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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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added as the source of tritiated vitamin B_6 (0.24–0.39 μCi/g of gel; 0.2–0.3 nmol/g of gel). Intrinsically enriched diets were prepared by blending perfused liver or muscle (raw or cooked) into the alginate gels at a 10–19% w/w level. This provided levels of [^{3}H]vitamin B_6 that were comparable to those [^{3}H]pyridoxine in control gels. The endogenous vitamin B_6 in gels containing liver was approximately 3.5–5 nmol/g of gel. Endogenous vitamin B_6 from muscle was 2.5–3.5 nmol/g of gel. In each experiment, unlabeled pyridoxine hydrochloride was added to the control gels to provide a level of total vitamin B_6 equivalent to that of gels containing liver or muscle.

All unlabeled forms of vitamin B_6 used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Pyridoxine hydrochloride (1.4 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). The [^{3}H]pyridoxine was judged to be greater than 94% pure by HPLC analysis with 5–6% unidentified. 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-$^{14}C_2$]Pyridoxine hydrochloride used in the liver feeding study was obtained from Amersham (23.6 mCi/mmol), while that employed in the muscle feeding study (58.7 mCi/mmol) was a gift of Hoffman-LaRoche (Nutley, NJ).

Perfusions. Tritiated liver tissue that was fed to the rats was intrinsically enriched with [^{3}H]vitamin B_6 via in situ vascular perfusion by the method of Mehansho et al. (1980). Rats were anesthetized with intramuscular ketamine hydrochloride (100 mg/kg). Proper oxygenation was accomplished by bubbling a gas mixture of 95% O_2 and 5% CO_2 into the perfusate reservoir throughout the perfusion period. Once the appropriate cannulations had been made, [^{3}H]vitamin B_6 was added to the recirculating perfusate. The [^{3}H]vitamin B_6 added to the perfusate was made via MnO_2 oxidation of [^{3}H]pyridoxine (Gregory and Kirk, 1977) and had the following distribution: 73% [^{3}H]pyridoxal, 16.1% [^{3}H]pyridoxine, 2.1% [^{3}H]-4-pyridoxic acid, 8.8% unidentified. Approximately half of the ^{3}H on the 4-methylene carbon would be lost in the oxidation of pyridoxine to pyridoxal (PL). Thus, the specific activity of [^{3}H]pyridoxal would be about 11% lower than that of [^{3}H]pyridoxine. Perfusions were conducted at 37 °C for 45 min with a flow rate of 8 mL/min and an arterial pressure of 100–120 mmHg.

Muscle tissue was intrinsically enriched with [^{3}H]vitamin B_6 via in situ vascular perfusions of the hind limb of the rat by the method of Ruderman et al. (1971) with some modification. The hind limbs of male rats were bilaterally perfused by cannulating the abdominal aorta slightly inferior to the iliac branches and the inferior vena cava above the point of the aortic cannulation. The tail vein was clamped, but no other vessels inferior to the abdominal aortic bifurcation were tied. The flow rate of the perfusate was 8 mL/min, and the arterial pressure was maintained at 100–120 mmHg. The perfusate was recirculated through the system at 37 °C. Tritiated vitamin B_6 prepared by oxidation of [^{3}H]PN with MnO_2 as previously described was added to the perfusate once the necessary cannulations had been made and recirculated for 75 min. Two different muscle perfusions were done to provide intrinsically labeled tissue for feeding experiments. The muscle 1 preparation was perfused with a mixture of 81% [^{3}H]PL and only 4% [^{3}H]PN while the muscle 2 preparation was perfused with 65% [^{3}H]PL and 29% [^{3}H]PN.

Tissue Preparation. The liver was homogenized in 3 mL of H_2O with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at $^3/_4$th speed for 60 s. The proteins in the homogenate were precipitated by rapidly adding $^1/_5$th volume of 10 M perchloric acid. After standing overnight at 4 °C, the sample was centrifuged at 7000*g* and the supernatant separated from the pellet. The pellet was washed with 35 mL of H_2O and recentrifuged prior to analysis by liquid scintillation spectrometry. The total liver radioactivity was determined as the sum of that in the perchloric acid supernatant and the aqueous wash. The supernatants were adjusted to pH 4.2–5.5 with 5 M KOH and samples taken for determination of radioactivity prior to filtration through a 0.45-μm nylon filter (Rainin Instrument Co., Woburn, MA). The solutions were then analyzed for [^{3}H]vitamin B_6 content by HPLC. Urine was filtered through a 0.45-μm nylon filter before analysis of [^{3}H]vitamin B_6 distribution using HPLC. Total carcass radioactivity was determined after the carcass was autoclaved and homogenized in a Waring blender. Feces and contents of the intestinal tract were homogenized in a Waring blender, and total radioactivity was determined. Plasma radioactivity was determined by direct analysis with no treatment prior to liquid scintillation spectrometry.

HPLC Equipment and Method. Separation of B_6 vitamers was accomplished by an ion-pair reversed-phase high-performance liquid chromatographic method with stepwise elution (Gregory and Feldstein, 1985). Chromatographic analyses were done with a solvent metering pump (Model 110A; Altex Scientific Berkeley, CA), loop injection valve (500-μL loop; Altex Model 905-42), a fluorometric detector (Model LS-5; Perkin-Elmer, Norwalk, CT), and an electronic integrator (Model 3388 A; Hewlett-Packard, Avondale, PA). Detection wavelengths were 295 nm for excitation and 405 nm for emission. A 4.6 × 250 mm precolumn pack with 37–53-μm silica (Whatman Inc., Clifton, NJ) provided mobile phase saturation with silica and thus prolonged the life of the Altex Ultrasphere-I.P. analytical column (4.6 × 250 mm, 5-μm octadecylsilica).

Two mobile phases were utilized in a stepwise elution procedure. Mobile phase A was 0.033 M phosphoric acid in deionized distilled water, pH 2.2, and contained 2.5% (v/v) 2-propanol, 4 mM octanesulfonic acid, and 4 mM heptanesulfonic acid. Mobile phase B was 0.033 M phosphoric acid, pH 2.2, with 17.5% (v/v) 2-propanol and no ion-pairing agent. Mobile phase A was pumped through the column for the first 3 min following injection, at which time a valve was switched to stop the flow of mobile phase A and start the flow of B, which was then pumped isocratically. HPLC analyses were performed 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; J. J. Baker Co., Jackson, TN) in a Beckman LS 2800 liquid scintillation counter in which channels were set for double-isotope counting. Appropriate quench curves and an external standard were used for conversion of counts per minute to disintegrations per minute with correction for spillover between channels. Replicate analyses yielded results that typically agreed within 5% for each vitamer.

Since pyridoxal phosphate (PLP) and pyridoxine phosphate (PNP) have similar retention times in this HPLC separation procedure, alkaline phosphatase treated samples were analyzed in addition to untreated samples. Following phosphatase treatment, the increase in radioactivity associated with the pyridoxine (PN) peak was assumed to represent PNP. Figure 1 shows a represent-

Table I. Radioisotopic Distribution in Test Meals Containing Intrinsically Enriched Liver or [^{3}H]Pyridoxine as the Source of [^{3}H]Vitamin B_6

	% distribution ^{3}H									
gel diet	PLP	PL	PMP	PM	PNP	PN	4-PA	3-min retentn[a]	other[b]	total radioact, μCi/g gel
control						94.6		5.4		0.337
raw liver	35.9	20.3	22.7		3.5	4.3	6.3	4.6		0.295
cooked liver	9.3	7.8	20.9	16.4	0.5	7.8	9.4	16.2	10.2	0.173

[a] Refers to HPLC retention time. [b] Other unidentified ^{3}H components.

Table II. Recovery of ^{3}H and ^{14}C in Tissues and Excreta of Rats Fed [^{3}H]Pyridoxine (Control) or Intrinsically Enriched Liver as a Source of [^{3}H]Vitamin B_6[a]

tissue or excreta	diet	% ingested isotope[b] ^{3}H	% ingested isotope[b] ^{14}C	rel isotop ratio,[c] % ^{3}H dose/% ^{14}C dose
liver	control	6.5 ± 0.4	8.1 ± 0.5	0.81 ± 0.02[a]
	cooked	3.5 ± 0.2	9.5 ± 0.4	0.37 ± 0.01[b]
	raw	6.2 ± 0.9	8.5 ± 0.9	0.72 ± 0.08[a]
carcass	control	23.0 ± 1.0	23.1 ± 1.5	0.99 ± 0.03[a]
	cooked	14.6 ± 1.4	22.8 ± 1.8	0.67 ± 0.09[a]
	raw	21.6 ± 2.4	22.9 ± 1.3	0.98 ± 0.16[a]
plasma[d]	control	0.80 ± 0.10	0.44 ± 0.04	1.85 ± 0.20[a]
	cooked	0.90 ± 0.11	0.51 ± 0.04	1.76 ± 0.13[a]
	raw	0.81 ± 0.05	0.45 ± 0.04	1.82 ± 0.22[a]
feces and intestinal contents	control	9.6 ± 0.5	8.7 ± 0.5	1.11 ± 0.03[a]
	cooked	33.3 ± 4.8	11.4 ± 1.4	2.87 ± 0.09[b]
	raw	17.0 ± 1.8	10.0 ± 0.6	1.72 ± 0.08[c]
urine	control	16.2 ± 1.3	15.7 ± 1.8	1.00 ± 0.01[a]
	cooked	13.0 ± 1.0	12.0 ± 1.1	1.10 ± 0.05[b]
	raw	23.2 ± 2.2	17.9 ± 1.4	1.30 ± 0.05[c]

[a] Values are means ± SEM, seven rats per group. All diets contained [^{14}C]pyridoxine for extrinsic enrichment. [b] The rats consumed the following amount of radioactivity in the test diets: control, 2.72 ± 0.14 μCi of ^{3}H, 1.35 ± 0.13 μCi of ^{14}C; cooked, 1.41 ± 0.14 μCi of ^{3}H, 1.35 ± 0.13 of μCi ^{14}C; raw, 1.72 ± 0.11 μCi of ^{3}H, 1.35 ± 0.09 μCi of ^{14}C (means ± SEM). [c] The relative isotopic ratios were evaluated by one-way analysis of variance and the Tukey honestly significant difference procedure for multiple comparisons. The ratio for cooked liver was compared to 73.5% of raw and control for liver, urine, carcass, and plasma to account for differences in oral dose caused by thermal effects (i.e., 26.5% destruction of [^{3}H]vitamin B_6 compounds). For each tissue or excreta, values followed by the same superscript letter were not significantly different at the 95% confidence level. No significant differences were noted between diets in relative concentration of ^{14}C of feces and intestinal contents ($P > 0.05$). [d] A plasma volume of 8 mL was assumed.

ative chromatogram from HPLC analysis of a rat liver from this study.

Analysis of Data. The data were evaluated by analysis of variance and the Tukey honestly significant difference procedure for multiple comparisons (Neter and Wasserman, 1974). The concentration of ^{3}H and ^{14}C in tissues and excreta was expressed as a percentage of oral dose. This facilitated direct comparisons of isotopic retention where differences in the dose existed. Further comparisons were made by calculation of a "relative isotopic ratio" (% of ^{3}H dose/% of ^{14}C dose) for tissues, urine, and feces. The relative isotopic ratio would be 1.0 if the extent of intestinal absorption and tissue retention of ^{3}H- and ^{14}C-labeled dietary components was equivalent. Chromatographically derived distributions of vitamin B_6 metabolites were expressed relative to the total extracted radioactivity (^{3}H or ^{14}C) for analysis of liver and diets and relative to total radioactivity for analysis of urine.

RESULTS

Bioavailability of Vitamin B_6 in Liver. Rat liver that had been intrinsically enriched with [^{3}H]B_6 vitamers was fed to rats to study the bioavailability of vitamin B_6 in raw and thermally processed liver. The concentration and distribution of [^{3}H]B_6 vitamers in the gelled diets containing perfused liver is shown in Table I. After thermal processing of the liver at 121 °C for 45 min, the total [^{3}H]vitamin B_6 existing as extractable tritiated PLP, PL, PNP, PN, pyridoxamine (PM), and PMP was 73.5% of the level in the raw livers, which was consistent with expected thermal losses.

One group of seven rats was fed an alginate gel containing [^{14}C]PN and raw [^{3}H]PL-perfused liver, a second

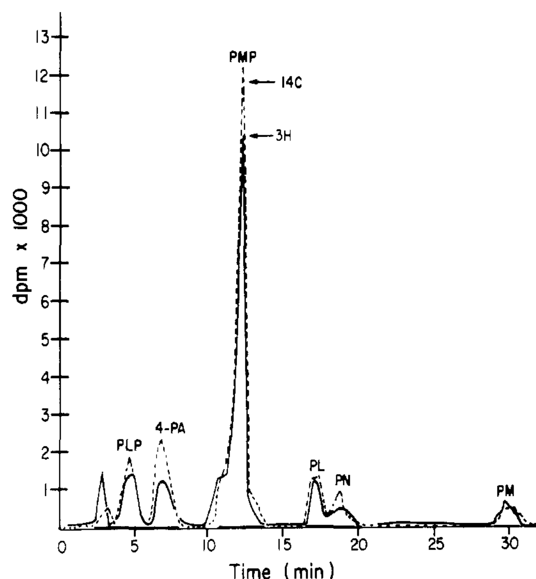

Figure 1. Typical chromatogram of HPLC analysis of radiolabeled B_6 vitamers in rat liver. Abbreviations: PLP, pyridoxal phosphate; 4-PA, 4-pyridoxic acid; PMP, pyridoxamine phosphate; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine.

group was fed [^{14}C]PN and cooked [^{3}H]PL-perfused liver, and a third group (control) was fed [^{14}C]PN and [^{3}H]PN. The total ^{14}C and ^{3}H consumed by each rat was determined, and the percentage of the consumed isotope present in various tissues was quantified (Table II). The percentage of ingested tritium in the liver, carcass, and urine of rats fed the cooked liver was 56.5%, 67.6%, and 56.0%,

Table III. Relative Concentration of Hepatic and Urinary Radiolabeled Forms of Vitamin B_6 in Rats Fed [^{3}H]Pyridoxine (Control) or Intrinsically Enriched Liver as a Source of [^{3}H]Vitamin B_6 (All Diets Contained [^{14}C]Pyridoxine for Extrinsic Enrichment)[a]

		major hepatic metabolites, %			urinary 4-PA and
diet	isotope	PLP	PMP	PL	lactone
control	^{3}H	14.0 ± 1.1	48.9 ± 6.6	17.8 ± 1.4	58.6 ± 1.9
	^{14}C	16.0 ± 2.7	63.0 ± 8.0	17.1 ± 1.6	60.0 ± 2.9
raw liver	^{3}H	16.1 ± 1.0	55.6 ± 6.1	17.5 ± 1.7	66.6 ± 1.7
	^{14}C	16.2 ± 1.2	67.6 ± 7.6	19.5 ± 1.9	67.7 ± 2.1
cooked	^{3}H	9.5 ± 3.1	62.2 ± 2.8	17.2 ± 2.0	59.6 ± 2.7
liver	^{14}C	10.5 ± 2.4	59.9 ± 6.4	21.4 ± 1.1	61.0 ± 2.0

[a] No significant difference between relative concentration of ^{3}H and ^{14}C for each B_6 vitamer among all dietary groups at the 95% confidence level determined by two-way analysis of variance. Values are means ± SEM, seven rats per group. Data are expressed as percentage of total hepatic or urinary radioactivity.

respectively, relative to that of the rats fed raw liver. It was stated previously that the cooking process reduced the vitamin B_6 content of cooked liver to 73.5% of the raw liver. If there were no effects of cooking on the bioavailability of vitamin B_6 remaining in the dietary liver, the concentration of ^{3}H in the carcass, urine, and liver of the rats fed cooked liver would be expected to be aout 73.5% of the levels of rats fed raw liver, assuming negligible intestinal absorption and/or tissue retention of thermal degradation products. The relative isotopic ratio (% of ^{3}H dose/% of ^{14}C dose) in the livers of the group fed cooked liver was significantly lower than 73.5% of the group fed raw liver. However, the relative isotopic ratio in the urine of the rats feed cooked liver was higher than 73.5% of that found for rats fed the diet containing raw liver. Therefore, heating of the liver appeared to have caused little or no reduction in the bioavailability of the vitamin B_6 remaining as monitored by tritium retention and excretion. The ^{3}H remaining in the feces of the rats fed cooked liver was significantly higher than that of the rats fed raw liver. This is suggestive of poor absorption of the thermal degradation products of [^{3}H]vitamin B_6 relative to the intact [^{3}H]vitamin B_6 or reduced digestibility of the thermally treated tissue proteins. Diet composition did not influence the utilization of the [^{14}C]-pyridoxine extrinsic label, as indicated by ^{14}C in feces and intestinal contents (Table II).

The results discussed above represent tritium retention in the animal but provide no information regarding the identity of the labeled compounds in tissue and excreta. To assess more completely the bioavailability of the dietary vitamin B_6, the distriution of [^{3}H]vitamin B_6 metabolites in the livers of the sacrificed animals was determined. The major ^{3}H metabolites found in the livers of all treatment groups were PMP, PLP, and PL, which accounted for over 90% of the ^{3}H present (Table III). Statistical comparisons showed no differences in relative distribution of ^{3}H- and ^{14}C-labeled vitamin B_6 metabolites within each dietary group. This is evidence of similar metabolism and utilization of the absorbed ^{3}H and ^{14}C B_6 vitamers irrespective of dietary treatment. Only slight differences in metabolite distributions were seen between dietary groups. Therefore, measures of bioavailability based on isotopic retention in the animal appear to be valid. These results also exclude potential biases in quantitation based on isotopic retention attributable to the in vivo retention of thermal degradation products of vitamin B_6.

Analysis of urine revealed that 50% or more of the ^{3}H or ^{14}C was in the form of 4-PA and its lactone for all treatment groups (Table III). No significant differences were observed in the percentage of urinary radioactivity as 4-PA and its lactone between isotopes across dietary treatments ($p > 0.05$). Because 4-PA is an end product of vitamin B_6 metabolism, the similarities observed further support the conclusions based on isotopic retention and excretion.

Bioavailability of Vitamin B_6 in Muscle. Rat hind limb muscle tissue that had been intrinsically enriched with tritiated B_6 vitamers was incorporated into test meals after thermal and/or chemical treatment. Five different test meals were employed for this study. Each contained [^{14}C]PN with only one of the following sources of [^{3}H]-vitamin B_6 (Table IV): (a) raw muscle 1; (b) cooked muscle 1; (c) cooked MSG treated muscle 1 (0.5% MSG); (d) cooked muscle 2; (e) ^{3}H-PN (no muscle). Monosodium glutamate (MSG), which is a common additive used in canned meats, was used as a variable in this study because of its potential for carbonyl–amine reactions with PLP and PL. The distribution of [^{3}H]B_6 vitamers in each of the four muscle test meals is shown in Table IV (raw muscle 2 was not fed to the rats but was shown for comparison of cooking effects on B_6 vitamer distribution). Thermal processing in the presence of MSG (0.5% w/w) did not markedly alter the distribution of remaining B_6 vitamers, as compared to processing muscle without MSG. No *N*-pyridoxylglutamic acid or *N*-(5-phosphopyridoxyl)-glutamic acid was detected in HPLC analyses. Two different perfused hind limb preparations were used in this study. Muscle 1 contained a higher percentage of ^{3}H in the form of PLP and PL than muscle 2, which was higher in PNP and PN. Muscle 1 was fed raw, cooked, and MSG treated while muscle 2 was fed only in the cooked state. The feeding of both muscles 1 and 2 made it possible to compare the bioavailabilities of vitamin B_6 in cooked tissue having different patterns of B_6 vitamers.

After the previously mentioned five sources of [^{3}H]- and [^{14}C]PN were administered, the percentage of ingested isotope found in the liver, carcass, urine, plasma, and feces was determined (Table V). The test meals containing cooked muscle 1, MSG-treated muscle 1, and cooked muscle 2 had 78.2%, 74.7%, and 65.4%, respectively, of

Table IV. Radioisotopic Distribution in Test Meals Containing [^{3}H]Pyridoxine or Intrinsically Enriched Muscle as the Source of [^{3}H]Vitamin B_6

	% distribution									
gel diet	PLP	PL	PMP	PM	PNP	PN	4-PA	3-min retentn[a]	other[b]	total radioact, μCi ^{3}H/g of gel
control						94.6		5.4		0.227
raw muscle 1	30.2	29.0	5.9		1.4	2.8	19.6	11.1		0.295
cooked muscle 1	14.2	9.1	15.5	9.3		4.4	13.4	19.6	12.8	0.295
cooked MSG muscle 1	14.3	2.8	16.1	10.3		6.9	15.6	17.4	15.2	0.295
raw muscle 2	14.0	16.8	9.9	2.4	35.3	12.7	4.1	5.0		c
cooked muscle 2	1.2	15.1	11.4	5.2	10.7	5.1	29.8	24.9		0.249

[a] Refers to HPLC retention time. [b] Other unidentified ^{3}H components. [c] Raw muscle 2 was not fed to rats. These data were included to show changes in B_6 vitamer distribution when raw muscle 2 was heated. Only cooked muscle 2 was fed.

Table V. Recovery of ^{3}H and ^{14}C in Rats Fed [^{3}H]Pyridoxine or Intrinsically Enriched Muscle as a Source of [^{3}H]Vitamin B_6[a]

tissue or excreta	diet	% of ingested isotope[b] ^{3}H	% of ingested isotope[b] ^{14}C	rel isotopic ratio,[c] % ^{3}H dose/% ^{14}C dose
liver	control	11.4 ± 1.4	6.9 ± 0.7	1.64 ± 0.12[a]
	muscle 1 (raw)	10.7 ± 0.9	8.3 ± 0.6	1.32 ± 0.16[a]
	muscle 1 (cooked)	5.1 ± 0.2	8.1 ± 0.3	0.64 ± 0.01[c,b]
	muscle 1 (MSG)	6.8 ± 0.2	8.7 ± 0.3	0.78 ± 0.03[c,b]
	muscle 2 (cooked)	5.3 ± 0.5	7.4 ± 0.5	0.72 ± 0.04[c,b]
carcass	control	26.4 ± 4.0	13.5 ± 2.0	1.96 ± 0.07[a]
	muscle 1 (raw)	26.5 ± 2.7	16.8 ± 2.1	1.58 ± 0.06[b]
	muscle 1 (cooked)	21.6 ± 1.4	17.8 ± 2.0	1.22 ± 0.05[b]
	muscle 1 (MSG)	20.0 ± 1.4	17.2 ± 1.1	1.17 ± 0.10[b]
	muscle 2 (cooked)	17.6 ± 1.4	14.6 ± 1.5	1.25 ± 0.18[b]
plasma[d]	control	0.70 ± 0.10	0.23 ± 0.07	3.50 ± 0.65[a]
	muscle 1 (raw)	0.54 ± 0.03	0.20 ± 0.01	2.64 ± 0.29[a]
	muscle 1 (cooked)	0.57 ± 0.04	0.18 ± 0.02	3.21 ± 0.24[a]
	muscle 1 (MSG)	0.62 ± 0.02	0.24 ± 0.02	2.64 ± 0.29[a]
	muscle 2 (cooked)	0.51 ± 0.04	0.18 ± 0.02	2.97 ± 0.16[a]
urine	control	22.8 ± 4.1	30.3 ± 6.2	0.77 ± 0.04[a]
	muscle 1 (raw)	25.7 ± 0.7	20.4 ± 2.0	1.28 ± 0.09[b]
	muscle 1 (cooked)	18.2 ± 0.9	18.3 ± 1.6	1.00 ± 0.05[b]
	muscle 1 (MSG)	17.4 ± 2.1	19.8 ± 1.8	0.87 ± 0.03[b]
	muscle 2 (cooked)	17.1 ± 0.7	16.6 ± 1.3	1.03 ± 0.05[b]
feces and intestinal contents	control	21.6 ± 8.1	12.2 ± 1.4	1.63 ± 0.57[a]
	muscle 1 (raw)	23.7 ± 3.1	10.4 ± 1.7	2.31 ± 0.14[a,b,c]
	muscle 1 (cooked)	31.8 ± 0.7	13.0 ± 1.2	2.49 ± 0.20[b,c]
	muscle 1 (MSG)	27.8 ± 2.6	14.9 ± 1.2	1.86 ± 0.04[a,b]
	muscle 2 (cooked)	33.6 ± 4.5	12.4 ± 0.9	2.67 ± 0.18[c]

[a] Values are means ± SEM, four rats per group. All diets contained [^{14}C]pyridoxine for intrinsic enrichment. [b] The rats consumed the following amounts of radioactivity in the test diets: control, 1.67 ± 0.13 μCi of ^{3}H, 1.10 ± 0.05 μCi of ^{14}C; raw muscle 1, 1.68 ± 0.08 μCi of ^{3}H, 2.08 μCi of ^{14}C; cooked muscle 1, 2.14 ± 0.03 μCi of ^{3}H, 1.24 ± 0.01 μCi of ^{14}C; cooked muscle 1 with MSG, 1.59 ± 0.03 μCi of ^{3}H, 1.09 ± 0.03 μCi of ^{14}C; cooked muscle 2, 1.56 ± 0.04 μCi of ^{3}H, 1.18 ± 0.05 μCi of ^{14}C. [c] The relative isotopic ratios were evaluated by one-way analysis of variance and the Tukey honestly significant difference procedure for multiple comparisons. For the liver, urine, and carcass, the relative isotopic ratios were compared as follows to account for thermally induced differences in the oral dose of [^{3}H]vitamin B_6: cooked muscle 1 compared to 78.2% of raw muscle 1; MSG compared to 74.7% of raw muscle 1; cooked muscle 2 compared to 65.4% of raw muscle 1. For each tissue or excreta, values followed by the same superscript letter were not significantly different at the 95% confidence level. No significant difference was found between diets in relative ^{14}C concentration of feces and intestinal contents ($P > 0.05$). [d] Assumed plasma volume of 8 mL.

the [^{3}H]B_6 vitamers found in raw muscle 1 at the time of feeding as a result of thermal destruction of B_6 vitamers. Bioavailability was assessed by comparing the relative isotopic ratio (% of ^{3}H dose/% of ^{14}C dose) for cooked muscle 1, MSG-treated muscle 1, and cooked muscle 2 ratios to 78.2%, 74.7%, and 65.4%, respectively, of the relative isotopic ratios of raw muscle. The relative isotopic ratio in the livers of the rats fed cooked muscle 1 was significantly lower ($P < 0.05$) than 78.2% of the relative isotopic ratio of the livers of the group fed raw muscle 1. However, the relative isotopic ratio of the carcass and urine of the group fed cooked muscle 1 was close to 78.2% of the ratio observed for the group fed raw muscle 1 and does not suggest incomplete bioavailability of [^{3}H]B_6 in the diet containing cooked muscle 1. The percentage of ingested ^{3}H in the feces and intestinal contents of rats fed the diets containing muscle was greater than observed for diets containing raw muscle. This result may reflect incomplete intestinal absorption of thermal degradation products of [^{3}H]vitamin B_6 and is consistent with observations regarding cooked liver. The extent of ^{14}C absorption, as indicated by ^{14}C in feces and intestinal contents, did not vary between diets (Table V).

The data concerning test diets containing the MSG-treated muscle 1 and cooked muscle 2 were similar to the results for cooked muscle 1. The ^{3}H retention in the livers of the rats fed MSG muscle 1 and cooked muscle 2, as reflected by the relative isotopic ratios, was less than 74.7% and 65.4% of that of the rats fed diets with raw muscle 1, respectively (74.7% and 65.5% represent the proportions of [^{3}H]B_6 vitamers in the MSG and muscle 2 test diets relative to the raw muscle 1 test meal), although not significantly less $P > 0.05$. The data for relative isotopic ratios of urine and carcass, for the MSG and muscle 2 test meals, reflect the similar bioavailability of [^{3}H]B_6 vitamers contained in these meals relative to the raw muscle test meal.

The major tritiated and [^{14}C]vitamin B_6 metabolites in the livers of these rats were found to be PMP, PLP, and PL, which accounted for at least 75% of the isotope present (Table VI). There was no consistent difference in the relative proportion of these B_6 metabolites among the treatments, other than a slightly higher percentage of hepatic [^{14}C]PMP.

The major tritiated and [^{14}C]vitamin B_6 metabolities found in the urine were 4-PA and its lactone (Table VI). Like the data concerning hepatic metabolites, the ^{3}H and ^{14}C urinary metabolities for the muscle test diets indicate that the radiolabeled B_6 vitamers were metabolized and utilized in a manner that was generally independent of the dietary source (Table VI).

DISCUSSION

Much of the research concerning the bioavailability of vitamin B_6 in foods has been done of rat bioassays with various quantitative indicators such as growth, plasma and liver PLP, and erythrocyte aminotransferase activity. When the rat is used in animal bioassay experiments involving vitamin B_6, possible bias due to coprophagy or direct absorption of B_6 vitamers synthesized by intestinal microflora must be considered. The results of Nguyen and Gregory (1983) indicated that rat bioassays of biologically available vitamin B_6 may be subject to a great deal of uncertainty when the test diet differs significantly from the reference diet with respect to carbohydrate or protein. Prevention of coprophagy has been found to be ineffective in significantly reducing microbial contribution of B_6 vi-

Table VI. Relative Concentration of Hepatic and Urinary Radiolabeled Forms of Vitamin B_6 in Rats Fed [^{3}H]Pyridoxine (Control) or Intrinsically Enriched Muscle as a Source of [^{3}H]Vitamin B_6 (All Diets Contained [^{14}C]Pyridoxine for Extrinsic Enrichment)[a,b]

diet	isotope	major hepatic metabolites, %			urinary 4-PA and lactone
		PLP	PMP	PL	
control	^{3}H	4.6 ± 0.6	50.1 ± 3.1	18.3 ± 3.6	53.9 ± 6.7
	^{14}C	6.0 ± 1.2	62.1 ± 5.1	14.6 ± 2.8	45.8 ± 4.6
muscle 1	^{3}H	9.0 ± 0.8	51.2 ± 1.5	16.0 ± 3.2	38.4 ± 2.7
(raw)	^{14}C	7.3 ± 0.5	57.3 ± 2.0	19.7 ± 3.0	53.3 ± 2.5
muscle 1	^{3}H	6.5 ± 2.5	53.7 ± 2.0	10.1 ± 2.0	48.5 ± 2.4
(cooked)	^{14}C	6.1 ± 0.7	64.2 ± 3.8	8.5 ± 2.4	48.5 ± 2.9
muscle 1	^{3}H	3.5 ± 0.9	53.1 ± 4.1	11.7 ± 0.8	51.8 ± 4.2
(cooked + MSG)	^{14}H	6.8 ± 1.8	62.1 ± 4.2	13.3 ± 1.9	48.9 ± 4.0
muscle 2	^{3}H	6.5 ± 1.7	55.0 ± 4.3	7.9 ± 1.7	52.8 ± 2.8
(cooked)	^{14}C	7.5 ± 1.6	61.8 ± 3.3	8.5 ± 1.4	40.2 ± 3.5

[a] Values are means ± SEM, four rats per dietary group. Data are expressed as percentage of total hepatic or urinary radioactivity. [b] For hepatic PLP and PL and urinary 4-PA and its lactone, no significant difference between relative concentration of ^{3}H and ^{14}C among all dietary groups ($P > 0.05$) was determined by two-way analysis of variance. For hepatic PMP, the overall difference between ^{3}H and ^{14}C concentrations was significant at $P < 0.001$.

tamers in rat bioassays (Gregory and Litherland, 1986).

The experimental protocol of the present study, which permits direct measurement of the absorption, metabolism, and retention of ^{3}H and ^{14}C dietary vitamin B_6 is unaffected by the ambiguities inherent in conventional bioassays. However, interpretation of the results should be made in light of the fact that the [^{3}H]B_6 vitamers within the cells of the perfused liver or muscle tissue in the test diet are not totally representative of endogenous B_6 vitamers of liver or muscle. The [^{3}H]B_6 vitamers incorporated in the liver and muscle would be expected to equilibrate more with the short-term pool of vitamin B_6 and less with the long-term pool (Johansson and Tiselius, 1973). Also, the percentage of ^{3}H existing as PLP relative to other vitamin B_6 metabolites in the perfused liver and muscle was lower than published values for endogenous PLP (Gregory and Feldstein, 1985).

Thermal processing of liver and muscle in these studies caused approximately 30% reduction in the levels of [^{3}H]B_6 vitamers. The thermal instability of PLP and PL and interconversion to PMP and PM found in this study were consistent with the results of other research (Polansky and Toepfer, 1969; Gregory and Hiner, 1983).

The bioavailability of [^{3}H]vitamin B_6 in the perfused liver after cooking was very similar to the [^{3}H]B_6 vitamers in the raw liver based on the relative isotopic retention in the liver, carcass, and urine. The control animals that received [^{3}H]PN had values for the relative isotopic ratio that were of similar magnitude to the group fed raw liver. The group fed cooked liver had a significantly higher relative isotopic ratio for the intestinal contents than either the control or the group fed raw liver, which indicates poor absorption of thermally induced [^{3}H]vitamin B_6 degradation products or [^{3}H]B_6 vitamers. The data concerning ^{14}C added extrinsically to each diet did not vary as a function of the treatment, which indicates that differences in gel composition did not influence the bioavailability of the added [^{14}C]PN.

The isotopic recovery in tissues and excreta was typically less than 80% for all treatment groups. This low recovery may be attributed largely to incomplete extraction of label from the carcass since a crude homogenate was used to provide an aliquot for quantitation of the isotopes. In addition, the head and neck of the animal were not included in the carcass homogenate, which would account for at least 10% of the total isotope.

Thermal processing and treatment with MSG had little or no effect on the bioavailability of [^{3}H]vitamin B_6 in muscle tissue based on retention of ^{3}H in the carcass and urinary content of ^{3}H relative to that of the extrinsically added dietary [^{14}C]pyridoxine. However, data concerning hepatic isotopic retention were indicative of slightly reduced bioavailability of [^{3}H]vitamin B_6 in the thermal- and MSG-treated muscle relative to raw tissue. Differences in protein digestibility between diets containing raw and cooked tissues may have been responsible for the observed differences in apparent bioavailability. The results for cooked muscle 1 showed stronger trends of reduced bioavailability of [^{3}H]B_6 vitamers relative to the raw muscle than those for cooked musle 2. Since muscle 1 had a higher percentage of [^{3}H]PLP than muscle 2, the reductive binding of PLP to proteins as a pyridoxylamino complex during thermal processing may have played a role in the observed lower apparent bioavailability of [^{3}H]B_6 vitamers in muscle 1 relative to muscle 2. It should be noted that the acidic extraction conditions used in preparation for HPLC analysis would not release PLP or PL bound to protein as pyridoxylamino complexes. Protein-bound pyridoxylamino compounds have been shown to exhibit approximately 50% bioavailability relative to the molar utilization of free PN (Gregory, 1980). A weak antagonistic effect has been observed only in vitamin B_6 deficient rats. (Gregory, 1980). Thus, it would be expected that protein-bound pyridoxylamino complexes would partially contribute to the biologically available [^{3}H]vitamin B_6 of cooked liver and muscle yet would not be detected in the HPLC analysis of extractable B_6 vitamers in the cooked tissues. The magnitude of this effect would depend on the digestibility of the protein.

The predominant [^{3}H]B_6 vitamers found in the liver 24 h after ingestion of diets containing either liver or muscle were PMP, PLP, and PL, accounting for 75% or more of the isotope found. Pyridoxamine phosphate, PLP, and PL have been previously found to represent more than 70% of [^{3}H]B_6 vitamers in the livers of rats 24 h or more after intraperitoneal injection of [^{3}H]PN (Segalman and Brown, 1981; Shane, 1982). The relative concentration of hepatic PLP in this study was markedly lower while PMP concentrations were higher than those reported previously. This difference may be due to the route of administration (in gel diet vs. injected) and the dietary protocol employed. Further studies are needed to resolve these effects.

The HPLC analysis of urinary ^{3}H metabolites in this study indicated that 4-PA and its lactone were the most prevalent compounds. This result is in agreement with previous studies involving the rat and human (Tillotson et al., 1966, Contractor and Shane, 1970; Kelsay et al., 1968). A relative isotopic ratio (% of ^{3}H dose/% of ^{14}C dose) different from 1.0 was found for isotopic retention in some of the tissues of control animals. The presence

of 21.9% of the ^{3}H on the 4-methylene group of pyridoxine purchased frm Amersham may account for these observations. The metabolism of [^{3}H]pyridoxine to pyridoxal or pyridoxic acid would result in loss of some or all of the ^{3}H in the 4′-position and generation of ^3H_2O (Contractor and Shane, 1970), which would equilibrate with total body water. This has been reported previously for metabolism of ^{3}H- and ^{14}C-labeled PN in the rat (Contractor and Shane, 1970; Johansson and Tiselius, 1973). Such an effect may have been responsible for the difference between the percentages of [^{3}H]PMP and [^{14}C]PMP observed in this study (Tables III and VI).

The data concerning hepatic and urinary [^{3}H]vitamin B_6 metabolites in this study are of nutritional interest because they provide information concerning the absorption and metabolism of dietary forms of vitamin B_6. More importantly, the data indicate that the ^{3}H and ^{14}C compounds absorbed by the animals were effectively utilized and metabolized independently of the dietary source and treatment. This finding is supportive of the use of isotopic retention data as indicators of the bioavailability of vitamin B_6 in studies such as this. In general, there was very good agreement among the response criteria employed in this study. With this single-dose protocol, the results can be interpreted without consideration of possible biases due to coprophagy or direct absorption of B_6 vitamers synthesized by intestinal bacteria. The results of this study suggest that isotopic enrichment procedures can be useful in the assessment of vitamin B_6 bioavailability.

ABBREVIATIONS USED

pyridoxine = PN
pyridoxal phosphate = PLP
pyridoxine phosphate = PNP
pyridoxamine phosphate = PMP
pyridoxal = PL
pyridoxamine = PM
pyridoxic acid = 4-PA

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