Preparation of Crystalline Pyridoxine 5′-Phosphate and Some of Its Properties

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Among the six forms of vitamin B_6, the only one that is not currently commercially available is pyridoxine 5′-phosphate. The methods that have been reported in the literature for its synthesis are fairly complicated and time consuming, and the product is of doubtful purity. In the present method, pyridoxine 5′-phosphate was synthesized from pyridoxal 5′-phosphate by reduction with $NaBH_4$. This simple reduction is complicated by the fact that the product forms a stable complex with the borate ions generated in the course of this process. The borate ions were removed by passing an alkaline solution of the products of reduction through 10 mL of Amberlite IRA-743 ion-exchange resin, which is specific for borate ions, and elution with water. The effluent was passed through 20 mL of Amerlite IRC-84 weak cation-exchange resin in the hydrogen form and concentrated, and after addition of acetone, white needlelike crystals of pure pyridoxine 5′-phosphate were obtained in 92% yield. It was free of a byproduct that is formed during this reduction. The ionization constants of pyridoxine 5′-phosphate and its stability in acidic, neutral, and alkaline solutions are reported.

In connection with our studies on the effect of various vitamin B_6 analogues on blood clotting (Sgoutas et al., 1983), PNP was needed for testing. Among the six forms of vitamin B_6 the only one that is not currently commercially available is PNP. Unfortunately, some companies sell pyridoxine phosphate (salt) that probably has been used, in place of the ester, by some investigators (Gustavson et al., 1966).

The synthesis of PNP was reported approximately 30 years ago (Heyl et al., 1951; Baddiley and Mathias, 1952; Peterson and Sober, 1954; Morrison and Long, 1958). All these methods are fairly complicated and time consuming, and the product is of doubtful purity. The deamination of PMP (Peterson and Sober, 1954) seems to be the method of choice (Vanderslice et al., 1980; Tryfiates and Sattsangi, 1982; Coburn and Mahuren, 1983). That method requires, for a 3-mmol scale, the passage of the reaction products through approximately 1000 mL of a weak cation-exchange resin, and in addition, there are claims (Osbond, 1964; Takeuchi et al., 1960) that the final product is a mixture of PNP and PN4P. PLP is commercially available and less expensive than PMP. The use of $NaBH_4$ for the reduction of aldehydes to the corresponding primary alcohols is well-known; the reduction proceeds generally rapidly and in most cases quantitatively in aqueous media. The idea of reducing PLP to PNP by $NaBH_4$ has been employed by a few investigators (Morrison and Long, 1958; Stock et al., 1966; Morino and Snell, 1967; Gregory, 1980; Viceps-Madore et al., 1983), but only in one case (Morrison and Long, 1958) are details of the synthesis reported. This reduction is complicated by the fact that the product, PNP, forms a stable complex with the sodium borate generated from $NaBH_4$ in the course of the reduction (Scheme I). A similar problem is confronted with the reduction of sugars by $NaBH_4$ to alcohols where the borate forms a complex with the reduced sugar. The borate is usually removed, as methyl borate, by repeated evaporation with methanol (Morrison and Long, 1958). The PNP–borate complex apparently does not break by passage of the reduced product through 100 mL of a weak cation-exchange resin (Morrison and Long, 1958).

In the present method, the borate is removed by passing the reduction product through 10 mL of Amerlite IRA-743 ion-exchange resin that is selective for borate anions. Also, the ionization constants of PNP were determined.

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EXPERIMENTAL PROCEDURES

Materials. Pyridoxal 5′-phosphate monohydrate, pyridoxamine 5′-phosphate monohydrochloride, pyridoxine hydrochloride, $NaBH_4$, Amberlite IRA-743, and Amberlite IRC-84 were from Sigma Chemical Co. All other reagents and chemicals were of the highest commercial grade available.

Preparation of Resins. Amberlite IRA-743 was washed repeatedly with absolute ethanol and then water. Then, it was washed sequentially with 1 M HCl, water, 1 M NaOH, and water. Amberlite IRC-84 was washed sequentially with 1 M NaOH, water, 1 M HCl, and finally water.

Preparation of Crystalline PNP. To a solution of 530 mg (2 mmol) of PLP in 8 mL of freshly made 0.5 M NaOH was added in portions and with mixing from time to time 30 mg (0.8 mmol) of $NaBH_4$. The dark yellow color of the initial solution became in 15 min light yellow. A few drops of acetone was added to destroy the excess of $NaBH_4$ and the solution left for 30 min. It was then made alkaline by adding two pellets of NaOH, left for a few minutes to dissociate the borate–PNP complex, and applied to a 1-cm i.d. column containing 10 mL of Amberlite IRA-743 ion-exchange resin in the free-base form. The effluent from the column was dropped into a 1-cm i.d. column containing 20 mL of Amberlite IRC-84 weak cation-exchange resin in the hydrogen form. After the 8 mL had been adsorbed in the resin, the columns were eluted with 30 mL of 0.5 M NaOH and then with 150 mL of glass-distilled water. The flow rate was 100 mL/h. The effluent from the second column was concentrated under vacuum at 40–50 °C to a small volume, and acetone was added until slight turbidity. After a few minutes, white, needlelike crystals appeared, were left for a while at room temperature, and then were placed in the refrigerator overnight to complete the crystallization: yield 458 mg (92%); mp 209–210 °C dec; UV [λ, nm (ϵ)] (0.1 M HCl) 290 (8540); (0.1 M NaOH) 245 (5960) and 310 (7160); (pH 7) 253 (3650) and 325 (7510).

The HPLC system and experimental conditions have been reported (Argoudelis, 1984). For this work an ISCO Model V[4] variable-wavelength detector was used.

Ionization constants were determined by potentiometric titration using a Beckman Model Century SS pH

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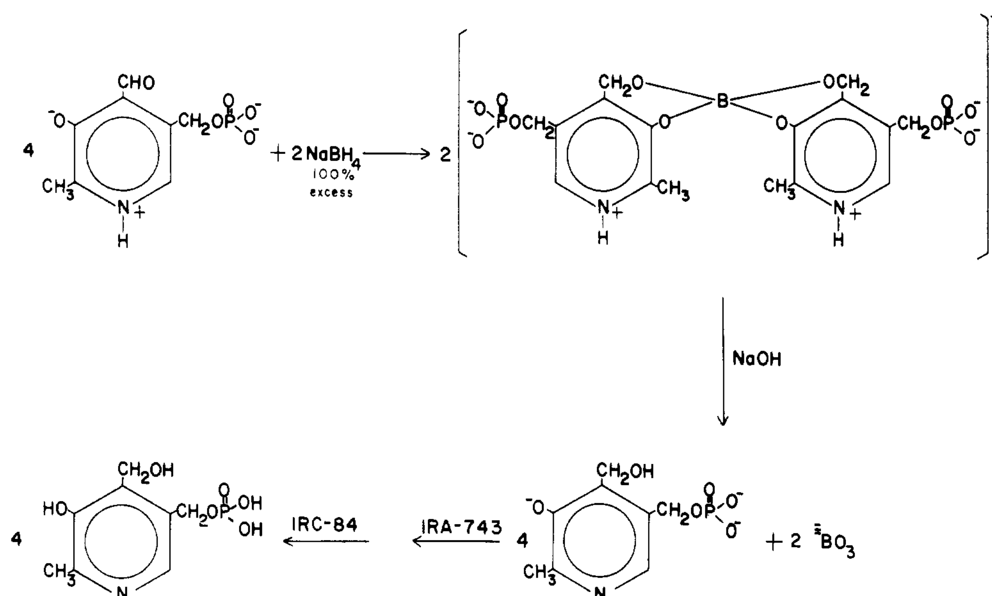
Scheme I



meter and a Fisher microprobe combination electrode at 0.01 M concentrations and 27 °C. Ionization constants were also determined by UV spectrophotometry using acetate or carbonate buffers at $I = 0.01$, at the analytical wavelength of 290 nm (phenolic) or 330 nm (pyridine nitrogen) and 7×10^{-5} M PNP. Both determinations of ionization constants were made by the method of Albert and Serjeant (1984). Commercial buffers were used to standardize the pH meter, and standard volumetric solutions of 0.1 N NaOH and 0.1 N HCl were from Anachemia Chemicals, Inc.

RESULTS AND DISCUSSION

The synthesis of PNP is depicted in Scheme I. The formation of the PNP–boric acid complex was ascertained from the UV spectrum obtained when an aliquot from the solution, taken after the reduction was completed, was placed in 0.1 M NaOH solution. Initially, the UV spectrum had a maximum at 298 nm (PNP–boric complex) and a shoulder around 305 nm (PNP) ($NaBH_4$ had been used in 60% Excess). The UV spectrum changed with time, and after 45 min the hydrolysis of the PNP–boric acid complex was completed, showing maxima at 310 and 245 nm (the expected spectrum of PNP in 0.1 M NaOH). There was no absorption at 388 nm indicative of completion of reduction of PLP.

It was found necessary to make the reduced solution strongly alkaline before applying it to the Amberlite IRA-743 resin; otherwise, an appreciable amount of PNP was left bound on the resin. The small excess of $NaBH_4$ must be destroyed since it passes through the resin. When the reduction was carried out under alkaline conditions, the isolated product had a light yellow color. The isolated crystalline PNP was free of PLP and PN as can be seen in the HPLC chromatogram presented in Figure 1.

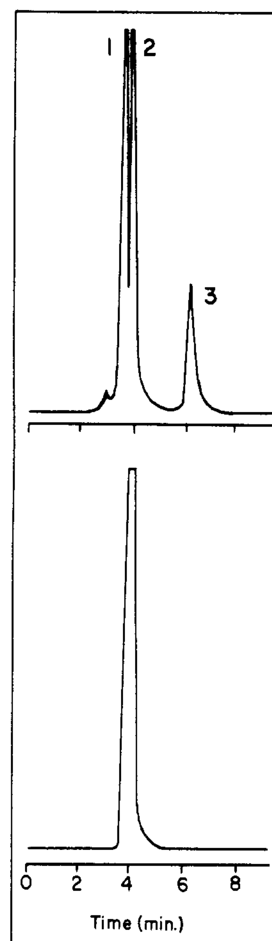


Figure 1. HPLC: (top) synthetic mixture consisting of PLP (1), PNP (2), and PN (3); (bottom) synthesized PNP. The small peak in front of PLP is an impurity present in commercial PLP. Column: Partisil-10SCX (250 × 4.60 mm) eluted with 0.1 M $NH_4H_2PO_4$ at 1 mL/min.

In view of the claims (Takeuchi et al., 1960) that PNP synthesized by the methods of either Heyel et al. (1951) or Peterson and Sober (1954) contained PN4P and since the chromatographic conditions used in this work might not separate the two isomers, the UV spectrum of the isolated crystals was taken in a phosphate buffer (pH 7) containing 2% boric acid. It has been reported (Scudi et al., 1940) that the PN–boric acid complex was formed only if the phenolic and 4′-hydroxymethyl groups were free. Therefore, PN4P cannot form the complex and consequently the λ_{max} at pH 7 of PN4P in the presence or absence of boric acid will be the same, i.e. 325 nm. It is assumed, of course, that the UV spectra of the two isomers will be very similar. The UV spectrum of the synthesized PNP crystals is shown in Figure 2 in pH 7 phosphate buffer containing 2% boric acid (broken line); the λ_{max} has shifted from 325 nm (without boric acid) to 298 nm. Since

Table I. Ionization Constants (pK_a Values)

compd	pK_1		pK_2		pK_3	
	titration	UV	titration	UV	titration	UV
pyridoxine	4.88 ± 0.05	4.87 ± 0.04	8.96 ± 0.03	9.02 ± 0.04	...	...
	4.72[a]	...	8.96[a]	...	...	...
	5.00[c]	5.00[b]	8.96[c]	8.97[c]		
	4.94[d]	...	8.89[d]			
pyridoxine 5'-phosphate	4.96 ± 0.05	4.98 ± 0.03	6.43 ± 0.04	...	9.69 ± 0.06	9.77 ± 0.03

[a] Harris et al. (1940). [b] Lunn and Morton (1952). [c] Williams and Neilands (1954). [d] Metzler et al. (1973).

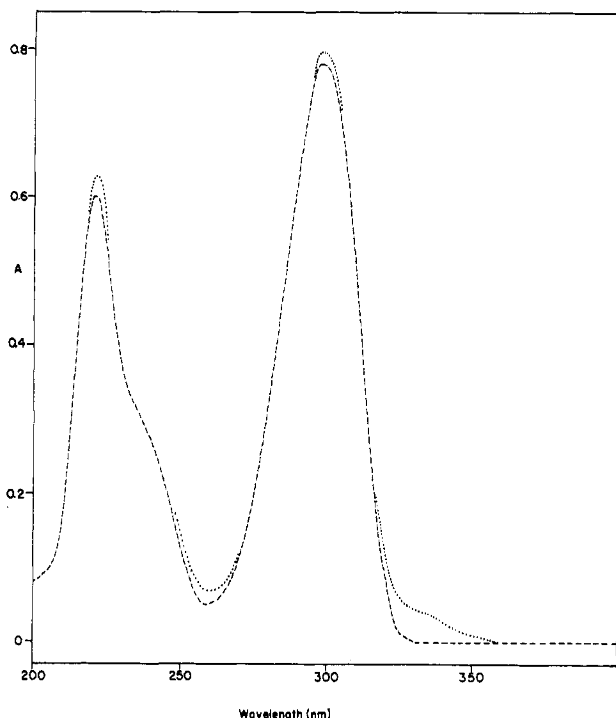


Figure 2. UV absorption spectrum: synthesized PNP at pH 7 in presence of 2% boric acid (---); a mixture of 4% PMP in PNP under the same condition (···).

PN4P was not available to see its effect on the UV spectrum of PNP in the same buffer, the UV spectrum of a mixture consisting of 4% PMP in PNP was taken (dotted line). The UV spectrum of PNP has a shoulder at 310 nm due to the presence of a small amount of PMP, which does not form a complex with boric acid, and thus its λ_{max} is not shifted. It was expected that a similar UV spectrum would have been obtained had the isolated PNP contained some PN4P as an impurity.

After the crystals of PNP were filtered off, an aliquot from the filtrate was injected into the HPLC system, and the chromatogram showed the presence of a byproduct absorbing at 290 nm that has not been identified yet.

The ionization constants of PNP and PN as determined by potentiometric titration and spectrophotometry are presented in Table I. There is very good agreement for the pK_a values determined by the two methods. There is also fairly good agreement for the pK_a values reported in the literature and those found in this work for PN. Apparently, the phosphate ester of PNP does not have an affect on the pK_1 value (phenolic), but it does have an effect on the pK_3 value (nitrogen), which is increased from 9.0 in PN to 9.7 in PNP, the highest of any of the six forms of vitamin B_6.

The stability of PNP under various conditions is shown in Table II. As expected PNP, a monoalkyl phosphate ester, was very stable in alkaline solutions. These results contradict those of Peterson and Sober (1954) who reported appreciable hydrolysis under similar alkaline conditions. It was thought that, perhaps, PNP was destroyed under those conditions, thus liberating inorganic phosphate. However, when similar aliquots of PNP solutions were injected into the HPLC system before or after heating the alkaline solutions at 100 °C for 16 h, the areas under the PNP peaks were very similar. The liberation of inorganic phosphate under alkaline conditions (Peterson and Sober, 1954) might be explained as being due to an impurity present in PNP prepared from PMP. As far as hydrolysis of PNP is concerned, it seems that it is very stable in alkaline solutions. All experiments were carried out under conditions of subdued light. In acidic conditions, it was found that the higher the concentration of the acid the lower the percentage of hydrolysis. When an aliquot from a known concentration mixture of PNP and PN was injected into the HPLC system, the ratio of the areas under the two peaks at 290 nm was found to be very close to the expected molar ratio of the two compounds.

Table II. Percentage Hydrolysis of PNP[a] to PN

conditions	%[b]
water 5 °C, 2 months	0.2
water 27 °C, 2 months	12
0.1 N H_2SO_4 25 °C, 5 months	1
0.1 N NaOH 25 °C, 5 months	0
water 100 °C, 16 h[c]	56
0.01 N H_2SO_4 100 °C, 16 h	57
0.1 N H_2SO_4 100 °C, 16 h	44
1 N H_2SO_4 100 °C, 16 h	24
0.01 N NaOH 100 °C, 16 h	0.4
0.1 N NaOH 100 °C, 16 h	0
1 N NaOH 100 °C, 16 h	0

[a] The concentration of PNP in the solutions was 1 mg/mL. [b] Determined from the ratio of the areas under the two peaks in HPLC. [c] In sealed ampules.

It is believed that the simple method reported in this paper for the preparation of pure crystalline PNP will facilitate the work of researchers interested in this compound.

Abbreviations Used: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PNP, pyridoxine 5'-phosphate; PN, pyridoxine; PN4P, pyridoxine 4'-phosphate; UV, ultraviolet; HPLC, high-performance liquid chromatography.

Registry No. PLP, 54-47-7; PNP, 447-05-2.

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Determination of Vitamin B_6 Bioavailability in Animal Tissues Using Intrinsic and Extrinsic Labeling in the Rat

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The effect of thermal processing on the bioavailability of vitamin B_6 in liver and muscle was examined by radioisotopic enrichment of these tissues. Rats were fed a single gelled test meal containing rat liver or muscle intrinsically enriched by vascular perfusion with [^{3}H]vitamin B_6 or a gelled test meal containing [^{3}H]pyridoxine (PN). Diets were extrinsically enriched with [^{14}C]PN to permit a direct comparison of enrichment methods. Absorption and metabolism were examined by analysis of tissues and excreta 24 h after the test meal had been consumed. The bioavailability of [^{3}H]B_6 vitamers in the raw tissues was equivalent to that of [^{3}H]PN in controls. Thermal processing of the tissues (121 °C, 45 min) induced destruction of 25–30% of the [^{3}H]B_6 vitamers and weakly reduced (≤10%) the utilization of the remaining [^{3}H]B_6 vitamers. The presence of monosodium glutamate (MSG) during thermal processing did not alter the results. The utilization of [^{14}C]PN was unaffected by diet composition. These data demonstrate the high bioavailability of vitamin B_6 in animal-derived foods and support the use of isotopic enrichment methods as an alternative to conventional bioassay procedures.

The vitamin B_6 content of many foods had been assessed by microbiological, chemical, and chromatographic techniques. This information has been used to evaluate the adequacy of vitamin B_6 dietary intakes of various population groups. However, to assess more fully the vitamin B_6 nutriture of a population, information about the expected extent of absorption and utilization of dietary vitamin B_6 should also be considered. The proportion of dietary B_6 vitamers that are absorbed and utilized in vitamin B_6 metabolism is referred to as the bioavailability of vitamin B_6. Vitamin B_6 bioavailability studies have been done by a variety of techniques such as bioassays with rats or chicks, intestinal perfusions, and human metabolic studies (Gregory and Ink, 1985). The effects of various dietary factors or treatments that could influence vitamin B_6 bioavailability such as fiber, protein, thermal processing, and storage conditions have been investigated previously. This research has provided information regarding the bioavailability of vitamin B_6, although interpretation of the results of such studies is frequently difficult. Additional studies that focus on the bioavailability of the vitamin are needed especially in light of reported marginal vitamin B_6 status in certain segments of the human population (Lonergan et al., 1975; Dempsey, 1978).

In this study, the bioavailability of the vitamin B_6 in animal products was examined on liver and muscle tissue that had been intrinsically enriched with radiolabeled forms of the vitamin and had received thermal and/or chemical treatment. This technique of intrinsic enrichment permits direct measurement of the absorption, metabolism, and retention of the tritiated B_6 vitamers from the diet without the ambiguities of conventional bioassays.

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MATERIALS AND METHODS

Animals and Diets. Male Sprague–Dawley rats (approximately 200 g) (Crl:CD(SD)BR) from Charles River Breeding Laboratories, Wilmington, MA, were individually housed in stainless-steel metabolism cages with wire-mesh floors and were fed a commercially nonpurified pelletted diet (#5001; Ralston Purina, St. Louis, MO) ad libitum. In addition, the rats were fed approximately 10 g of 1% (w/w) calcium alginate gel (Kelco Co., San Diego, CA), which contained 17 g of sucrose/100 g of gel. This gel was fed to the rats between 9:00 and 11:00 a.m. each day. The nonpurified diet was removed from the cage for several hours until the rat had consumed most of the gel.

After 1 week of conditioning in this way, the rats were fed a weighed alginate gel (typically 5–7 g) containing the appropriate source of radiolabeled vitamin B_6. Two hours after the gel had been fed, any gel remaining in the feed compartment of the cage was removed and weighed, after which the nonpurified diet was supplied ad libitum. The rats were decapitated 24 h after the radiolabeled gel had been fed. Livers were rapidly excised and frozen along with carcass (bones, hair, skin, kidney, muscle, etc.), intestinal contents (including cecal contents), plasma, feces, and urine for subsequent analysis.

All experimental gels were prepared with [^{14}C]pyridoxine added as an extrinsic label (0.15–0.3 μCi/g of gel; 4–6 nmol/g of gel). For control diets, [^{3}H]pyridoxine was

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