Rao, M. P.; Sethuram, B.; Rao, T. N. *Indian J. Chem., Sect. A* **1979**, *17A*, 52.
Saunders, B. C.; Holmes-Siedle, A. G.; Stark, B. P. "Peroxidase"; Butterworths: Washington, DC, 1964; pp 10–25.
Smissman, E. E.; Corbett, M. D. *J. Org. Chem.* **1972**, *37*, 1847.
Smith, M. R.; Gorrod, J. W. In "Biological Oxidation of Nitrogen"; Gorrod, J. W., Ed.; Elsevier/North-Holland Biomedical Press: Amsterdam, 1978; pp 65–70.
Weisburger, J. H.; Weisburger, E. K. *Pharmacol. Rev.* **1973**, *25*, 1.
You, I.; Bartha, R. *J. Agric. Food Chem.* **1982**, *30*, 274.
Zeyer, J.; Kearney, P. C. *Pestic. Biochem. Physiol.* **1982**, *17*, 215.

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Relative Biological Activity of Nonphosphorylated Vitamin B-6 Compounds in the Rat

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The relative biological activity for rats of pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) was examined in research concerning the quantitation of rat bioassays for vitamin B-6. With typical basal diets containing 19.8% casein, PM and PL elicited slightly lower growth and feed efficiency responses and essentially equivalent plasma pyridoxal 5′-phosphate (PLP) concentration, relative to PN. In contrast, PL and PM were markedly less active on the basis of erythrocyte aspartate aminotransferase activity and in vitro stimulation by exogenous PLP. Similar results were observed when high-protein (50% casein) basal diets were used for growth, feed efficiency, and plasma PLP indexes. The relative differences in biological activity were much less pronounced with low-protein (5% casein) diets. The specific activity of intestinal aromatic aminotransferase, which has been reported to be increased in severe vitamin B-6 deficiency, was not suitable for bioassay quantitation. The results of this study indicate the need to be cognizant of differences in the apparent biological activity of the various B-6 vitamers in rat bioassays employing PN dose–response curves. These results also support the validity of plasma PLP as an index of vitamin B-6 status.

Vitamin B-6 is the generic name for a family of compounds having similar biological activity. Although pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) are the only two B-6 vitamers presently known to be active coenzyme forms of the vitamin, various B-6 vitamers can be interconverted both enzymatically (Snell and Haskell, 1971) and nonenzymatically in the presence of catalysts [e.g., Metzler et al. (1954), Matsuo (1957), and Lui et al. (1981)]. Numerous studies have been conducted with pyridoxine (PN) hydrochloride as a standard in the quantitation of biologically available vitamin B-6 in food [e.g., Yen et al. (1976), Gregory and Kirk (1978), Gregory (1980a), and Tarr et al. (1981)]. The vitamin B-6 of animal-derived foods is comprised largely of PLP and PMP, with small amounts of PL, PM, and PN (Vanderslice et al., 1980; Gregory et al., 1981), while the majority of the vitamin in plant tissues appears to occur as PN (Polansky et al., 1964; Polansky, 1969). The use of PN as a standard in animal bioassays is based on the greater stability of this vitamer and, to some extent, the assumed equivalent biological response to PN and the other vitamin B-6 compounds.

Although a great deal of information has been reported on differences in responses of a number of microorganisms to various B-6 vitamers (Toepfer and Lehmann, 1961; Haskell and Snell, 1970; Guilarte et al., 1980% Gregory, 1982), data concerning the relative response of animal species to different forms of vitamin B-6, especially as influenced by diet, are rather limited. An influence of the intestinal microflora on the apparent relative activity of B-6 vitamers has been suggested by several studies. Sarma et al. (1946) reported equivalent growth-supporting activity in rats for PN, PM, and PL when administered in solution per os or injected intraperitoneally; however, PL and PM elicited a lower growth-promoting response when fed in the diet. Similar results were obtained for dietary and intraperitoneally injected PLP. These findings were confirmed in subsequent experiments with rats (Linkswiler et al., 1951) and chicks (Waibel et al., 1952). In these early studies growth was the only criterion used for the evaluation of the relative potency of the B-6 vitamers. Lower apparent activity of dietary PL and PM for rats was suggested in further research in which B-6 vitamer concentrations and the activity of aspartate aminotransferase and alanine aminotransferase in several tissues were determined (Brin and Thiele, 1967; Thiele and Brin, 1968). The degree of enzyme stimulation by in vitro addition of PLP and plasma PLP concentration were not determined, however.

The purpose of the present study was to evaluate the relative activity of the nonphosphorylated B-6 vitamers when fed in semipurified diets under conditions of typical rat bioassays. Of primary interest was a comparison of the relative activity of the vitamers as determined by several routinely used biochemical indicators of vitamin B-6 nutriture. A second objective as to evaluate the relative activity of the B-6 vitamers as a function of dietary protein content.

MATERIALS AND METHODS

Animals and Diets. Male weanling Crl:CD(SD)BR Sprague-Dawley rats (Charles River Breeding Laborator-

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Table I. Composition of the Basal Diet for Experiment I

ingredient	%
vitamin-free casein[a]	19.8
sucrose	59.9
cellulose[a]	10.0
corn oil	5.0
vitamin mix[b,c]	1.1
mineral mix[d]	4.0
DL-methionine[a]	0.2
total	100.00

[a] ICN Pharmaceuticals, Inc. [b] Vitamin mix (ICN Pharmaceuticals, Inc.) provided the following per kg of diet: vitamin A, 9900 units; vitamin D, 1100 units; α-tocopherol, 55 mg; choline chloride, 825 mg; menadione, 25 mg; niacin, 50 mg; riboflavin, 11 mg; calcium pantothenate, 33 mg; thamin, 0.22 mg, folic acid, 0.99 mg; vitamin B-12, 0.015 mg; biotin, 0.22 mg. [c] Diets were fortified as appropriate with PN, PM, or PL as their hydrochloride forms (Sigma Chemical Co.) in aqueous solution, which provided a total addition of 50 mL of H_2O/3 kg of diet. [d] Mineral mix from Teklad Test Diets was the Wesson-modified Osborne-Mendel mixture. Composition: calcium carbonate, 21.00%; copper sulfate pentahydrate, 0.039%; ferric phosphate, 1.47%; manganous sulfate, 0.02%; magnesium sulfate, 9.00%; potassium aluminum sulfate, 0.009%; potassium chloride, 12.00%; potassium dihydrogen phosphate, 31.00%; potassium iodide, 0.005%; sodium chloride, 10.50%; sodium fluoride, 0.05%; tricalcium phosphate, 14.90%. $ZnCO_3$ was added to provide 15 ppm of Zn.

ies) were housed individually in stainless steel cages with wire mesh floors in a temperature-controlled room and maintained on a 12-h light cycle. Feed and water were given ab libitum. Feed intake was determined 2–3 times a week and weight gains were recorded weekly.

In experiment I, all rats were fed a vitamin B-6 deficient basal diet (Table I) for a 2-week depletion period, following which they were subdivided into groups of 9–10 animals fed either a standard diet (basal diet supplemented with 0.59, 1.78, 3.55, and 7.10 nmol of added pyridoxine (PN)/g of basal feed) or a diet containing PL or PM added at a concentration of 3.55 nmol/g of basal diet. After a 3-week period on the supplemented diets, the rats were sacrificed by decapitation. Blood was collected from the cervical vessels into heparin-coated glass tubes and centrifuged a 1000*g* for 15 min to separate plasma, which was stored at −20 °C until assayed for PLP concentration. The erythrocytes were washed twice with 0.9% NaCl solution and stored at −20 °C until assayed for erythrocyte aspartate aminotransferase (AspAT) activity. The small intestine of each rat was rapidly excised and packed in crushed ice. Intestinal contents were removed by perfusion with 5 mM potassium phosphate–0.25 M sucrose (pH 7.5). Each whole small intestine was homogenized in 10 mL of the same buffer for 10 s with a Brinkmann Polytron at setting 7 at 0–2 °C. The homogenates were centrifuged for 30 min at 10000*g* at 2 °C, and the supernatants were stored at −20 °C until assayed for aromatic aminotransferase activity and protein concentration. Livers were rapidly excised, quick frozen, and stored at −20 °C until analyzed for PLP concentration.

In experiment II, three basal diets containing 5, 19.8, and 50% casein were used during the 2-week depletion period (Table II). Endogenous vitamin B-6 contents of the low-, normal-, and high-protein basal diets were 1.24, 0.71, and 1.42 nmol/g of feed, respectively, by microbiological assay using *Saccharomyces uvarum* (Haskell and Snell, 1970). The rats of each protein group were subsequently divided into subgroups of eight to nine animals and fed diets supplemented with PN at 0.59, 1.78, 3.55, and 7.10 nmol/g or PL or PM at 3.55 nmol/g. Plasma was collected as in the first experiment.

Table II. Composition of the Basal Rat Diets Used in Experiment II

ingredient	% low protein	% normal protein	% high protein
vitamin-free casein[a]	5.0	19.8	50.0
sucrose	75.3	60.5	30.3
cellulose[b]	9.4	9.4	9.4
corn oil	5.0	5.0	5.0
vitamin mix[c]	1.1	1.1	1.1
mineral mix[d]	4.0	4.0	4.0
DL-methionine[a]	0.2	0.2	0.2

[a] ICN Pharmaceuticals, Inc. [b] Alphacel, ICN Nutritional Biochemicals Corp. [c] ICN Nutritional Biochemicals Corp. See Table I for composition and method of vitamin B-6 fortification. [d] Wesson-modified Osborne-Mendel mixture, ICN Nutritional Biochemicals Corp. See Table I for composition. $ZnCO_3$ was added to provide 15 ppm of Zn.

All rat diets were stored in sealed containers at 2 °C until used.

Enzymatic Assays. *Erythrocyte AspAT Activity and Its in Vitro Stimulation by PLP.* Erythrocyte AspAT activity and its in vitro stimulation by exogenous PLP were assayed by the Calbiochem kinetic spectrophotometric procedure (1976). This assay procedure has been validated for hemolysates in terms of time-course linearity and linearity of rate as a function of enzyme concentration (Gregory, 1980a; Gregory and Kirk, 1978; Nguyen et al., 1981). It also was found to yield results that correlated closely with those of a continuous-flow procedure (Skala et al., 1981), which we performed using fluorometric detection. Distilled water was added to frozen erythrocytes in 1:1 volume ratio, and the solutions were mixed to hemolyze the erythrocytes and centrifuged to sediment cell debris. The supernatants were diluted approximately 1:10 with distilled water and kept on ice during the spectrophotometric assay. AspAT activity was determined at 30 °C with commercially prepared reagents (GOT Super StatPack, Calbiochem) with a Gilford Model 250 spectrophotometer (Gilford Instrument Laboratories, Inc.). Each sample was monitored for a minimum of 5 min. Serial dilutions of a composite hemolysate were analyzed to determine the linear range of the assay. For the determination of the stimulation of AspAT activity by the in vitro addition of PLP, 0.9 mL of each hemolysate was mixed with 0.1 mL of 8.2 mM PLP and incubated at 30 °C for 30 min prior to assay. Determination of hemoglobin in the hemolysates was based on the cyanomethemoglobin method of Crosby et al. (1954), using horse hemoglobin (Pentex Biochemicals) as the standard.

Plasma PLP Assay. Plasma PLP was determined by the method by Sloger and Reynolds (1980), using L-tyrosine apodecarboxylase (TDC) from *Streptococcus faecalis* (Sigma Chemical Co.). The apoenzyme was purified by the method recommended by Lumeng et al. (1981) prior to use. Plasma samples were deproteinized with trichloroacetic acid (TCA) and diluted with a 0.9% NaCl solution to a concentration of 5–10 ng of PLP/mL. After incubation of the deproteinized samples at 32 °C for 15 min and removal of particulate matter by centrifugation, TCA was removed from the sample solution by ethyl either extraction. Residual ether was evaporated under a nitrogen flow. Reaction mixtures contained 50–200 μL of sample extract–0.1 M sodium citrate buffer to bring the volume of the sample plus buffer to 300 μK, 100 μL of diluted apoenzyme, and 1 mL of L-[*carboxyl*-^{14}C]tyrosine solution (59 mCi/mmol, Amersham Corp. diluted with 500

Table III. Rat Responses to Diet Containing Various Forms of Vitamin B-6 in Experiment I[a,b]

	fortification, nmol/g	rat growth, g/21 days	feed efficiency, g of growth/g of feed	AspAT activity, milliunits/mg of Hb[c]	AspAT % stimulation, %	plasma PLP, pmol/mL	aromatic aminotransferase, milliunits/mg of protein[c]	liver PLP, nmol/g
PN	0.59	25.4 ± 2.4	0.174 ± 0.013	3.02 ± 0.18	104.9 ± 5.4	3.6 ± 1.2	1.46 ± 0.16	14.1 ± 1.1
	1.78	55.6 ± 5.6	0.289 ± 0.019	4.47 ± 0.58	101.5 ± 10.0	6.0 ± 1.0	2.35 ± 0.38	14.6 ± 1.2
	3.55	96.3 ± 2.7a	0.380 ± 0.010a	7.79 ± 0.62a	57.4 ± 8.0a	11.7 ± 0.8a	1.09 ± 0.09a	15.4 ± 1.0a
	7.10	121.2 ± 2.5	0.418 ± 0.017	9.36 ± 0.75	50.3 ± 8.2	22.6 ± 1.1	1.83 ± 0.21	20.2 ± 3.1
PL	3.55	89.2 ± 6.2a	0.365 ± 0.010a	5.33 ± 0.31b	98.0 ± 4.2b	8.1 ± 0.9a	1.16 ± 0.15a	14.9 ± 3.0a
PM	3.55	86.3 ± 2.6a	0.372 ± 0.013a	4.92 ± 0.44b	111.0 ± 7.5b	11.7 ± 3.1a	1.96 ± 0.38b	16.5 ± 1.5a

[a] $\bar{X}$ ± SEM; n = nine to ten rats per group. [b] Within each column, values for PN, PM, or PL at 3.55 nmol/g followed by the same letter were not significantly different at the 95% confidence level. [c] One unit of enzyme activity would yield 1.0 μmol of product/min under the described assay conditions.

Table IV. Calculated Relative Biological Activity of Pyridoxamine and Pyridoxal in Experiment I[a,b]

	relative activity by assay parameter (PN = 1.0)				
vitamer	growth	feed efficiency	AspAT activity	AspAT % stimulation	plasma PLP
PM	0.86 ± 0.03	1.12 ± 0.04	0.24 ± 0.02[c]	0.17 ± 0.01[c]	0.99 ± 0.26
PL	0.92 ± 0.06	1.05 ± 0.03	0.47 ± 0.02[c]	0.29 ± 0.01[c]	0.75 ± 0.08[c]

[a] $\bar{X}$ ± SEM; n = nine rats per group. [b] Biological activity calculated for each diet from the respective pyridoxine dose-response curves. A value of 1.0 would indicate full activity of PM or PL, relative to that predicted from the PN response curve, when present in the diet at 3.55 nmol/g. [c] Significantly different from 1.0 as determined by the two-tailed t test ($p < 0.05$).

mL of 0.1 M sodium citrate, 62.5 mL of 0.15 N HCl, and 0.7525 g of L-tyrosine to an activity level of 0.107 μCi/mL). the reaction tubes were equipped with plastic center wells (Kontes, Inc.) containing chromatography paper impregnated with methylbenzethonium hydroxide (Sigma Chemical Co.). The reaction was stopped after 20 min at 32 °C by injecting 1 mL of 5 N HCl into each tube. The trapped $^{14}CO_2$ in the center wells was counted for 3 min in 3 mL of scintillation fluid (Ready-Solv NA premixed cocktail, Beckman Instruments) with a liquid scintillation counter (Beckman Model LS9000). The efficiency of the counting system was 97.75 ± 0.06%. Plasma concentrations were determined by using a standard curve obtained from duplicate standard reaction mixtures containing 0–2.5 ng of PLP/tube. Samples were analyzed at two concentration levels, each in duplicate, or at a single concentration in triplicate. Recovery values for PLP added to plasma were typically 95–100%. The extraction and assay procedure were carried out in subdued light.

Intestinal Aromatic Aminotransferase Assay. Aromatic aminotransferase activity was determined essentially as described by Noguchi et al. (1976). Product quantification was based on the arsenate-catalyzed formation of aromatic 2-keto acid–borate complexes that are measured spectrophotometrically (George et al., 1967). Assay mixtures (1.6 mL) containing 10 mM phenylalanine, 5 mM α-ketoglutarate, 0.1 M potassium phosphate buffer (pH 8.0), and 0.2 mL intestinal homogenate were incubated routinely for 60 min at 37 °C. Phenylpyruvate formation was linear with respect to time and enzyme concentration under these conditions. Protein was determined by the method of Lowry et al. (1951).

Liver PLP Determination. Liver PLP was determined by the high-performance liquid chromatographic method of Gregory (1980b), which is based on perchloric acid extraction, conversion to the PLP semicarbazone derivative, reversed-phase separation, and fluorometric quantitation.

Microbiological Assay of Vitamin B-6. Samples of basal diets and fecal samples were analyzed for total vitamin B-6 content by the method of Haskell and Snell (1970). An inoculum of *S. uvarum* no. 4228 (ATCC 9080) was prepared with 20 mL of pyridoxine Y medium (Difco Laboratories) to which 40 ng of PN had been added. Duplicate samples, weighing 3 g, were autoclaved in 180 mL of 0.44 N HCl at 121 °C and 15 psi for 2 h. One of the duplicate samples was fortified with PN prior to autoclaving to provide 30 ng of added PN/mL of extract for the determination of recovery values. The extracts were adjusted to pH 4.5, diluted to 250 mL, and filtered prior to addition to growth medium. Inoculated mixtures were incubated at 30 °C for 15–18 h and analyzed at three different concentrations (0.05, 0.10, and 0.15 mL/5-mL assay tube), each concentration in triplicate. Triplicate standard solutions of PN, ranging from 0 to 8 ng of PN/5 mL of medium, were used for a standard curve.

Statistical Analysis of Data. Differences in rat responses to PN, PL, and PM in the three basal diets in experiments I and II were determined by two-way analysis of variance and Tukey's method for pairwise comparisons (Neter and Wasserman, 1974).

In experiment I, the biological activity of PM and PL was calculated based on a PN dose–response standard curve. A two-tailed t test was used to determine the statistical significance of the differences in biological activity between PN and the two other forms of vitamin B-6. The slopes of all vitamin B-6 dose–response curves were analyzed by linear regression.

RESULTS

Rat responses to diets supplemented with PN, PL, and PM from experiment I are summarized in Table III. The nonzero slopes of the PN dose–response curves ($p < 0.05$) allowed the use of these standard curves in the determination of the biological activity of PM and PL from the experimental diets (Table IV). Liver PLP concentration was only weakly related to the dietary vitamin B-6 level; thus, it was not suitable for quantitation of the relative activity of PM and PL under the conditions of this experiment. Liver PLP values were similar between diets containing equimolar amounts of the vitamers. Although no significant differences in biological activity between the various dietary groups were observed with growth and feed data, AspAT activity and its in vitro stimulation by ex-

Table V. Rat Responses to Pyridoxine, Pyridoxamine, and Pyridoxal as a Function of Basal Diet Protein Level (Experiment II)

protein level	B-6 vitamer added, nmol/g	response[a,b] growth, g/21 days	feed efficiency, g of growth/g of feed	plasma PLP, pmol/mL
low (5%)	PN, 0.59	2.1 ± 1.6	0.02 ± 0.01	5.7 ± 0.7
	PN, 1.78	8.1 ± 1.8	0.06 ± 0.01	12.1 ± 1.1
	PN, 3.55	14.0 ± 2.5a	0.10 ± 0.01a	16.2 ± 1.8a
	PN, 7.10	14.5 ± 1.4	0.11 ± 0.01	78.0 ± 5.1
	PM, 3.55	13.4 ± 1.5a	0.10 ± 0.01a	15.0 ± 2.0a
	PL, 3.55	13.4 ± 2.0a	0.10 ± 0.01a	12.8 ± 2.1a
normal (19.8%)	PN, 0.59	7.7 ± 2.8	0.06 ± 0.02	2.6 ± 0.4
	PN, 1.78	34.9 ± 3.2	0.22 ± 0.02	4.3 ± 0.6
	PN, 3.55	85.6 ± 4.7a	0.36 ± 0.02a	15.1 ± 1.8a
	PN, 7.10	121.3 ± 10.1	0.39 ± 0.02	65.5 ± 8.2
	PM, 3.55	58.8 ± 2.8b	0.30 ± 0.01b	12.9 ± 1.0a
	PL, 3.55	70.5 ± 5.1b	0.33 ± 0.02ab	12.4 ± 3.0a
high (50%)	PN, 0.59	−2.0 ± 2.4	−0.02 ± 0.03	2.7 ± 0.7
	PN, 1.78	17.8 ± 2.2	0.14 ± 0.02	4.1 ± 1.1
	PN, 3.55	57.9 ± 3.3a	0.33 ± 0.01a	10.5 ± 2.2a
	PN, 7.10	92.9 ± 5.1	0.40 ± 0.03	70.2 ± 7.0
	PM, 3.55	38.3 ± 1.6b	0.22 ± 0.01b	12.9 ± 2.0a
	PL, 3.55	47.1 ± 6.2b	0.16 ± 0.02c	9.9 ± 0.9a

[a] $\bar{X}$ ± SEM; seven to nine animals per group. [b] Within each protein level and for each response criterion, values for PN, PM, or PL at 3.55 nmol/g followed by the same letter were not significantly different at the 95% confidence level.

ogenous PLP indicated significantly lower vitamin B-6 activity of PM and PL relative to that predicted from the PN dose–response curves. The relationship between the small intestinal aromatic aminotransferase activity and dietary levels of PN, as shown in Table III, was not significant ($r = 0.001$).

Responses to PN, PL, and PM as a function of dietary protein levels in experiment II are presented in Table V. in view of the results of experiment I, plasma PLP was the only biochemical indicator employed in the second experiment. Dose–response relationships for PN in 19.8% protein diets were similar to those observed in experiment I for growth, feed consumption, feed efficiency, and plasma PLP. The slopes of dose–response curves for PN in high- and low-protein basal diets were lower than those for the 19.8% protein basal diets.

An evaluation of the response parameters for diets containing 3.55 nmol/g PN, PM, and PL by two-way analysis of variance showed significant interaction between the protein level and the form of the vitamin for rat growth and feed efficiency. Because of this interaction, the comparison of the responses to the three vitamers at the 3.55 nmol/g level was made separately for each dietary protein concentration (Table V). At the low protein level, no significant differences in responses to these B-6 vitamers were detected. At the normal and high protein levels PN consistently elicited higher growth and feed efficiency than PL or PM ($p < 0.05$). PL yielded higher feed efficiency than PM only at the high protein level. Differences in plasma PLP between rats fed the different B-6 vitamers were not significant at any basal diet protein level. For each B-6 vitamer, differences between the normal- and low-protein diets with respect to growth response and feed efficiency were significant at the 99% confidence level. Differences in responses between the normal- and high-protein diets were significant ($p < 0.01$) for growth when PL was fed, for growth and feed efficiency when PM was fed, and for all response criteria except plasma PLP when PN was the form of dietary vitamin B-6.

DISCUSSION

In many studies of the bioavailability of vitamin B-6, the determination of biologically available vitamin B-6 in test diets has been based on the comparison of responses of rats fed test diets to those of rats fed standard diets fortified with PN. There is a need to examine more closely the relative response of rats to various forms of vitamin B-6 because the bioavailability of vitamin B-6 in test diets could be underestimated if the rat responded less to PL or PM and only a PN dose–response standard curve was used. The aldehyde and amine forms of the vitamin and their phosphorylated derivatives are commonly found in many foods. Responses of the rat to different B-6 vitamers are not well established, especially as related to changes in diet composition. Although the phosphorylated vitamers were not examined in this study, it is anticipated that their molar response values would be similar to those of the nonphosphorylated compounds in view of their enzymatic dephosphorylation in the small intestine (Hamm et al., 1979; Mehansho et al., 1979).

The bioassay protocol used in this study involved a comparison of a single dose level of PM and PL added to rat diets. The concentration chosen, 3.55 nmol/g (0.6 μg of free base/g), represents the midpoint of typical PN dose–response curves in many rat bioassays for vitamin B-6. Maximal rat growth is obtained in such assays with approximately 6.8–7.5 nmol of PN/g of diet or 130–150 nmol of PN/day (Lumeng et al., 1978; Gregory, 1980c). While more comprehensive results would be obtained by using a multilevel design with slope-ratio analysis, the approach used in this study permitted a comparison of activities in the range that is used for most bioassay quantitation. Proportionally similar results for PM and PL would be anticipated over a broader dosage range.

Brin and Thiele (1967) suggested that the lower response of rats to dietary PL and, especially, PM was due partially to the instability of these compounds during refrigerated storage of semipurified diets, although no data were presented. This hypothesis was based on analysis of diets that were stored for 2 months after their feeding experiment was concluded. Limited analysis of diets in our laboratory by microbiological and HPLC methods has indicated negligible degradation of the vitamers when stored for approximately 6 weeks at 2 °C (J. F. Gregory and S. A. Litherland, unpublished experiments). Previous research has indicated half-life values for PM and PL of 35 and 46 days, respectively, when stored in powdered model food systems at 37 °C and 0.6 water activity

(Gregory and Kirk, 1978). In view of the comparatively slow degradation of these vitamers during storage under relatively severe storage conditions, high stability of PL and PM during storage at 2 °C would be expected.

Response criteria used in this study of biological activity of vitamin B-6 included growth and feed efficiency as well as activity parameters for vitamin B-6 dependent enzymes and the concentration of PLP in liver and plasma. Although growth is a sensitive indicator of biologically available vitamin B-6 for an animal fed suboptimal levels of the vitamin, vitamin B-6 dependent enzyme activities and plasma PLP data give more specific indexes of vitamin B-6 utilization. Correlations between AspAT activity, its in vitro stimulation by PLP, and vitamin B-6 nutriture have been reported by various researchers (Raica and Sauberlich, 1964; Brin and Thiele, 1967; Bayoumi and Rosalki, 1976). Recently Lumeng et al. (1978) and Li and Lumeng (1981) suggested that activation factors of AspAT were not as reproducible and sensitive an indication of vitamin B-6 status as the level of PLP in plasma since the aminotransferases tightly bind the cofactor and the degree of enzyme unsaturation increases only in relatively severe deficiency. In this study, different degrees of vitamin B-6 deficiency were included to obtain a standard PN dose–response curve. At the level of 3.55 nmol of added B-6 vitamer/g of basal diet, the AspAT activity parameter was found to be sufficiently sensitive to detect differences in biological activity of the three B-6 vitamers. By this parameter PL and PM were found to have a biological activity equivalent to less than 50% of the activity of PN. The difference in biological activity of PL and PM relative to that of PN was more pronounced with the AspAT stimulation criterion (Table IV). As previously observed (Lumeng et al., 1978; Gregory, 1980a,c) liver PLP is not a sensitive indicator of vitamin B-6 status over the range of dietary vitamin B-6 concentration employed in the limited duration of this bioassay. Liver PLP is therefore not suitable for bioassay quantitation under these conditions.

Noguchi and Tokada (1980) reported the apparent induction of an aromatic aminotransferase in the small intestine of vitamin B-6 deficient rats, which suggested its possible use as a specific indicator of vitamin B-6 status in animal bioassays. The absence of a significant correlation between intestinal aromatic aminotransferase activity and dietary vitamin B-6 under these typical bioassay conditions precludes the use of this enzyme activity as an assay criterion.

Plasma PLP concentration has been recommended as a good indicator of vitamin B-6 status because of its sensitivity to vitamin B-6 intake and its correlation with tissue vitamin B-6 stores (Lumeng et al., 1978). By this criterion, PM, PL, and PN were found to exhibit little or no difference in apparent biological activity in the present study. This was in contrast to the marked differences in response to the vitamers as reflected by AspAT activity and in vitro stimulation values. These results strongly support the validity of plasma PLP as an indicator of vitamin B-6 status for rat bioassay quantitation. An examination of the PN response standard curve shows that plasma PLP rises dramatically above the dietary level of 4.0–4.5 nmol of added PN/g of diet for all three protein levels (Table V). Observed values for this response parameter are consistent with values previously reported for deficient rats (Sloger et al., 1978) and are much lower than values reported for rats fed diets containing higher levels of vitamin B-6 (Sloger and Reynolds, 1980). The linear dose–response relationship for plasma PLP of experiment I, in contrast to the nonlinearity observed in experiment II, suggests that minor differences in vitamin B-6 nutriture may strongly influence the rate or extent of plasma PLP synthesis at dietary vitamin B-6 levels approaching the requirement. The other routinely used bioassay criteria, erythrocyte AspAT activity and coenzyme stimulation, varied as a linear function of the logarithm of dietary vitamin B-6 level (Table III), as observed in previous studies [e.g., Gregory and Kirk (1978) and Gregory (1980c)]. In spite of the consistent linearity of such AspAT dose–response curves and the relative ease of analysis for the AspAT parameters, the markedly lower relative activity of PM and PL by the AspAT criteria is a limitation of their use for bioassay quantitation.

Results of experiment II show that rat responses to the three nonphosphorylated forms of vitamin B-6 are dependent on the protein level in the diet. For all three forms of vitamin B-6, there was a moderate decrease in rat growth related responses to vitamin B-6 in high-protein diets and a much greater decrease in responses to vitamin B-6 in low-protein diets as compared to responses of rats on normal dietary protein level, whereas the slope of plasma PLP response curves increased with increasing protein content. These results reflect the interaction between dietary protein intake and the role of B-6 coenzymes in protein metabolism. This effect has been discussed more fully in a recent paper involving the use of these data for the determination of the bioavailability of vitamin B-6 in selected foods as a function of diet composition (Nguyen and Gregory, 1983).

All response criteria indicated no significant differences in rat responses to the tested B-6 vitamers with the 5% dietary protein level. When the level of protein in the diets was increased to either 20% or as high as 50% of the total diet, growth-related parameters indicated higher responses to PN in the diet relative to PL and PM (Table V). It has been suggested in earlier work that intestinal microorganisms may preferentially utilize PL and PM in the diet, leading to decreased availability of and therefore decreased rat responses to dietary PL and PM (Waibel et al., 1952; Davies et al., 1959). Analysis of fecal vitamin B-6 indicated no differences between total vitamin B-6 in fecal samples of low- and high-protein basal diets (1.95 ± 0.12 and 1.71 ± 0.24 nmol of vitamin B-6/g of sample, respectively), regardless of dietary vitamin B-6 concentration. Fecal vitamin B-6 represents both unabsorbed dietary vitamin B-6 and endogenous vitamin B-6, the amount of which is greatly influenced by intestinal microbial activity. Unfortunately, observed fecal vitamin B-6 values do not indicate relative amounts of dietary and endogenous vitamin B-6 and thus cannot provide an insight on whether decreased rat responses to PL and PM relative to that to PN were due to a preferential utilization of dietary PL and PM by the microorganisms in the gut. To elucidate the cause of such decrease in response would require further study.

In summary, these results illustrate the need for careful selection of biochemical response criteria used for quantitation of rat bioassays for biologically available vitamin B-6. Under the conditions of these experiments, plasma PLP values exhibit a fairly uniform response to the nonphophorylated B-6 vitamers and, thus, would provide more accurate bioassay results than erythrocyte AspAT measurements.

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Registry No. Pyridoxine, 65-23-6; pyridoxal, 66-72-8; pyridoxamine, 85-87-0; vitamin B_6, 8059-24-3; pyridoxal 5′-phosphate,

54-47-7; aspartate aminotransferase, 9000-97-9; aromatic amino acid aminotransferase, 37332-38-0.

LITERATURE CITED

Bayoumi, R. A.; Rosalki, S. K. *Clin. Chem. (Winston-Salem, N.C.)* **1976**, *22*, 327–335.
Brin, M.; Thiele, V. F. *J. Nutr.* **1967**, *93*, 213–221.
Calbiochem LaJolla, CA, 1976, Document No. L03023.
Crosby, W.; Munn, J. I.; Furth, F. W. *U.S. Armed Forces Med. J.* **1954**, *5*, 693–703.
Davies, M. K.; Gregory, M. E.; Henry, K. M. *J. Dairy Res.* **1959**, *26*, 215–220.
George, H.; Turner, R.; Gabay, S. *J. Neurochem.* **1967**, *14*, 841–845.
Gregory, J. F. *J. Food Sci.* **1980a**, *45*, 84–86.
Gregory, J. F. *Anal. Biochem.* **1980b**, *102*, 374–379.
Gregory, J. F. *J. Nutr.* **1980c**, *110*, 995–1005.
Gregory, J. F. *J. Nutr.* **1982**, *112*, 1643–1647.
Gregory, J. F.; Kirk, J. R. *J. Food Sci.* **1978**, *43*, 1801–1815.
Gregory, J. F.; Manley, D. B.; Kirk, J. R. *J. Agric. Food Chem.* **1981**, *29*, 921–927.
Guilarte, T. R.; McIntyre, P. A.; Isan, M. F. *J. Nutr.* **1980**, *110*, 954–958.
Hamm, M. W.; Mehansho, H.; Henderson, L. M. *J. Nutr.* **1979**, *109*, 1552–1559.
Haskell, B. E.; Snell, E. E. *Methods Enzymol.* **1970**, *18A*, 512–519.
Li, T. L.; Lumeng, L. "Methods in Vitamin B-6 Nutrition"; Plenum Press: New York, 1981; pp 289–296.
Linkswiler, H.; Baumann, C. A.; Snell, E. E. *J. Nutr.* **1951**, *43*, 565–573.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
Lui, A.; Minter, R.; Lumeng, L.; Li, T. K. *Anal. Biochem.* **1981**, *112*, 17–22.
Lumeng, L.; Lui, A.; Li, T. K. "Methods in Vitamin B-6 Nutrition"; Plenum Press: New York, 1981; pp 57–67.
Lumeng, L.; Ryan, M. P.; Li, T. K. *J. Nutr.* **1978**, *108*, 545–553.
Matsuo, Y. *J. Am. Chem. Soc.* **1957**, *79*, 2016–2019.
Mehansho, H.; Hamm, M. W.; Henderson, L. M. *J. Nutr.* **1979**, *109*, 1542–1551.
Metzler, D. E.; Ikawa, M.; Snell, E. E. *J. Am. Chem. Soc.* **1954**, *76*, 648–652.
Nguyen, L. B.; Gregory, J. F. *J. Nutr.* **1983**, in press.
Nguyen, L. B.; Gregory, J. F.; Damron, B. L. *J. Nutr.* **1981**, *111*, 1403–1420.
Noguchi, T.; Okuno, E.; Minatogawa, Y.; Kido, R. *Biochem. J.* **1976**, *155*, 107–115.
Noguchi, T.; Tokada, T. J. *Biol. Chem.* **1980**, *255*, 9844–9547.
Neter, J.; Wasserman, W. "Applied Linear Statistical Models"; Richard D. Irwin, Inc.: Homewood, IL, 1974.
Polansky, M. M. *J. Am. Diet. Assoc.* **1969**, *54*, 118–121.
Polansky, M. M.; Murphy, E. W.; Toepfer, E. W. *J. Assoc. Off. Agric. Chem.* **1964**, *47*, 750–753.
Raica, N.; Sauberlich, H. E. *Am. J. Clin. Nutr.* **1964**, *15*, 67–72.
Sarma, P. S.; Snell, E. E.; Elvehjem, C. A. *J. Biol. Chem.* **1946**, *165*, 55–63.
Skala, J. H.; Waring, P. P.; Lyons, M. F.; Rusnak, M. G.; Alletto, J. S. "Methods in Vitamin B-6 Nutrition"; Plenum Press: New York, 1981; pp 171–202.
Sloger, M. S.; Reynolds, R. D. *J. Nutr.* **1980**, *110*, 1517–1524.
Sloger, M. S.; Scholfield, L. G.; Reynolds, R. D. *J. Nutr.* **1978**, *108*, 1355–1360.
Snell, E. E.; Haskell, B. E. *Compr. Biochem.* **1971**, *21*, 47–71.
Tarr, J. B.; Tamura, T.; Stokstad, L. R. *Am. J. Clin. Nutr.* **1981**, *34*, 1328–1337.
Thiele, V. F.; Brin, M. *J. Nutr.* **1968**, *94*, 237–242.
Toepfer, E. W.; Lehmann, J. *J. Assoc. Off. Agric. Chem.* **1961**, *44*, 426–430.
Vanderslice, J. T.; Maire, C. E.; Doherty, R. F.; Beecher, G. R. *J. Agric. Food Chem.* **1980**, *28*, 1145–1149.
Waibel, P. E.; Cravens, W. W.; Snell, E. E. *J. Nutr.* **1952**, *48*, 531–538.
Yen, J. T.; Jensen, A. H.; Baker, D. H. *J. Anim. Sci.* **1976**, *42*, 866–870.

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Isolation and Identification of Volatile Compounds from Fried Chicken

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Volatile flavor compounds were isolated from 150 lb of fried chicken by a specially designed apparatus. The isolated volatile flavor compounds were subjected to extensive gas chromatographic fractionation, and the pure fractions obtained were identified by gas chromatography–mass spectrometry. A total of 130 compounds were identified. The compounds identified in the volatiles of fried chicken included hydrocarbons, alcohols, aldehydes, ketones, acids, esters, pyrazines, pyridines, thiazoles, thiazolines, oxazoles, oxazolines, thiophenes, pyrroles, furans, a trithiolane, a trithiane, and thialdine.

Wilson and Katz (1972) reviewed the literature on chicken flavor and listed 178 compounds that had been reported in the volatiles from cooked chicken. Using GC–MS, Wilson and Katz (1972) identified 47 compounds in the volatiles of stewing chicken. The majority of these were saturated and unsaturated alcohols, aldehydes, and ketones. Janney et al. (1974) identified additional compounds, mainly aldehydes, ketones, and hydrocarbons, in freshly cooked fried chicken. In the analysis of boiling chicken broth, Horvat (1976) identified 53 compounds as its volatile constituents. A most recent review by Ramaswamy and Richards (1982) revealed that more than 250 compounds have been identified in the volatiles of poultry meat.

The present paper reports the isolation and systematic characterization of the volatile flavor constituents of fried chicken.

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EXPERIMENTAL SECTION

Isolation of the Volatile Compounds from Fried Chicken. Fresh chicken breasts, with bones, purchased

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