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# Determination of Pyridoxine β-Glucoside Bioavailability Using Intrinsic and Extrinsic Labeling in the Rat

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A major form of vitamin $B_6$ in plant-derived foods is 5′-*O*-(β-D-glucopyranosyl)pyridoxine (PN-β-glucoside). In this study, the bioavailability of vitamin $B_6$ as tritiated PN-β-glucoside in purified form and in intrinsically enriched alfalfa sprouts was examined relative to $^3H$- and $^{14}C$-labeled pyridoxine (PN). Twenty-four hours after administration of the isotopes in a single test meal, isotopic contents of tissues and excreta and distribution of vitamin $B_6$ metabolites were determined. The extent of intestinal absorption of PN-β-glucoside was approximately half that of PN. PN-β-glucoside, when fed in purified form or in sprouts, was partially hydrolyzed in vivo and utilized vitamin $B_6$. Over 80% of urinary $^3H$ derived from dietary PN-β-glucoside was in the form of the intact glucoside, however. These results indicate incomplete bioavailability of vitamin $B_6$ as a glucose conjugate.

The vitamin $B_6$ content of various plant-derived foods has been determined following hydrolytic treatment to release bound forms of the vitamin. This process may not be reflective of the digestive process in the human gastrointestinal tract, and the analytical results may not represent the amount of vitamin available for metabolic utilization. A conjugated form of pyridoxine and glucose (pyridoxine β-glucoside) has been isolated from rice bran and identified as 5′-*O*-(β-D-glucopyranosyl)pyridoxine (Yasumoto et al., 1977). The amount of vitamin $B_6$ present as a glycosidic conjugate has been determined in several foods and was found to represent 50% or more of the total vitamin $B_6$ in some fruits, vegetables, and legumes (Kabir et al., 1983a). Tsuji et al. (1977) reported that chemically synthesized pyridoxine β-glucoside both is biologically available as vitamin $B_6$ to rats that are $B_6$ deficient and is permeable to everted sacs of rat small intestine. In contrast, Kabir et al. (1983b) reported an inverse relationship between the glycosylated vitamin $B_6$ content of a food and its vitamin $B_6$ bioavailability in humans as indicated by measurement of urinary 4-pyridoxic acid and plasma pyridoxal phosphate.

The purpose of the present study was to investigate the distribution of tritium in the tissues and excreta of rats fed [$^3H$]pyridoxine β-glucoside in a purified form or in alfalfa sprouts and to identify any [$^3H$]vitamin $B_6$ compounds resulting from metabolism of pyridoxine β-glucoside. Thus, the bioavailability of pyridoxine β-glucoside in the rat could be determined on the basis of the metabolism and retention of the compound and its presence in the excreta. A unique aspect of this study was the intrinsic enrichment of a plant-derived food, alfalfa sprouts, for the evaluation of vitamin $B_6$ bioavailability.

## 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 pelletted diet (#5001, Ralston Purina, St. Louis, MO) ad libitum. In addition, the rats were fed daily approximately 10 g of 1% w/w calcium alginate gel and Kelco Co., San Diego, CA) that was made 17% w/w sucrose. This gel was fed to the rats between 9:00 a.m. and 11:00 a.m. each day. Rat chow pellets were removed from the cage until the rat had consumed most of the gel. After 6–7 days of conditioning the rats were fed a weighed portion of an alginate gel (8 g) containing the appropriate sources of radiolabeled vitamin $B_6$. Two hours after the gel had been fed, any gel remaining in the cage was removed and the pelletted chow 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, intestinal contents, plasma, and urine for subsequent analysis.

All experimental gels were extrinsically enriched with [$^{14}C$]pyridoxine (0.246 μCi/g, 4.20 nmol/g). The control diet was also enriched with [$^3H$]pyridoxine as the source of tritiated vitamin $B_6$ (0.243 μCi/g, 0.170 nmol/g). Alfalfa sprouts, which had been intrinsically enriched with [$^3H$]-pyridoxine as described later, served as a source of [$^3H$]-PN-β-glucoside in crude or purified form for enrichment of gels. The gelled diets were formulated with tritium sources as follows: purified [$^3H$]PN-β-glucoside, 0.253 μCi/g, 4.7 nmol/g; 12.5% w/w raw intrinsically enriched alfalfa sprouts, 0.201 μCi/g, 5.0 nmol/g; 12.5% w/w intrinsically enriched alfalfa sprouts heated in an autoclave at 121 °C for 20 min, 0.240 μCi/g, 5.80 nmol/g. All of these data refer to microcuries or nanomoles per gram of hydrated alginate gel as fed to the rats.

All unlabeled forms of vitamin $B_6$ used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). [$^3H$]Pyridoxine hydrochloride (1.4 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). The distribution of tritium reported by the manufacturer was as follows: methyl, 61.9%; 5-methylene, 2.0%; 4-methylene, 21.9%; C-6, 11.9%. [4,5-$^{14}C$]Pyridoxine hy-

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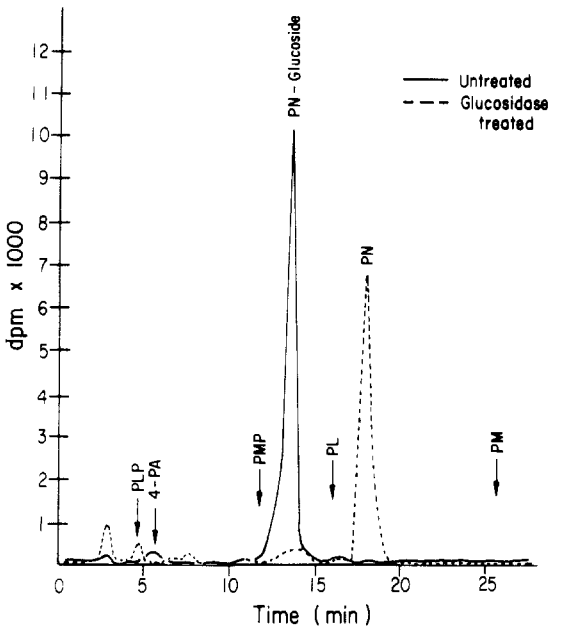


**Figure 1.** Typical HPLC chromatogram of tritiated compounds associated with [$^3$H]pyridoxine β-glucoside extracted and purified from alfalfa sprouts propagated in the presence of [$^3$H]pyridoxine. The sample was analyzed before (—) and after (- - -) treatment with β-glucosidase. Abbreviations used: pyridoxine, PN; pyridoxal phosphate, PLP; pyridoxine phosphate, PNP; pyridoxamine phosphate, PMP; pyridoxal, PL; pyridoxamine, PM; pyridoxic acid, 4-PA.

drochloride (58.7 mCi/mmol) was a gift of Hoffman-LaRoche (Nutley, NJ).

**Propagation of Alfalfa Sprouts and Synthesis of Pyridoxine β-Glucoside.** Ten grams of alfalfa seeds was soaked in water overnight. The excess water was poured off, and 1 mL of $H_2O$ containing 173 μCi of [$^3$H]pyridoxine (PN) was added. The sprouting of the alfalfa seeds was continued for 4 days in the dark with addition of enough $H_2O$ to keep the sprouts moist. After 4 days, the sprouts, which now weighed 20 g, were washed thoroughly with $H_2O$ to remove any $^3$H remaining on the outside of the sprouts. Ten grams of sprouts was homogenized in 10 mL of $H_2O$, followed by the addition of 100% (w/v) trichloroacetic acid (TCA) to a concentration of 7% (w/v) and centrifugation. The [$^3$H]PN-β-glucoside in the supernatant was partially purified with a high-performance liquid chromatographic (HPLC) procedure that will be described later in this section. The retention time of the PN-β-glucoside was about 15 min. The [$^3$H]PN-β-glucoside was further purified by applying the HPLC-pooled fractions to a column packed with Bio-Rad AG 50W-X8 (7 mm × 10 cm; Bio-Rad Laboratories, Richmond, CA) in the $Na^+$ form and equilibrated with 0.033 M $NaH_2PO_4$ (pH 2.2). The sample was loaded onto the column, rinsed 10 mL of the equilibration buffer, and eluted with a gradient generated by two connected flasks of equal size, one containing 25 mL of 0.05 M $NaH_2PO_4$ (pH 7.0) and the other 25 mL of 0.25 M $NaH_2PO_4$ (pH 7.0). The former flask was connected directly to the column. The identity and purity of the PN-β-glucoside were verified by treating an aliquot of the pooled fractions from the ion-exchange column with β-glucosidase (Sigma Chemical Co., St. Louis, MO; type I) and noting that the HPLC retention time for the one major peak of radioactivity was now 17.5 min corresponding to that for PN (Figure 1). An untreated aliquot exhibited a single peak of radioactivity with an HPLC retention time of 15 min that was not associated with any $B_6$ vitamer and was assumed to be the intact PN-β-glucoside. The radiochemical purity of this compound, which was later confirmed to be PN-β-glucoside, was greater than 98%. These pooled chromatographic (Bio-Rad AG 50W-X8) fractions were used as the source of [$^3$H]PN-β-glucoside for incorporation into alginate gels to be fed to the rats. The specific radioactivity of the purified product was approximately 54 mCi/mmol, which reflects isotopic dilution with endogenously synthesized PN-β-glucoside.

The remaining intact sprouts that were not used in the purification of [$^3$H]PN-β-glucoside were used to prepare a homogenate using 6 mL of $H_2O$/g of sprouts. This homogenate was incorporated into an alginate gel and fed to rats in the raw state and after being heated in an autoclave for 20 min at 121 °C.

**HPLC Equipment and Method.** Separation of $B_6$ vitamers was accomplished by an ion-pair reversed-phase high-performance liquid chromatographic method (Gregory and Feldstein, 1985). Chromatographic analysis were performed with a Minipump solvent delivery pump (Model 396, Laboratory Data Control, Riviera Beach, FL), loop injection valve (500-μL loop; Altex Model 905-42, Berkeley, CA), a fluorometric detector (Model LS-5, Perkin-Elmer, Norwalk, CT), and an electronic integrator (Model 3388A, Hewlett-Packard, Avondale, PA). The wavelengths used for fluorometric detection were 295 nm for excitation and 405 nm for emission. The Minipump was depulsed as described by Stewart (1977) using high-pressure components.

Two mobile phases were utilized in a stepwise elution procedure with a Techsphere-Ultra 5 C-18 column (HPLC Technology, Palos Verdes Estates, CA) providing the stationary phase. Mobile phase A was 0.033 M phosphoric acid in deionized distilled water, pH 2.2, and contained 2.5% 2-propanol, 4 mM octanesulfonic acid, and 4mM heptanesulfonic acid. Mobile phase B was also 0.033 M phosphoric acid, pH 2.2, with 17.5% 2-propanol and no ion-pairing agent. Mobile phase A was pumped through the column for the first 3 min following injection of the sample at which time a valve was switched, shutting off the flow of mobile phase A and starting the flow of B, which was pumped until completion of the run. HPLC analyses were done at ambient temperature at a flow rate of 1.0 mL/min. Unlabeled vitamin $B_6$ standards were injected along with each radioactive sample to verify retention times of $B_6$ vitamers. Fractions of 0.5 mL were collected in liquid scintillation vials and counted after mixing with 5 mL of scintillation fluid (Aqualyte Plus, J. T. Baker Co., Jackson, TN) in a Beckman LS 2800 liquid scintillation counter in which channels were set for double isotope counting and H number used to determine efficiency.

Since pyridoxal phosphate (PLP) and pyridoxine phosphate (PNP) have retention times that are similar (5 min), and also pyridoxamine phosphate (PMP) and 4-pyridoxic acid lactone (13 min), samples treated with alkaline phosphatase were analyzed in addition to untreated samples. The increase in radioactivity associated with PN and the radioactivity remaining with a retention time of 13 min in the enzyme-treated sample were assumed to represent PNP and 4-pyridoxic acid lactone, respectively. One milliliter of sample was combined with 1.5 mL of a 0.1 M Tris buffer, pH 11.0, which was also 0.1 M in glycine. Approximately 0.2 mg of alkaline phosphatase (0.8 U; Sigma Chemical Co.; type V) was added, and the mixture was incubated at 37 °C for 15 h. Prior to injection on the HPLC column the samples were made 7% (w/v) in TCA and were centrifuged. An aliquot of the supernatant was then analyzed.

**Table I. Distribution of Radioactivity among Forms of Vitamin $B_6$ in Test Meals[a]**

| | | % distribution[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 min[b] unident | PLP | 4-PA | PMP | 4-PA lactone | PN-glucoside | PL | PN | PM |
| control | $^3H$ | 11.2 | 1.2 | 0.6 | 0.4 | 0.7 | 1.9 | 2.1 | 81.7 | 0.4 |
| | $^{14}C$ | 2.4 | 0.8 | 0.4 | 1.0 | 1.2 | 0.6 | 4.8 | 86.1 | 2.8 |
| glucoside | $^3H$ | 0.9 | 0.2 | 0.1 | 0.3 | 1.5 | 88.1 | 1.9 | 6.4 | 0.2 |
| | $^{14}C$ | 2.2 | 0.8 | 0.4 | 0.7 | 0.9 | 0.9 | 3.5 | 84.8 | 2.0 |
| raw sprouts | $^3H$ | 14.6 | 1.9 | 0.5 | 3.0 | | 41.7 | 5.4 | 30.6 | 2.4 |
| | $^{14}C$ | 2.6 | 0.5 | 0.2 | 1.3 | | 1.1 | 4.7 | 87.2 | 2.5 |
| cooked sprouts | $^3H$ | 15.3 | 2.0 | 3.0 | 1.8 | 2.5 | 48.2 | 4.1 | 20.7 | 5.0 |
| | $^{14}C$ | 2.1 | 0.8 | 1.3 | 0.6 | 0.8 | 1.2 | 5.7 | 84.1 | 4.4 |

[a] Abbreviations: PLP, pyridoxal phosphate; 4-PA, 4-pyridoxic acid; PMP, pyridoxamine phosphate; PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine. [b] Refers to HPLC retention time.

Enzymatic treatment of samples was also done to confirm the identity of any PN-β-glucoside peaks. An increase in radioactivity associated with PN after β-glucosidase treatment was confirmation of the identity of the glucoside (Figure 1). The glucosidase incubation was done by making the sample 0.1 M in $Na_3PO_4$, adjusting to pH 5.0, and incubating for 18 h at 37 °C after adding 0.1 mg of β-glucosidase (EC 3.2.1.21; 3 U; Sigma Chemical Co.; type II)/mL of sample. TCA treatment of the mixture prior to HPLC analysis was done as described for the phosphatase incubations.

**Tissue Preparation.** Liver (1 g) was homogenized in 3 mL of $H_2O$ with a Polytron homogenizer (Brinkman Instruments, Westburg, NY) at three-fourths speed for 60 s. The homogenate was made 7% in TCA (v/v) and was centrifuged for 10 min at 12000*g*. The supernatant was filtered through a 0.45-μm nylon filter from Rainin Instrument Co. (Woburn, MA), and aliquots were taken for determination of labeled $B_6$ vitamers using HPLC. The pellet was washed with 4 mL of $H_2O$, homogenized, and centrifuged. Aliquots of both supernatants were analyzed for radioactivity for the calculation of total hepatic radioactivity. Total carcass radioactivity was determined after the carcass was autoclaved and homogenized in a Waring blender, treated with an equal volume of 2 N $HClO_4$, allowed to stand overnight at 4 °C, and centrifuged for 20 min at 12000*g*. The radioactivity in the supernatant and a wash of the pellet was determined. Feces and intestinal contents were homogenized in a Waring blender and centrifuged, and total radioactivity was determined. Radioactivity in plasma and urine was quantitated by direct analysis with no treatment prior to liquid scintillation spectrometry. Urine samples were filtered through a 0.45-μm filter described previously before HPLC analysis.

All isotopic quantitation was done via liquid scintillation spectrometry as described previously.

**Statistical Analysis.** Isotopic concentrations in tissues and excreta were expressed as percentage of oral dose to compensate for minor differences in $^3H$ and $^{14}C$ in the diets. The results were evaluated by two-way analysis of variance to permit an assessment of the effects of diet, isotope, and their interaction. A relative isotopic ratio (% of $^3H$ dose/% of $^{14}C$ dose) was used to facilitate a comparison of the isotopes. These data were analyzed by one-way analysis of variance. The Tukey procedure for multiple comparisons was employed in all analyses. All statistical procedures were conducted as described by Neter and Wasserman (1974).

## RESULTS

Table I shows the $^3H$ distribution of $B_6$ compounds present in each of the four test meals as determined by HPLC analysis. Rats fed cooked and raw sprouts received 48.2% and 41.7%, respectively, of the dietary $^3H$ in the form of [$^3H$]PN-β-glucoside with 20.7% and 30.6% of the remaining $^3H$ in the form of [$^3H$]PN. The glucoside-fed group received 88% of the $^3H$ in the form of PN-β-glucoside and only 6.4% as [$^3H$]PN. More than 80% of the $^3H$ fed to the control group and $^{14}C$ fed to all groups was in the form of PN.

The total $^{14}C$ and $^3H$ consumed by each rat was determined and the isotopic distribution in tissues and excreta determined (Table II). The rats fed the purified glucoside, raw sprouts, or cooked sprouts retained significantly less $^3H$ in the liver carcass and plasma than the rats fed [$^3H$]PN. The isotopic retention observed for each group (percentage of oral dose) was used to calculate a relative isotopic ratio (% of $^3H$ dose/% of $^{14}C$ dose). This ratio was used to facilitate comparisons between the various forms of dietary [$^3H$]vitamin $B_6$ and the [$^{14}C$]pyridoxine present in all diets. The relative isotopic ratio in the urine of the group fed PN-β-glucoside was more than 4 times that observed for the control group. The relative isotopic ratio in the urine of the raw and cooked sprout-fed groups was about twice that in the urine of the control group. Data concerning the concentration of urinary metabolites (presented later in this section) provide clarification with regard to vitamin $B_6$ bioavailability.

The relative isotopic ratio found in the carcasses of rats fed the glucoside, raw sprouts, or cooked sprouts was significantly less than that of in the control group. These data are evidence of reduced bioavailability (i.e., less in vivo retention) of PN-β-glucoside.

Comparison of the $^3H$ and $^{14}C$ recovered in the urine, tissues, or plasma of the control group indicated that the two isotopes do not exhibit identical retention patterns. Factors that may have contributed to this are considered in the Discussion.

The $^3H$ remaining in the feces and intestinal contents of rats fed the glucoside, raw, and cooked sprouts, was about twice that of the control group. The glucoside-fed group had $^{14}C$ remaining in the feces and intestinal contents that was similar to the level observed for $^3H$. In contrast, the relative isotopic ratio (% of $^3H$ dose/% of $^{14}C$ dose) for the group fed PN-β-glucoside was significantly higher than the control and is reflective of reduced rates of absorption for PN-β-glucoside relative to PN. This reduced absorption was most pronounced in the sprout-fed groups, which indicates that PN-β-glucoside in the sprouts exhibited lower bioavailability than the purified PN-β-glucoside, which was not presented to the rats within plant tissue.

The relative isotopic ratio in the plasma of the rats fed PN-glucoside, raw sprouts, or cooked sprouts was lower than that in the plasma of controls and again is evidence for incomplete bioavailability of the PN-β-glucoside.

The data concerning liver, urine, and carcass discussed previously represent $^3H$ and $^{14}C$ that has been absorbed by the animal from the gastrointestinal tract, some of which is retained in the animal over a 24-h period and the

**Table II. Recovery and Relative Isotopic Retention of $^3H$ and $^{14}C$ in Tissues and Excreta[a]**

| tissue or excreta | diet | % ingested isotope[b] | | rel isotop ratio[c] (% $^3H$ dose/% $^{14}C$ dose) |
|---|---|---|---|---|
| | | $^3H$ | $^{14}C$ | |
| liver | control | 13.2 ± 0.8[a] | 9.4 ± 0.3[a] | 1.40 ± 0.05[a] |
| | glucoside | 5.8 ± 0.5[b] | 9.8 ± 1.0[a] | 0.61 ± 0.05[b] |
| | raw sprouts | 6.4 ± 0.6[b] | 10.0 ± 1.0[a] | 0.65 ± 0.04[b] |
| | cooked sprouts | 5.4 ± 0.3[b] | 10.1 ± 0.5[a] | 0.54 ± 0.02[b] |
| carcass | control | 37.3 ± 1.3[a] | 25.8 ± 1.2[a] | 1.46 ± 0.08[a] |
| | glucoside | 19.4 ± 2.1[b] | 31.4 ± 3.3[a] | 0.62 ± 0.04[b] |
| | raw sprouts | 24.3 ± 1.9[b] | 33.6 ± 2.1[a] | 0.74 ± 0.08[b] |
| | cooked sprouts | 23.6 ± 2.4[b] | 30.0 ± 1.9[a] | 0.78 ± 0.03[b] |
| plasma[d] | control | 0.9 ± 0.04[a] | 0.3 ± 0.01[a] | 3.06 ± 0.08[a] |
| | glucoside | 0.3 ± 0.01[b] | 0.4 ± 0.03[a] | 0.75 ± 0.07[b] |
| | raw sprouts | 0.6 ± 0.14[c] | 0.3 ± 0.03[a] | 1.91 ± 0.13[c] |
| | cooked sprouts | 0.7 ± 0.12[c] | 0.3 ± 0.02[a] | 2.10 ± 0.11[c] |
| urine | control | 11.8 ± 1.3[a] | 21.2 ± 1.5[a] | 0.55 ± 0.03[a] |
| | glucoside | 39.5 ± 7.3[b] | 16.8 ± 4.5[a] | 2.31 ± 0.29[b] |
| | raw sprouts | 28.3 ± 1.8[bc] | 26.8 ± 4.0[a] | 1.06 ± 0.01[c] |
| | cooked sprouts | 24.6 ± 2.3[ac] | 21.4 ± 3.3[a] | 1.14 ± 0.04[bc] |
| feces[e] | control | 9.6 ± 1.0[a] | 16.8 ± 1.1[a] | 0.56 ± 0.03[a] |
| | glucoside | 17.3 ± 3.1[a] | 17.5 ± 2.1[a] | 0.95 ± 0.10[b] |
| | raw sprouts | 25.6 ± 5.3[a] | 18.1 ± 4.3[a] | 1.47 ± 0.05[c] |
| | cooked sprouts | 22.3 ± 3.9[a] | 17.1 ± 2.9[a] | 1.30 ± 0.03[c] |

[a] Values are means and SEM, five rats per group. Rats in the respective dietary groups consumed the following quantities of radioactivity (means ± SEM): control, 1.73 ± 0.05 μCi of $^3H$, 2.17 ± 0.06 μCi of $^{14}C$; PN-glucoside, 1.68 ± 0.10 μCi of $^3H$, 2.10 ± 0.13 μCi of $^{14}C$; raw sprouts, 1.78 ± 0.07 μCi of $^3H$, 2.08 ± 0.11 μCi of $^{14}C$; cooked sprouts, 1.83 ± 0.08 μCi of $^3H$, 2.17 ± 0.09 μCi of $^{14}C$. [b] Data for $^3H$ and $^{14}C$ recovery within each tissue or excreta were analyzed by two-way analysis of variance and the Tukey procedure for multiple comparisons. Significant diet X isotope interactions were observed for liver, carcass, plasma, and urine ($P < 0.01$). Within each column ($^3H$, $^{14}C$) of each category of tissue or excreta analyzed values followed by the same superscript letter were not significantly different ($P > 0.05$). [c] Within each category of tissue or excreta analyzed, values followed by the same superscript letter were not significantly different ($P < 0.05$) as determined by one-way analysis of variance and the Tukey procedure. [d] Calculation based on 8 mL of plasma/rat. [e] Includes intestinal contents.

rest excreted in the urine. These data do not reflect the extent of metabolic utilization of the various forms of dietary vitamin $B_6$. Therefore, to assess more completely the bioavailability of the labeled dietary forms of vitamin $B_6$, the [$^3H$]- and [$^{14}C$]vitamin $B_6$ metabolites in the liver and urine were determined. The major $^3H$ and $^{14}C$ metabolites in the livers of all treatment groups were PMP, PLP, and PL, representing about 75% of the $^3H$ and $^{14}C$ present (Table III). There were no significant differences between the treatment of groups in the relative proportions of these metabolites. No [$^3H$]PN-β-glucoside was present in the livers of the rats fed PN-β-glucoside, raw sprouts, or cooked sprouts. This suggests that [$^3H$]PN-β-glucoside is metabolized to a limited extent by the rat, which yields a distribution of [$^3H$]vitamin $B_6$ retained in the liver that is indistinguishable from the pattern of $^{14}C$ metabolites. Therefore, direct comparison of the relative isotopic concentrations and relative isotopic ratios appearing in the liver for the four treatment groups is a valid indicator of bioavailability.

The relative concentrations of the major vitamin $B_6$ metabolites in the urine are also shown in Table III. The control animals showed approximately 40% of urinary $^3H$ and $^{14}C$ as the major urinary metabolite 4-PA and its lactone, with no evidence of [$^{14}C$]PN-β-glucoside. In contrast to the data regarding hepatic metabolites, there were sharp contrasts between $^3H$ and $^{14}C$ in urinary metabolites in the glucoside- and sprout-fed groups. The $^3H$ in the urine of the fed rats fed PN-β-glucoside was found to be predominantly in the form of intact [$^3H$]PN-β-glucoside with no [$^{14}C$]PN-β-glucoside present. The proportion of [$^3H$]-4-PA was significantly less than the control group. The urine from groups fed raw and cooked sprouts contained about two-thirds of the $^3H$ as intact [$^3H$]PN-β-glucoside, which reflected the lower concentration of $^3H$ in the form of [$^3H$]PN-β-glucoside in the diets containing sprouts. No $^{14}C$ was associated with the [$^3H$]PN-β-glucoside HPLC fractions of urine from groups fed sprouts.

The above results for the urine indicate that PN-β-glucoside entering the general circulation as a result of absorption from the gastrointestinal tract is utilized as vitamin $B_6$ much less effectively than PN. The bioavailability of PN-β-glucoside was markedly less on the basis of isotopic retention, excretion, and metabolism.

## DISCUSSION

The intrinsic labeling of foods has greatly facilitated our understanding of the bioavailability of various inorganic nutrients, although application of these techniques to the study of vitamins has been minimal. Inadequate enrichment of plant tissues with organic nutrients often occurs as a result of limited absorption, extensive metabolic degradation, or both. We found that propagation of alfalfa sprouts in the presence of isotopically labeled pyridoxine served as a highly effective way of preparing an intrinsically enriched plant-derived food for use in nutritional studies and as a substrate for further purification of labeled PN-β-glucoside. This procedure was used in a previous

**Table III. Relative Concentration of Radiolabeled Forms of Vitamin $B_6$ in Rat Liver and Urine[a]**

| diet | isotope | major hepatic metabolites,[b] % | | | urinary radioactivity,[c] % | |
|---|---|---|---|---|---|---|
| | | PLP | PMP | PL | 4-PA and lactone | PN-glucoside |
| control | $^3H$ | 9.2 ± 0.7 | 57.1 ± 1.1 | 10.2 ± 0.4 | 41.1 ± 1.3[a] | nd |
| | $^{14}C$ | 9.2 ± 0.7 | 59.2 ± 1.0 | 9.6 ± 0.8 | 37.7 ± 0.4[ab] | nd |
| glucoside | $^3H$ | 12.0 ± 1.7 | 51.0 ± 1.8 | 13.4 ± 2.6 | 6.0 ± 1.0[c] | 85.2 ± 3.6 |
| | $^{14}C$ | 11.7 ± 1.4 | 54.4 ± 2.3 | 14.7 ± 3.2 | 37.7 ± 2.9[ab] | nd |
| raw sprouts | $^3H$ | 10.9 ± 0.6 | 51.3 ± 2.3 | 11.0 ± 1.7 | 12.1 ± 1.6[c] | 58.2 ± 9.2 |
| | $^{14}C$ | 10.5 ± 6.4 | 56.3 ± 2.0 | 10.2 ± 1.6 | 35.4 ± 2.7[ab] | nd |
| cooked sprouts | $^3H$ | 9.7 ± 0.6 | 53.8 ± 2.2 | 10.9 ± 1.3 | 11.7 ± 1.4[c] | 66.8 ± 1.5 |
| | $^{14}C$ | 9.2 ± 0.2 | 60.7 ± 2.5 | 10.3 ± 1.3 | 32.1 ± 2.5[b] | nd |

[a] Values are means ± SEM, five rats per group. Abbreviations: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; PL, pyridoxal; 4-PA, 4-pyridoxic acid; PN-glucoside, pyridoxine β-glucoside. nd = not detected. [b] Data for each metabolite were analyzed by two-way analysis of variance and the Tukey procedure for multiple comparisons. No significant effects of diet or isotope on hepatic percentages of PLP, or PL ($P > 0.05$). The effect of isotope ($^3H$ vs. $^{14}C$) on hepatic PMP was significant at $P < 0.05$. [c] A significant diet × isotope interaction was observed for 4-PA and lactone ($P < 0.01$). Values followed by the same superscript letter were not significantly different ($P > 0.05$). For urinary PN-glucoside, a significant difference between groups fed purified PN-glucoside vs. those fed raw or cooked sprouts ($P < 0.05$) was noted.

study for the preparation of milligram quantities of unlabeled PN-β-glucoside (Gregory and Ink, 1985).

The results of this study strongly suggest that PN conjugated to glucose in the form of PN-β-glucoside has low biological availability as vitamin $B_6$ in the rat. The retention of $^3H$ in the liver and carcass of the glucoside-fed group was 50% or less of the control levels, with little difference in distribution of hepatic metabolites between the two groups. The predominant [$^3H$]$B_6$ vitamers in the liver 24 h after ingestion of the test meals were PMP, PLP, and PL, which accounted for more than 70% of the $^3H$. This is in general agreement with previous studies that found [$^3H$]PMP and PLP to be the major $B_6$ vitamers in the livers of rats 24 h or more after administration of [$^3H$]PN (Segalman and Brown, 1981; Shane, 1982). The higher ratio of PMP to PLP for both isotopes than observed in the previous studies (Segalman and Brown, 1981; Shane, 1982) may be attributable to the different modes of administration (oral vs. injected). No [$^3H$]PN-β-glucoside was in the livers of the groups fed the glucoside or alfalfa sprouts. This is indicative of rapid clearance or metabolism of the glucoside from the tissues. The observation of [$^3H$]$B_6$ vitamers in the livers of groups fed PN-β-glucoside or alfalfa sprouts indicates partial hydrolysis of the glucoside. Hydrolysis of PN-β-glucoside by the intestinal microflora appears likely, although the precise site of hydrolysis cannot be determined from this study.

Urinary excretion of $^3H$ in the group fed purified PN-β-glucoside was over threefold greater than observed in the control group. In addition, 85% of the $^3H$ in the urine of the glucoside group was found intact as PN-β-glucoside. These results indicate that PN-β-glucoside can be absorbed in the gut and, upon entry into the general circulation, cleared efficiently by the kidney and excreted largely in intact form. Since no [$^{14}C$]PN-β-glucoside was detected in the urine, in vivo synthesis of this conjugate is unlikely.

If the percentage of ingested $^3H$ found as hepatic PMP or as 4-PA and its lactone in the urine is used as a measure of bioavailability, PN-β-glucoside is estimated to have 40% or less of the bioavailability of PN in the rat. This result is in agreement with the findings of Kabir et al. (1983b) that showed incomplete bioavailability of vitamin $B_6$ from foods with glycosylated vitamin $B_6$ for human subjects. These investigators also reported the presence of intact glycosylated vitamin $B_6$ in the urine. The results of Tsuji et al. (1977), however, are not in general agreement with the findings of this study. These investigators reported that PN-β-glucoside will permeate in vitro everted gut sacs of rats, which is in support of our results, but found no evidence of reduced bioavailability of PN-β-glucoside based on restoration of urinary xanthurenic acid and liver enzymic activities to normal in vitamin $B_6$ deficient rats fed PN-β-glucoside for 12 days. When the rat is used in animal bioassay experiments involving vitamin $B_6$, possible bias due to coprophagy or direct absorption of microbially synthesized or metabolized vitamers must be considered (Nguyen and Gregory, 1983). However, differences in microbial metabolism of PN-β-glucoside probably do not account for the differences in bioavailability found for PN-β-glucoside in the study by Tsuji et al. (1977) as compared to this study since they also reported evidence of in vivo hydrolysis of PN-β-glucoside to free PN in the rat.

A relative isotopic ratio greater than 1.0 was found for isotope retention in the liver and carcass of control animals while the ratio was less than 1.0 in the urine of control animals. The presence of 21.9% of the $^3H$ on the 4-methylene group of [$^3H$]PN purchased from Amersham may account for these observations. Metabolism of [$^3H$]PN to PL or 4-PA would result in loss of $^3H$ in the 4-position and generation of $^3H_2O$. The equilibration of $^3H_2O$ generated in the animal with total body $H_2O$ would result in greater retention of $^3H$ and less urinary excretion relative to $^{14}C$ in the control animals. Similar differences between $^3H$- and $^{14}C$-labeled PN have been reported previously (Contractor and Shane, 1970; Johansson and Tiselius, 1973).

Data from this study indicate that PN-β-glucoside has incomplete vitamin $B_6$ bioavailability in the rat via an experimental approach that permits direct measurement of the absorption, metabolism, and retention. Through the use of $^3H$ and $^{14}C$ forms of dietary vitamin $B_6$, the results of this study are unaffected by the ambiguities inherent in conventional bioassays. The implications of these findings could be significant in the assessment of the vitamin $B_6$ nutriture of a human population if it is found that the human and rat are similar with regard to the metabolism of PN-β-glucoside. Recent analyses have determined that a considerable amount of the plant-derived vitamin $B_6$ is present as PN-β-glucoside (Kabir et al., 1983a; Gregory and Ink, 1985). Of interest was the observation that thermal processing had little effect on the concentration of PN-β-glucoside in the alfalfa sprouts (Table I). This is consistent with the previously observed stability of the glycosidic bond (Gregory and Ink, 1985) as well as stability of the pyridoxine moiety of PN-β-glucoside.

The structure of a water-soluble pyridoxine conjugate in rice bran was identified as 5′-*O*-(β-D-glucopyranosyl)-pyridoxine by Yasumoto et al. (1977). Subsequently, an enzyme catalyzing the formation of this compound with pyridoxine and UDP-glucose as substrates was isolated from pea and Chinese cabbage seedlings (Tadera et al., 1979). Thus, it appears that this conjugate of pyridoxine is likely to have widespread occurrence in foods of plant origin. The results of Kabir et al. (1983a) do, in fact, indicate the presence of a glucosidic linkage of vitamin $B_6$ in a wide variety of plant-derived foods. However, the structure of the glucosidic conjugates was not determined. Research was conducted in this laboratory to determine the precise identity of the vitamin $B_6$ glucoside(s) in plants and the extent of distribution. These results indicated a widespread occurrence of pyridoxine glucoside in plants in the form of 5′-*O*-(β-D-glucopyranosyl)pyridoxine on the basis of NMR identification and HPLC fluorometric quantitation (Gregory and Ink, 1985). Current research in this laboratory is directed toward determination of the extent of utilization of PN-β-glucoside in human beings.

**Registry No.** PN-β-glucoside, 63245-12-5; $T_2$, 10028-17-8; $^{14}C$, 14762-75-5; vitamin $B_6$, 65-23-6; pyridoxal phosphate, 54-47-7; 4-pyridoxic acid, 82-82-6; pyridoxamine phosphate, 529-96-4.

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# Solid-Phase Radioimmunoassay of Ochratoxin A in Serum

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A solid-phase radioimmunoassay (RIA) for the determination of ochratoxin A in serum was developed. [$^{14}$C]Ochratoxin A, with a specific activity of 130 Ci/mol, was used as tracer. Antibody was obtained by repeated injection of ochratoxin A–bovine serum albumin conjugate in rabbits. A rapid sample cleanup was achieved on a Sep-Pak $C_{18}$ cartridge. Immobilization of antibody on different solid phases was tested. RIA with antibody coupled to protein A–Sepharose CL-4B allowed a detection of ochratoxin A in serum as low as 0.4 ng/mL. Recoveries in the 0.4–20 ng/mL range were 83.4–87.5% with standard deviations of 0.9–2.1%.

## INTRODUCTION

The ochratoxins are a group of isocoumarin-containing toxic secondary metabolites produced by a number of fungal species of the *Aspergillus* and *Penicillium* genera. Ochratoxin A, the most toxic of this mycotoxin series, has been found in a number of agricultural commodities. Because of its association with mold-induced porcine nephropathy (Krogh, 1978) and with endemic Balkan nephropathy (Krogh et al., 1977), a fatal human kidney disease, several assay procedures of ochratoxin A in cereals have been developed (Lee and Chu, 1984; Rousseau et al., 1985).

One of the main problems in the analysis of cereals is the sampling of the suspected commodities. In very large parcels the moldiness mostly appears in so called "hot spots", which are isolated contamination zones. Since homogenization of the whole parcel is not possible, samples must be taken under well-defined conditions. Statistical analyses have shown that 5 kg of fine cereals to 25 kg of nuts are required to have a reasonable chance of encountering contaminated parts of a heterogeneous parcel (Dickens and Whitaker, 1982).

To overcome this problem, analysis of the toxin in biological samples (serum, urine, kidney) of slaughter animals, especially pigs, fed with the suspected cereals was suggested. Consequently the presence of ochratoxin A in serum and kidneys of pigs has been reported after detection by means of high-performance liquid chromatography (HPLC) (Bauer et al., 1984) and thin-layer chromatography (TLC) (Sandor, 1984). These results indicate that a broad survey of biological samples of slaughter animals is necessary. Ochratoxin A determinations by TLC and HPLC often require an extensive cleanup and consequently are time consuming. Immunoassay procedures have several advantages: they are very sensitive and specific and allow rapid determination of a large number of samples. Hence, an RIA for the detection of ochratoxin A in serum was developed in our laboratory.

As solid-phase systems offer several advantages, the binding of rabbit antibodies against ochratoxin A on various coated glass beads and on protein A–Sepharose CL-4B was thoroughly compared. Derivatized glass beads are often used for immobilization of proteins and enzymes (White and Kennedy, 1980; Slegers et al., 1984).

The characteristic biological property of protein A is its ability to interact with IgG molecules. Since the interaction of protein A and IgG does not involve the antigen-binding portion of the immunoglobulin molecule, it is possible to develop a competitive RIA with protein A–Sepharose CL-4B as solid phase, which to our knowledge, has not yet been employed.

## EXPERIMENTAL SECTION

**Materials.** Ochratoxin A was obtained from Janssen Chimica (Beerse, Belgium) and RIALUMA (cumene) from Lumac/3M (Schaesberg, The Netherlands). Ochratoxin B was supplied by Dr. E. Creppy (Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France). Certified serum samples (one negative and three positives) of pigs were supplied by Dr. B. Hald (Royal Veterinary and Agricultural University, Copenhagen, Danmark). Protein A–Sepharose CL-4B and Sephadex G-25 columns were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Controlled-pore glass beads of 500-Å pore size (80–100 mesh) were purchased from Pierce Chemical Co. (Rockford, IL), and Sep-Pak $C_{18}$ cartridges were from Waters Associates (Millford, MA). Silanes were a gift of Union Carbide (Brussels, Belgium).

**Apparatus.** Scintillation counting was performed with a Packard Tri-Carb Model 3390 liquid scintillation spectrometer.

**Synthesis of [$^{14}$C]Ochratoxin A.** [$^{14}$C]Ochratoxin A was synthesized by methods previously described (Rousseau et al., 1984). Its specific acitvity was 130 Ci/mol.

**Production of Ochratoxin A Antiserum.** Synthesis of ochratoxin A–bovine serum albumin conjugate was

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