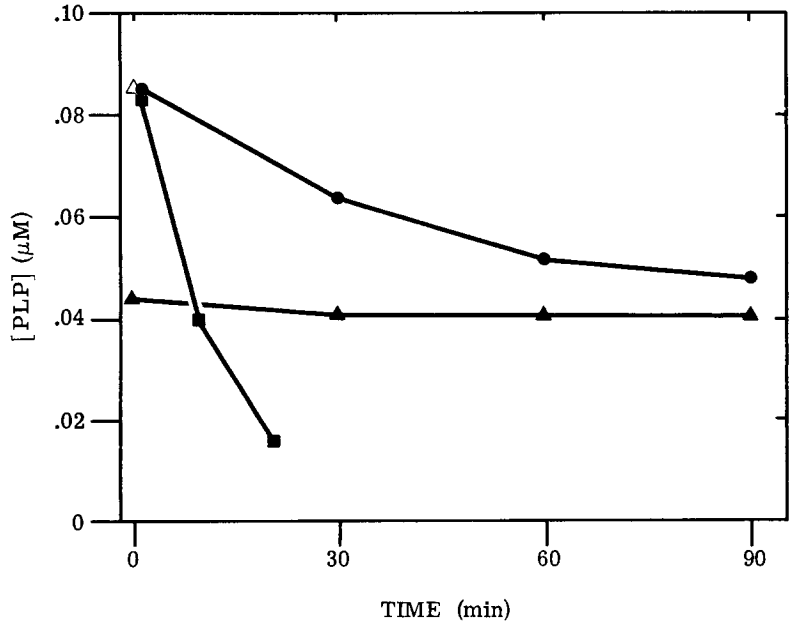

Fig. 4 Effect of dialysis and plasma membrane-associated phosphatase on the PLP content of hepatic cytosol. (△) undialyzed cytosol, 1.5 mg protein; (▲-▲) dialyzed cytosol plus plasma membranes, 0.32 mg protein; (●-●) dialyzed cytosol and 0.4 nmoles PLP plus plasma membranes; (■-■) 0.8 nmoles PLP plus plasma membranes. Triethanolamine-HCl buffer, 50 mM (pH 7.4) and $MgCl_2$, 5 mM in a final volume of 10 ml.

to maintain the constancy of the cellular content of PLP in the face of large excesses of the concentration of its precursors. When the PLP concentration exceeds the binding capacity of cellular proteins, excess, free PLP is rapidly hydrolyzed. The factors which regulate hepatic PLP metabolism are therefore similar to those which control the PLP content of serum: PLP in serum is bound to albumin and exists in balance as a dynamic pool between hepatic synthesis and PLP degradation (14).

The nature of the PLP-binding proteins in hepatic cytosol is unknown. In skeletal muscle, glycogen phosphorylase has been identified as the principal binding protein for PLP (15). The many PLP-dependent enzymes undoubtedly account for a substantial portion of the bound PLP in liver and exchange of PLP between apoenzymes has been reported to occur (16). Whether other proteins such as the anion-binding proteins in liver (17) participate as well in PLP-

binding is currently under investigation. Studies are also in progress to define the nature of the PLP-hydrolyzing activity of liver. Preliminary data indicate that it is largely accountable by the plasma membrane-associated alkaline phosphatase (18).

Acknowledgement: The technical assistance of Jen Yu and Cheryl Cosner is gratefully acknowledged. Supported by the Veterans Administration and the U. S. Army Medical Research and Development Command (DADA 17-72-C-2132).

References

1. Bain, J. A. and Williams, H. L. (1960). In Inhibition in the Nervous System and Gamma-aminobutyric Acid (Roberts, E., ed.) pp. 275-293, Pergamon Press, New York.
2. Shapiro, S., Enser, M., Pugh, E. and Horecker, B. L. (1968) Arch. Biochem. Biophys. 128, 554-562.
3. Snell, E. E. and Haskell, B. E. (1971) In Comprehensive Biochemistry (Florkin, M. and Stotz, E. H., eds.) Vol. 21, pp. 47-67, Elsevier Publishing Co., New York.
4. Wada, H. and Snell, E. E. (1961) J. Biol. Chem. 236, 2089-2095.
5. Turner, J. M. (1961) Biochem. J. 80, 663-668.
6. Andrews, M. J. and Turner, J. M. (1966) Nature 210, 1159.
7. Hogeboom, G. H. (1955) In Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 1, pp. 16-19, Academic Press, New York.
8. Ray, T. K. (1970) Biochim. Biophys. Acta 196, 1-9.
9. Ingebretsen, W. R., Jr. and Wagle, S. R. (1972) Biochem. Biophys. Res. Comm. 47, 403-410.
10. Minard, F. N. (1967) J. Neurochem. 14, 681-692.
11. Lumeng, L. and Li, T. K. (1974) J. Clin. Invest. 53, 693-704.
12. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) J. Biol. Chem. 177, 751-766.
13. National Research Council (1962) Nat. Acad. Sci. USA publ. 990, 51-95.
14. Lumeng, L., Brashear, R. E., and Li, T. K. (1974) J. Lab. Clin. Med. 84, 334-343.
15. Krebs, E. G. and Fischer, E. H. (1964) Vitamins Hormones 22, 399-410.
16. Churchich, J. E. (1970) Biochem. Biophys. Res. Comm. 40, 1374-1379.
17. Levi, A. J., Gatmaitan, Z., and Arias, I. M. (1969) J. Clin. Invest. 48, 2156-2167.
18. Li, T. K. and Lumeng, L. (1974) Fed. Proc. 33, 1546.