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Identification and Quantification of Pyridoxine–β-Glucoside as a Major Form of Vitamin B_6 in Plant-Derived Foods[1]

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A high-performance liquid chromatographic procedure was adapted to the fluorometric measurement of a glucoside conjugate of vitamin B_6 compounds in foods. 5′-*O*-β-D-Glucopyranosylpyridoxine was identified by HPLC and NMR methods as the major glycosylated form of the vitamin. This conjugate, which has been shown to exhibit incomplete metabolic utilization as vitamin B_6, was found to comprise 5–70% of the total vitamin B_6 in selected fruits and vegetables. Pyridoxine–β-glucoside was not detected in animal-derived foods including meats, human milk, and cow's milk.

The existence of conjugated or "bound" forms of vitamin B_6 in various foods of plant origin has been suggested by the results of several studies. Yasumoto et al. (1977) isolated and identified 5′-*O*-β-glucopyranosylpyridoxine from rice bran. These workers evaluated the biological activity of the synthetic pyridoxine–β-glucoside (PN–glucoside) and reported that the compound was well absorbed in vitro and was well utilized as vitamin B_6 in bioassays with deficient rats (Tsuji et al., 1977). Kabir et al. (1983a) devised an indirect microbiological assay procedure for the quantitation of β-glycosylated forms as well as total vitamin B_6 in foods. The results of this assay and studies of vitamin B_6 bioavailability in human subjects suggested that β-glycosylated forms of the vitamin were not biologically available (Kabir et al., 1983b). In contrast to the results of Tsuji et al. (1977), we have recently shown that PN–glucoside is well absorbed but undergoes little metabolic utilization in rats (Ink et al., 1986).

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A high-performance liquid chromatographic method was recently developed in our laboratory that is suitable for the measurement of the biologically active B_6 vitamers in foods and other biological materials (Gregory and Feldstein, 1985). The objectives of the present study were as follows: (a) to extend this HPLC method to the quantification of the principal β-glucoside conjugate(s) of vitamin B_6 in foods; (b) to identify the principal glycosylated form(s) of vitamin B_6 in foods; (c) to determine the concentration of total and glycosylated vitamin B_6 in selected foods.

MATERIALS AND METHODS

Reagents and Samples. Pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and 4′-deoxypyridoxine (4-dPN) were obtained as their hydrochloride salts from Sigma Chemical Co. (St. Louis, MO). Pyridoxal 5′-phosphate, 4-pyridoxic acid, and pyridoxamine 5′-phosphate also were obtained from Sigma. Water was purified for chromatographic use with a Milli-Q system (Continental Water Systems Corp., El Paso, TX). 2-Propanol (HPLC Grade) and sulfosalicyclic acid (Certified ACS) were obtained from Fisher Scientific Co. (Pittsburgh, PA). 1-Octanesulfonic acid (sodium salt) was obtained from Eastman Kodak Co. (Rochester, NY). All other chemicals were of analytical grade.

Tritiated pyridoxine hydrochloride (G-^{3}H; 1.67 Ci/mmol; Amersham Corp., Arlington Heights, IL), which was found by HPLC to have a radiochemical purity of 95%, was used without further purification. The isotopic distribution reported by the manufacturer was as follows: methyl, 61.9%; 5-methylene, 2.0%; 4-methylene, 21.9%; C-6, 11.9%.

All food samples were purchased locally. Samples of human milk (approximately 30 mL of foremilk) were obtained from six local donors ranging in stage of lactation from 1–8 months. These women included three who consumed a typical mixed diet (meats, poultry, fish, fruits, cereals, vegetables, dairy products) and three who were lacto-ovo vegetarians. Several donors consumed a daily vitamin supplement providing 2 mg of pyridoxine hydrochloride.

Determination of PN–Glucoside in Foods. Extraction using sulfosalicylic acid (SSA) and purification of sample extracts by anion-exchange chromatography were performed by methods developed by Vanderslice et al. (1981) as described in our previously reported HPLC procedure (Gregory and Feldstein, 1985). Solid samples (2 g) were mixed with 9 mL of 5% (w/v) SSA and 1 mL of a solution of 4-dPN (typically 120 nmol/mL in 5% SSA) and then homogenized with a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY) for 45 s at power setting 7. Ten milliliters of methylene chloride was added, and the mixture was blended for 20 s at power setting 5. The homogenates were centrifuged at 9000*g* for 15 min at 2 °C, after which an aliquot of the aqueous layer was removed.

Milk samples (3 mL) were blended in 10-mL centrifuge tubes with 1 mL of 20% (w/v) SSA and 0.15 mL of 120 nmol/mL 4-dPN with a Polytron for 45 s at power 7. The mixtures were mixed again on a Vortex mixer after the addition of 4 mL of methylene chloride for 20 s and then centrifuged.

Preparative anion-exchange chromatography was performed with a column packed with Bio-Rad AG2-X8 (Bio-Rad Laboratories, Richmond, CA), essentially as described by Vanderslice et al. (1981), to remove SSA and purify the sample extracts. The biologically active B_6 vitamers, 4-dPN, and PN-glucoside eluted as a single fluorescing peak near the void volume (retention time approximately 4 min). The eluate either was analyzed directly or was subjected to enzymatic hydrolysis prior to HPLC analysis. For enzymatic hydrolysis of PN–glucoside, 0.9 mL of the AG2-X8 eluate was diluted with 0.1 mL of 1.0 M sodium phosphate, adjusted to pH 5.0 with 1 N NaOH, and then 0.05 mL of a solution of β-glucosidase in water was added (2 mg of protein, 60 U; Sigma Chemical Co.; type I). The mixtures were incubated for 5 h in a 37 °C water bath and then deproteinated by addition of 0.07 mL of 100% (w/v) trichloroacetic acid.

The HPLC analytical system, which was identical with that described previously (Gregory and Feldstein, 1985), consisted of two solvent metering pumps (Model 110A; Altex Scientific, Berkeley, CA), a gradient controller (Altex Model 410), injection valve (altex Model 905-40 with 50-μL loop), and a fluorometric detector (Model LS-5; Perkin-Elmer Corp., Norwalk, CT) equipped with a 20-μL flow cell. An excitation wavelength of 330 nm (15-nm bandpass), an emission wavelength of 400 nm (20-nm bandpass), and a response factor of 4 were employed. An electronic integrator (Model 3388A; Hewlett-Packard, Avondale, PA) recorded and integrated chromatographic data, initiated and reset solvent programs, and controlled programmed valve switching between solvent A2 and B (described below). The analytical column was a Perkin-Elmer 3 × 3 (3 μm octadecylsilyl, 3-cm length × 4.6-mm i.d.). Fluorometric detection was enhanced by using the postcolumn phosphate–bisulfite reagent (Coburn and Mahuren, 1983), as previously described (Gregory and Feldstein, 1985). The 1.0 M potassium phosphate reagent containing 1 mg/mL sodium bisulfite (pH 7.5) was pumped into the column eluate stream at 0.2 mL/min (Model 196-31 pump; Milton-Roy Co., St. Petersburg, FL).

The following HPLC mobile phases were employed: solvent A1, 0.033 M potassium phosphate and 8 mM octanesulfonic acid (pH 2.2); solvent A2, 0.033 M potassium phosphate, 8 mM octanesulfonic acid, and 2.5% (v/v) 2-propanol (pH 2.2); solvent B, 0.033 M potassium phosphate, 6.5% 2-propanol (pH 2.2). The separation was peformed at ambient temperature by using a linear gradient (1.8 mL/min total flow rate) from 100% solvent A1 to 100% solvent A2 in 12 min, followed by a programmed switch to 100% solvent B 15 min after injection. After elution of PM, approximately 10 min was required for reequilibration prior to injection of the next sample. A blank gradient was run daily as a check for trace contamination of the water with vitamin B_6 of microbial origin. Methanol was used as the storage solvent. Quantitation was done relative to the response of the internal standard 4′-deoxypyridoxine. All phases of this procedure were performed under gold fluorescent lights, and samples were shielded with aluminum foil to minimize photochemical degradation of vitamin B_6 compounds.

Isolation of PN–Glucoside from Alfalfa Sprouts. Dry alfalfa seeds (8 g) were soaked in water overnight, then washed with water, and drained. Pyridoxine hydrochloride (25 mg) was dissolved in several milliliters of water and the resultant added to the moist seeds. The seeds were maintained in an open container in the dark at ambient temperature (ca. 22 °C) for 7 days. Water was added daily as needed to maintain hydration. After 7 days a total of 68 g of alfalfa sprouts was obtained.

For the purification of PN–glucoside, 12 g of alfalfa sprouts was minced and homogenized in 3 mL of water with a Polytron. Trichloroacetic acid was immediately added to yield a final concentration of 7% (w/v). The homogenate was centrifuged 20 min at 12000g at 4 °C. The

trichloroacetic acid was removed from the supernatant containing PN-glucoside by repeated extraction with diethyl ether, followed by evaporation of traces of ether under a stream of nitrogen gas.

PN-glucoside was isolated by preparative HPLC by a binary step-gradient method similar to that reported previously (Gregory and Feldstein, 1985). The preparative separation was performed on a Techsphere Ultra 5C18 column (5 μm octadecylsilyl, 5-mm i.d. × 25-cm length; HPLC Technology, Palos Verdes Estates, CA), and the following mobile phases were utilized: solvent A, 0.033 M potassium phosphate, 8 mM octanesulfonic acid, 2.5% (v/v) 2-propanol (pH 2.2); solvent B, 0.033 M potassium phosphate, 14% (v/v) 2-propanol (pH 2.2). The column was equilibrated in solvent A. After injection of 900 μL of the alfalfa extract, the column was eluted with solvent A for 5 min, followed by continued isocratic elution with solvent B for 40 min. Fractions were collected, and those containing PN-glucoside were pooled. In contrast to the analytical separation in which PN-glucoside eluted between PL and PN, PN-glucoside eluted between PMP and PL in the preparative procedure. This preparative HPLC was repeated until all of the extract had been purified.

Isolated PN-glucoside was further purified to remove the components of the HPLC mobile phase. A column (0.7-cm i.d. × 10-cm length) was packed with Bio-Rad AG50W-X8 (100–200 mesh, ammonium form) and equilibrated in 0.033 M ammonium phosphate, pH 2.2. The entire volume of pooled PN-glucoside (13 mL) was applied to the column and then washed with 10 mL of 0.05 M ammonium acetate (pH 4) followed by a convex gradient from 25 mL of 0.05 M ammonium acetate (pH 4) and 25 mL of 0.25 M ammonium acetate (pH 4). Elution was accomplished by using a convex gradient of 25 mL of 0.25 M ammonium acetate (pH 4) and 25 mL of 0.25 M ammonium acetate (pH 7). Fractions were monitored for the presence of the glucoside by HPLC. PN-glucoside eluted within the first 25 mL of the gradient. The fractions containing PN-glucoside were pooled and lyophilized. The yield of PN-glucoside was approximately 400 μg of PN equivalents (2.4 μmol) or 9% relative to the PN added (based on 12 g of sprouts). Analytical HPLC of the purified glucoside indicated the absence of PN and other B_6 vitamers.

Preparation of Tritiated PN-Glucoside in Alfalfa Sprouts. In order to evaluate the conjugation process and to determine the fate of PN-glucoside during extraction and extract purification, alfalfa sprouts (3 g of dry seeds) were grown as described above except in the presence of tritiated PN (2.0 μCi, 1.2 nmol). The tritiated PN was added after hydration of the seeds, and water was added daily as needed over the 5-day growth period. Three replicates of this procedure were performed. The total distribution of vitamin B_6 compounds was determined by extraction with SSA, purification on the AG2-X8 column, and analytical HPLC (Perkin-Elmer 3 × 3 column, ternary gradient) with fluorometric detection. The distribution of radiolabeled B_6 vitamers was determined by HPLC (Techsphere Ultra 5C18 column, binary gradient) followed by liquid scintillation spectrometry of collected fractions using Aqualyte scintillation fluid (J. T. Baker Chemical Co., Jackson, TN). Conversion of counts per minute to disintegration per minute was done by using efficiency determined from the observed H number and quench curves.

Characterization of PN-Glucoside by NMR Spectroscopy. Pooled fractions of unlabeled PN-glucoside from ion-exchange chromatography (approximately 300 μg of PN equivalents) were lyophilized to dryness. The sample was dissolved in 5 mL of deuterium oxide (99.6 atom %) and again lyophilized to achieve equilibration. The deuteriated sample was then dissolved in 1 mL of deuterium oxide containing 1 mg of sodium (trimethylsilyl)tetradeuteriopropionate (TSP; Wilmad, Buena, NJ), and the NMR spectra were recorded.

NMR spectra were obtained at 25 °C on a Nicolet NT-300 system (Nicolet Instrument Corp., Madison, WI) with a superconducting wide-bore spectrometer operated in the Fourier transform mode at 300 MHz. The 70-kG field was locked on the deuterium signal, with data acquisition in the double-precision mode due to the limited amount of sample. Spectra were recorded at a 90° angle with a sweep width of 2500 Hz.

RESULTS

Determination of PN-Glucoside. The previously reported ion-pair HPLC method for the determination of vitamin B_6 compounds in foods and other biological materials (Gregory and Feldstein, 1985) was examined to determine its suitability for the measurement of PN-glucoside. The gradient elution scheme previously described achieved separation of PN-glucoside from the other vitamers. Occasionally, a nonlinear gradient was used when improved resolution of the glucoside from PL and PN was required. PN-glucoside eluted as a fluorescing peak between PL and PN in all analyses.

Evaluation of the molar response of PN-glucoside in purified form and that present in plant extracts was performed by measuring peak areas relative to the area of the internal standard before and after enzymatic hydrolysis. These results, as illustrated in Figure 1, indicated equivalent molar fluorescence of PN-glucoside and PN. Therefore, the concentration of the glucoside may be routinely determined by measurement of the PN-glucoside peak area with quantification relative to the area of the PN standard, which eliminates the need for a purified PN-glucoside standard.

Enzymatic hydrolysis served as a convenient diagnostic tool in verifying the identity of the glucoside peak. The chromatograms of Figure 1 illustrate the effect of enzymatic hydrolysis and show representative separations. The lack of interferences was verified by the fact that, in all plant-derived samples examined, no peaks having the retention time of PN-glucoside were observed following β-glucosidase treatment.

The area of the PN-glucoside peak was linearly related to the amount injected over the entire range examined (10 pmol–100 nmol/injection). This range of linearity is consistent with that observed with other forms of vitamin B_6 in this analytical system (Gregory and Feldstein, 1985). The limit of detection (signal/noise 3) for the determination of PN-glucoside was approximately 1 pmol/50-μL injection, which corresponds to 0.06 nmol/g of sample. The linear response characteristics and high sensitivity are suitable for the measurement of PN-glucoside at levels occurring in a wide variety of foods and other biological materials.

The overall precision of the preparative and analytical chromatographic systems was evaluated through repetitive analysis of a standard mixture at levels routinely used in the assay (Table I). The observed relative standard deviation (RSD) for PN-glucoside (3.77%) was similar to that of other B_6 vitamers (2.46–5.04%).

The results of the analysis of various fruits, vegetables, and milk are shown in Table II. Analysis of replicate samples of various foods yielded RSD values for all vitamers that were generally greater than those observed for

Table I. Within-Run Precision of Preparative and Analytical Chromatographic Systems Determined by Five Replicate Analyses of a Mixed B_6 Vitamer Solution[a]

vitamer	concn, nmol/mL	RSD, %	vitamer	concn, nmol/mL	RSD, %
PLP	10	3.06	PN–glucoside	1.3	3.77
PMP	4	2.46	PN	5	5.04
PL	5	3.68	PM	2	3.69

[a] Abbreviations: RSD, relative standard deviation; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PN–glucoside, 5′-*O*-β-D-glucopyranosylpyridoxine; PN, pyridoxine; PM, pyridoxamine.

standards (Table II). This may reflect the added variance of extraction as well as possible heterogeneity of the samples. PN–glucoside was detected in all plant-derived samples and ranged from 5.5% of the total vitamin B_6 in bananas to 70.1% in carrots among the common foods analyzed. The complete distribution of vitamin B_6 compounds in many of these plant-derived foods has not been previously determined.

Evaluation of chromatograms (e.g., Figure 1) before and after treatment with β-glucosidase indicated that PN was the only significant glycosylated form of the vitamin in the samples analyzed. As observed in the previous study (Gregory and Feldstein, 1985), effective sample purification by the anion-exchange system (Vanderslice et al., 1980) as well as the specificity of the fluorometric detection yielded chromatograms that were largely free of non B_6 components. Of the samples analyzed to date from both plant and animal sources, the only interference found was an incompletely resolved peak that precluded measurement of PLP in peanut butter.

A peak that eluted slightly before PN–glucoside was observed in all samples of cow's milk and human milk. Repeated analysis of milk samples before and after enzymatic treatment in the presence and absence of added PN–glucoside confirmed that the pyridoxine–β-glucoside was not endogenously present in human or cow's milk (Table I). In view of the low limit of detection for PN–glucoside (1 pmol/injection), the presence of PN–glucoside at levels of 3–5% of the total vitamin B_6 in human milk could be readily detected. Enzymatic treatment of milk extracts to which exogenous PN–glucoside had been added yielded complete hydrolysis and formation of PN; however, enzymatic treatment did not alter the area or retention time of the unidentified peak in any of the milk samples.

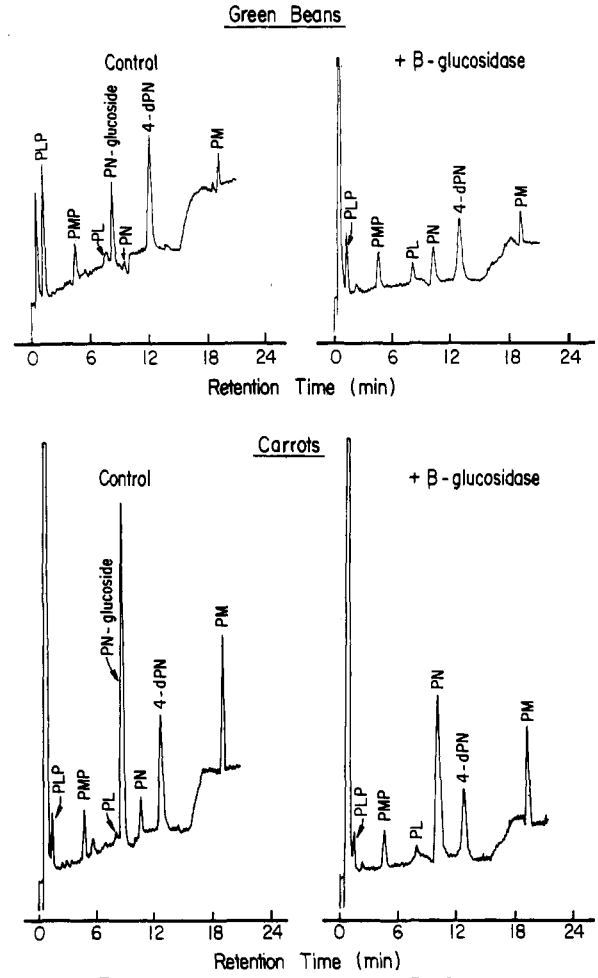

Figure 1. Typical chromatograms from HPLC analysis of green beans and carrots before and after treatment with β-glucosidase.

The HPLC data for total vitamin B_6 and the percentage of PN–glucoside were compared with previously published data concerning vitamin B_6 in these foods (Table III). General agreement between the HPLC results for total vitamin B_6 found in this study with the previously published data supports the validity of the HPLC procedure. Large differences were observed between the values for PN–glucoside percentage found in this study and the total glycosylated vitamin B_6 reported by Kabir et al. (1983a). Differences in total vitamin B_6 and percentage PN–

Table II. Concentration of PN-Glucoside and Other Forms of Vitamin B_6 in Selected Foods[a]

sample		PLP	PMP	PL	PN–glucoside	PN	PM	total B_6
raw broccoli	mean	5.82	1.65	0.85	5.62	1.91	0.15	16.0
	SD	1.32	0.38	0.18	0.84	0.71	0.04	1.8
peanut butter	mean	int	1.19	1.21	7.15	2.18	0.71	12.4
	SD		0.30	0.33	1.58	0.51	0.16	1.7
bananas	mean	3.20	0.29	0.41	1.18	12.5	4.02	21.6
	SD	0.24	0.06	0.13	0.22	1.13	0.40	1.3
raw green beans	mean	4.17	1.24	0.93	9.30	nd	0.21	15.9
	SD	0.34	0.23	0.28	0.44		0.02	0.7
raw carrots	mean	2.15	0.71	0.47	18.3	2.91	1.56	26.1
	SD	0.72	0.01	0.05	2.66	0.84	0.26	2.9
orange juice	mean	nd	1.04	0.32	5.99	1.04	0.27	8.67
	SD		0.02	0.09	0.16	0.02	0.04	0.19
cow's milk	mean	0.363	0.290	2.09	nd	nd	0.262	3.01
	SD	0.080	0.038	0.05			0.032	0.11
human milk[b]	mean	0.308	nd	0.645	nd	nd	0.039	0.992
	SD	0.093		0.062			0.001	0.112

[a] Values are given in nanomoles/gram for solids and nanomoles/milliliter for liquid foods. The orange juice was reconstituted from frozen concentrate. ND, not detected; int, PLP not measured in peanut butter due to interfering peaks. All data were derived from four independent extractions and analyses of single samples. [b] Milk sample was from a donor consuming a mixed diet and a daily 2-mg supplement of pyridoxine hydrochloride.

Table III. Comparison of HPLC Results with Previously Published Data

sample		this study	previous publications: Kabir et al.	Orr	Coburn and Mahuren
raw broccoli	total B_6, nmol/g	16.0	10.0	11.5	
	% PN-glucoside	35.1	nd (65)		
bananas	total B_6, nmol/g	21.6	18.5	30.2	
	% PN-glucoside	5.5	3		
raw green beans	total B_6, nmol/g	15.9	3.55	4.7	
	% PN-glucoside	58.5	10 (28)		
raw carrots	total B_6, nmol/g	26.1	10.1	8.9	
	% PN-glucoside	70.1	51		
orange juice	total B_6, nmol/g	8.67	2.54	2.1	
	% PN-glucoside	69.1	37		
cow's milk	total B_6, nmol/g	3.01	0.3	2.4	2.09
	% PN-glucoside	nd	nd		
human milk	total B_6, nmol/g	0.992		0.592	0.888
	% PN-glucoside	nd			

[a] Previously published data from Kabir et al. (1983a), Orr (1969), and Coburn and Mahuren (1983). The values in parentheses of Kabir are for canned products. The data of Coburn and Mahuren were obtained by HPLC analysis. Other analyses were based on microbiological growth assay.

Table IV. Concentration of Endogenous and Tritiated Vitamin B_6 Compounds in Alfalfa Sprouts

form of vitamin B_6	expt[a]	PLP	PMP	PL	PN-glucoside	PN	PM	total B_6
endogenous	1 (nmol/g)	1.95	1.81	2.37	38.9	1.96	0.86	47.9
	1 (% dist)	4.1	3.8	4.9	81.3	4.1	1.8	
tritiated	1 (% dist)				67.5	32.5		
tritiated	2 (% dist)	2.8		9.0	72.6	12.5	3.1	
tritiated	3 (% dist)	2.0	1.2	5.3	70.8	19.3	1.4	

[a] Refers to analysis of three independent preparations of alfalfa sprouts in the presence of a tracer amount of tritiated PN. % dist refers to the percentage distribution of endogenous or tritiated B-6 vitamers. Only tritiated compounds were analyzed in experiments 2 and 3.

glucoside between these studies may reflect variation in stage of maturity, varietal differences, and environmental conditions during propagation and after harvest. A direct comparison between the HPLC method and the microbiological procedure of Kabir et al. is required to resolve these questions.

The propagation of alfalfa sprouts served as a convenient means of preparing a plant tissue under controlled conditions for evaluation of B_6 vitamer distributions and as a source of PN-glucoside for further characterization. Quantification of extracted radioactivity indicated 73–97% uptake of the tritiated PN. Similar patterns of B_6 vitamer distribution indicated that the metabolism and conjugation of the labeled PN paralleled that of the endogenous vitamin B_6 (Table IV). Because the pattern of radiolabeled B_6 vitamers was determined by direct analysis of crude extracts and the endogenous B_6 vitamers were determined by HPLC following anion-exchange purification, the similarity of these results is evidence of high recovery of PN-glucoside in the preparative phase of the analytical procedure. High recovery values (>90%) were also observed for purified PN-glucoside added to food samples prior to extraction and HPLC analysis.

Identification of PN-Glucoside. The NMR spectrum of PN-glucoside dissolved in deuterium oxide is shown in Figure 2. The spectrum was obtained by using a double-precision data acquisition technique with a one-pulse experiment. Signals at δ 5.03, 5.01, 4.69, and 4.66 represent doublets of the 5′-methylene protons while the signal at δ 4.98 was derived from the 4′-methylene protons. The 5-methylene protons of underivatized (nonglycosylated) pyridoxine have been reported to elicit NMR signals at a higher field than the 4-methylene protons (Korytnyk and Paul, 1965). The signals of these methylene protons would be markedly altered by glycosylation at either position, which would be indicative of the site of the glycosidic linkage. The spectrum of PN-glucoside in this study

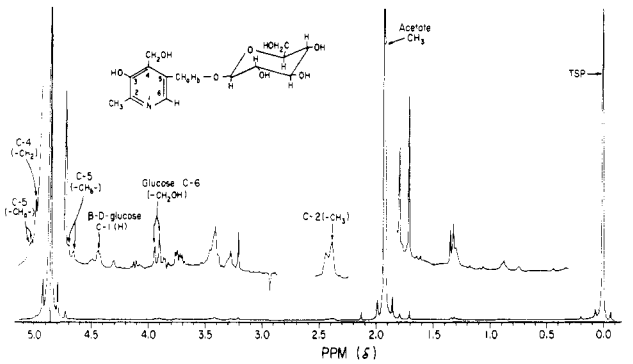

Figure 2. NMR spectrum of PN-glucoside in deuterium oxide.

(Figure 2) was suggestive of a 5′-glycosylation. In addition, comparison of the observed spectrum to those reported for acetylated derivatives of pyridoxine 4′-*O*-β-glucoside and 5′-*O*-β-glucoside (Suzuki et al., 1979) provided further evidence that the glucosidic linkage of the compound isolated in this study was associated with the 5′-methylene carbon of pyridoxine.

The signal at δ 4.44 represents the anomeric proton of glucose. Spectra of methyl-β- and methyl-α-D-gulosiduronic acid have shown that the anomeric proton of the β orientation is in an axial shielded position while that of the α-glycoside is equatorially oriented with a signal (δ 5.38) at a lower field than that of the β-glycoside (δ 4.56; Penman and Sanderson, 1972). Thus, the high-field position (δ 4.44) of the anomeric proton in this spectrum indicates a β-glucosidic linkage. The observed coupling constant (approximately 6 Hz) of the δ 4.44 signal also is evidence of a trans-diaxial arrangement of the anomeric proton and the proton of the adjacent (C-2) carbon, which is similar to the spectrum of methyl-β-D-gulosiduronic acid (Penman and Sanderson, 1972). The signal at δ 2.38 is derived from the methyl protons at the 2-position of PN. Signals found at δ 3.2–3.8 are associated with hydroxy-

methyl and other protons of glucose. The sharp signals at δ 1.92 and 0 were due to residual acetate from the isolation procedure and the TSP reference, respectively.

DISCUSSION

The results of the studies reported here indicate that 5′-*O*-β-D-glucopyranosylpyridoxine (PN–glucoside) represents a substantial proportion of the vitamin B_6 in many fruits and vegetables. The identity of this conjugated form of vitamin B_6 was verified by (a) conversion of the glucoside to PN by treatment with β-glucosidase and (b) NMR spectra indicating conjugation to the 5′-position of PN by an *O*-β-glycosidic linkage.

These studies have established the applicability of reversed-phase ion-pair HPLC to the determination of PN–glucoside in foods. As shown in this study and in previous research (Gregory and Feldstein, 1985), this HPLC method permits an accurate and highly sensitive determination of vitamin B_6 compounds in foods and biological materials. Factors contributing to the validity of the method include an effective sample extraction and ion-exchange purification procedure (Vanderslice et al., 1980), high-efficiency chromatographic HPLC separation, sensitive fluorometric detection facilitated by postcolumn addition of strongly buffered bisulfite solution (Coburn and Mahuren, 1983), and individual quantification of vitamin B_6 compounds using 4′-deoxypyridoxine as an internal standard. The precision, sensitivity, and recovery observed for PN–glucoside observed here were comparable to those of other vitamin B_6 compounds.

With the exception of bananas, in which the PN–glucoside exists as a minor constituent, the plant-derived samples examined contained approximately 35–80% of their vitamin B_6 as PN–glucoside (Tables III and IV). PN–glucoside was not detected in liver or muscle samples from rats, cattle, or swine. Recent studies in our laboratory concerning the metablism of PN–glucoside have indicated that β-glucosylation of B_6 vitamers does not occur in vivo in the rat (Ink et al., 1986), and no evidence of the formation of other conjugated forms of the vitamin has been seen. The absence of PN–glucoside from milk suggests that the primary route of elimination of dietary PN–glucoside from the body is by urinary excretion, as recently reported (Ink et al., 1986), with little or no secretion by the lactating mammary gland in the human and the cow. It was particularly noteworthy that PN–glucoside was not detected in any of the human milk samples, regardless of the diet of the donor (mixed vs. lacto-ovo vegetarian). The data for vitamin B_6 compounds in human milk were from replicate analysis of a single sample (Tables II and III). These data (total concentration and distribution of B_6 vitamers) were similar to results from analysis of samples from other donors.

The measurement of glycosylated forms of vitamin B_6 has been done previously by differential microbiological assay after hydrolytic treatments (Kabir et al., 1983a; Yasumato et al., 1976). Kabir et al. (1983a) examined various fruits and vegetables and reported results for β-glycosylated vitamin B_6 that differed somewhat from those found in this study. Underestimation of glycosylated vitamin B_6 in samples containing endogenous β-glucosidase activity may have been a limitation of their assay, as suggested by the data of Table III. The use of sulfosalicylic acid as an extractant in this study rapidly inactivates hydrolytic enzymes and, thus, minimizes these losses. Yasumoto et al. (1976) also utilized a microbiological assay procedure for the examination of "bound" forms of vitamin B_6 in various cereal grains and seeds. Unidentified forms of the vitamin that became active for the assay organism following β-glucosidase treatment were detected in all samples analyzed in that study. Ion-exchange chromatographic analysis of enzymatic hydrolysis products indicated that PN was the major form of vitamin B_6 released by β-glucosidase treatment (Kabir et al., 1983a; Yasumato et al., 1976). HPLC analysis of enzymatically hydrolyzed samples in this study (Figure 1) directly confirmed these observations. Similar results were also reported by Nelson et al. (1977) concerning the analysis of an unidentified bound form of vitamin B_6 in orange juice.

The existence of two minor esterified derivatives of PN–glucoside pea seedlings has been recently reported. Tadera et al. (1983) found a PN–glucoside in which the C-6 position of the glucopyranosyl moiety was esterified to 3-hydroxy-3-methyl-4-carboxybutanoic acid, while in later studies they found the analogous malonyl ester (Tadera et al., 1985). Further research is needed to determine the quantitative significance of these esterified forms of PN–glucoside in foods, although the results of the present study suggest that such derivatives of PN–glucoside are negligible in common plant-derived foods. Research has shown that a glucosyltransferase of pea seedlings exhibits high specificity for uridinediphospho-(UDP)glucose as the glucose donor in the conjugation of PN in vitro (Tadera, 1982). The source of the acyl moiety in the esterified derivatives is unclear at this time.

A major implication of this research involves the nutritional properties of PN–glucoside. Research employing radiolabeled PN–glucoside in rats indicated little in vivo utilization in vitamin B_6 metabolism (Ink et al., 1986). The urinary clearance of PN–glucoside was nearly 4 times faster than for nonglycosylated vitamin B_6, while intact PN–glucoside represented 85% of urinary labeled vitamin B_6 compounds. Low apparent bioavailability also was reported by Kabir et al. (1983b) in studies of the utilization by human subjects of vitamin B_6 from several foods. Most information concerning the content of vitamin B_6 in foods has been derived by microbiological growth assays using vitamin B_6 dependent yeasts (Toepfer and Polansky, 1970). The procedure recommended for samples of plant origin require heating of the sample in HCl at 121 °C in 0.44 M HCl for 2 h (AOAC, 1970), which induces complete hydrolysis of PN–glucoside as well as phosphorylated forms of the vitamin. Thus, microbiological assay procedures such as these would overestimate the biologically available vitamin B_6 in foods. The extent of hydrolysis as influenced by other combinations of time, temperature, and acid concentration have not been examined. Earlier microbiological assays based on extractions with dilute sulfuric acid (Atkin et al., 1943) presumably would yield at least partial hydrolysis, although this has not been determined. The HPLC method reported here, which utilizes much milder extraction conditions and permits a direct measurement of PN–glucoside, would yield data that could be interpreted with regard to the low bioavailability of PN–glucoside.

The high concentration of PN–glucoside in many plant-derived foods suggests a widespread incomplete bioavailability of the vitamin in human diets, which could not be evaluated accurately by current food composition tables. The absence of PN–glucoside from meats and milk is further evidence of high bioavailability of the vitamin in these foods. The potential existence of PN–glucoside or a derivative in human milk from women ingesting large proportions of their vitamin B_6 in the conjugated form requires further study.

In summary, 5′-*O*-β-D-glucopyranosylpyridoxine has been found to be a common component of the vitamin B_6

of plant-derived foods. The HPLC procedure reported here permits a direct assessment of the concentration of this compound and represents the first instrumental method that provides results that would be relavant to the bioavailability of vitamin B_6 in foods.

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Application of in Vitro Methods To Assess the Nutritive Value of Leaf Protein Concentrates

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Sixteen leaf protein concentrates (LPC) were prepared from different crops by different processes and either freeze dried or oven dried in the 1983–1985 seasons. Total lysine and chemically available lysine of these samples were estimated. Biological assay parameters such as N retention and apparent digestibility were evaluated. Total lysine and chemically available lysine show good correlation with results from biological assay. Predicted biological nutritive values show good agreement with those from in vitro studies.

Processing conditions affect the nutritive value of food proteins. Rat assays are the best methods of nutritive value evaluation. Some in vitro methods such as digestibility with proteolytic enzymes (Buchnan, 1969; Saunders et al., 1973), growth of tetrahymena (Lexander et al., 1970; Smith and Pena, 1977), and microbial availability of essential amino acids (Henry and Ford, 1965) were used for nutritive evaluation of LPC. Shurpalekar et al. (1966) and Bickoff et al. (1975) using protein efficiency ratio assays with rats found considerable losses of nutritive value on thermal drying of LPC curd as compared with freeze drying. Henry (1964), who determined the biological value and true digestibility of LPC samples, found that hot-air drying in particular reduced the true digestibility of LPC. Byers (1971) found that damage to the LPC occurred during the heat coagulation stage, particularly to its lysine content. Proteins with lysine as a first limiting amino acid might, therefore, be subject to more severe reductions in nutritive value than would those deficient in methionine (Knipfel et al., 1975).

Biological nutritive evaluation methods are costly and time consuming. Hence, attempts are made to find quicker and simple chemical methods to predict the biological nutritive value parameters. In LPC preparations methionine is usually the first limiting amino acid. In some LPC preparations, due to low availability lysine could be the second deficient or limiting amino acid (Ohshima, 1985). Thus, digestibility and availability of methionine and lysine are the chief factors that govern the nutritive value. Processing conditions affect both sulfur amino acids and lysine in the same way. Since methods for determination of available lysine have worked out well for studies on factors affecting the nutritive value of LPC, a mathematical relation between available lysine and in vivo rat assay parameters such as apparent digestibility and N retention will be highly useful.

In order to evolve a suitable mathematical relationship between chemically available lysine and apparent diges-

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