Changes in vitamin B-6 status indicators of women fed a constant protein diet with varying levels of vitamin B-6¹⁻⁵

Christine M Hansen, James E Leklem, and Lorraine T Miller

ABSTRACT  Changes in vitamin B-6 status indicators were evaluated in vitamin B-6-replete subjects. Ten young women consumed diets providing 85 g protein/d and 1.03, 1.33, 1.73, and 2.39 mg vitamin B-6/d for 12 or 15 d during four successive diet periods; in a second study, six women were fed diets providing 85 g protein/d and 0.84, 1.14, and 2.34 mg vitamin B-6/d for 10 or 12 d during three successive diet periods. Vitamin B-6 status indicators showing significant differences among intakes included urinary excretion of 4-pyridoxic acid and total vitamin B-6, pyridoxal 5'-phosphate and total vitamin B-6 in plasma, and xanthurenic acid excretion after a 2-g L-tryptophan load. Significant correlations were found between vitamin B-6 intake and 4-pyridoxic acid, total vitamin B-6, plasma pyridoxal 5'-phosphate, plasma total vitamin B-6, erythrocyte alanine aminotransferase percentage stimulation and postload excretion of xanthurenic acid and volatile amines (kynurenine plus acetylkynurenine). Depending on the indicator, between 20% and 70% of the subjects had inadequate values for 4-pyridoxic acid, total vitamin B-6, plasma pyridoxal 5'-phosphate, and erythrocyte alanine aminotransferase percentage stimulation at a vitamin B-6 intake of 1.33 mg/d (0.016 mg vitamin B-6/g protein). A ratio of dietary vitamin B-6 to protein > 0.016 mg/g is required for adequate vitamin B-6 status in women.  Am J Clin Nutr 1997;66:1379–87.

KEY WORDS  Vitamin B-6, pyridoxal 5'-phosphate, 4-pyridoxic acid, tryptophan load test, aminotransferases, feeding studies, requirements, women

INTRODUCTION

The present recommended dietary allowance (RDA) of vitamin B-6 for women of 1.6 mg/d is based on a dietary ratio of 0.016 mg vitamin B-6 to 1 g protein (1). The research on which this recommendation is based (1, 2) was done primarily in men (3–5). The studies done in women focused on oral contraceptive users (6–9), but also included normal control women. Other than these types of investigations, the few studies that have been done in healthy women (not pregnant or using oral contraceptives) were depletion-repletion studies, which used a return to predepletion values to determine when vitamin B-6 status had normalized (10–12). Also, the ratios of dietary vitamin B-6 to protein used in these studies varied over a wide range.

According to data from the second National Health and Nutrition Examination Survey, 40% of black females and 33% of white females consumed < 0.015 mg vitamin B-6/g protein, suggesting women may be at risk for inadequate vitamin B-6 status (13). Data from the third National Health and Nutrition Examination Survey show that half of all females have a vitamin B-6 intake that is < 82% of the RDA (1.31 mg/d), and half of black females have an intake that is < 73% of the RDA (1.18 mg/d) (14). Thus, there is a need to investigate the vitamin B-6 status of women consuming a ratio of vitamin B-6 to protein less than that used to set the RDA for vitamin B-6.

To assess accurately the requirement for vitamin B-6 in normal adult women, the values of several vitamin B-6 status indicators at varying vitamin B-6 intakes and ratios of vitamin B-6 to protein should be determined (15). The objective of the two studies described here was to evaluate the extent of changes in vitamin B-6 status indicators in women fed a controlled diet of constant protein and varied amounts of vitamin B-6 over a normal range of intakes by using several direct and indirect measures of vitamin B-6 status. An additional objective was to examine the interrelationships among the indexes of vitamin B-6 status under controlled metabolic conditions.

SUBJECTS AND METHODS

Subjects

The subjects, 10 in study 1 and six in study 2, were healthy women, between the ages of 21 and 39 y, who were nonsmokers and not using oral contraceptives. They were recruited from the local community. Subjects were screened for history of intestinal, renal, or metabolic disorders that could affect absorption, metabolism, or excretion of vitamin B-6 (16) and for previous vitamin supplementation. None of the subjects was taking vitamin B-6 supplements regularly for 3 mo before their participation in the studies. Three subjects who reported occasional multivitamin use had not taken any supplements for ≥ 3 mo.

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³ Paper no. 10 590 from Oregon Agricultural Experiment Station.
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Received June 16, 1997. Accepted for publication June 27, 1997.

wk before the beginning of the studies. Normal results from blood chemistry tests—including hematocrit, hemoglobin, glucose, protein, and lipids—and from xylose absorption and liver function tests (17) were required for selection. Subjects were instructed to maintain their usual activity level throughout the study. The study protocol was approved by Oregon State University’s Committee for the Protection of Human Subjects and informed consent was obtained from each subject.

**Experimental design**

In study 1, the first experimental period was 15 d long to allow for adaptation to the diet and was followed by three successive 12-d experimental periods. Because some of the blood samples from this study were lost because of a freezer failure before the plasma total vitamin B-6 and red blood cell (RBC) pyridoxal 5'-phosphate (PLP) analyses were completed, a second study was done. In study 2, there were three successive experimental periods, 12, 10, and 10 d long, respectively. We used periods of this duration because previous research in our laboratory showed that several indexes of vitamin B-6 status plateau by 7–8 d at vitamin B-6 intakes of 1.6–2.3 mg/d (18–21).

The basal diet used in both studies (Table 1) was prepared and served in our metabolic kitchen and provided ~8368 kJ (2000 kcal). Additional energy was supplied to the subjects by hard candy, soft drinks, sugar, margarine, and salad dressing to maintain the subjects’ body weights. In study 1, the basal diet fed throughout the four experimental periods provided 1.03 mg (6.09 μmol) vitamin B-6 and 85 g crude protein (g N × 6.25) per day. Vitamin B-6 intake was increased in periods 2, 3, and 4 by oral administration of an equally divided dose of pyridoxine hydrochloride solution at breakfast and dinner to provide a total of 1.33, 1.73, and 2.39 mg (7.87, 10.24, and 14.14 μmol) vitamin B-6 per day, respectively. Ratios of vitamin B-6 to protein for the four successive periods were 0.012, 0.016, 0.020, and 0.028 mg/g.

The basal diet in study 2 was the same as in study 1, except that a toasted-oat breakfast cereal (Quaker Oats, Chicago) was substituted for shredded wheat (Kraft Foods, White Plains, NY) at breakfast. On the basis of microbiological analysis in our laboratory, 30 g shredded wheat provides 0.07 mg vitamin B-6 and toasted-oat cereal, 0.08 mg vitamin B-6. The basal diet in study 2 provided 85 g crude protein/d and 0.84 mg (4.96 μmol) vitamin B-6/d. The difference in vitamin B-6 content of the diet between study 1 and study 2 was due to a difference in the analyzed vitamin B-6 content of the animal products (determined after the feeding part of each study). In the second and third periods an oral pyridoxine hydrochloride solution increased the intake to 1.14 and 2.34 mg (6.74 and 13.83 μmol) vitamin B-6/d, respectively. This resulted in ratios of vitamin B-6 to protein of 0.010, 0.013, and 0.028 for the three experimental periods.

In study 1, an oral 2-g L-tryptophan load (four tablets of 500 mg each) was administered with breakfast on day 15 of period 1, day 12 of periods 2 and 3, and day 11 of period 4. We determined that the tablets contained an average of 500 ± 10 mg L-tryptophan as follows: two tablets from each of four different bottles of L-tryptophan tablets (Bi-Mart, Eugene, OR) were dissolved in 0.1 mol HC1/L and the ultraviolet spectra was compared with a spectrum obtained from pure L-tryptophan (Sigma Chemical, St Louis). Because of the reported cases of eosinophilia in people taking tryptophan (22) and the subsequent recall of tryptophan from retail stores, the tryptophan load test was not done in the second study.

**Sample collection and analyses**

Composites of the daily diet were made weekly during both studies and analyzed for vitamin B-6 with *Saccharomyces uvarum* by using a procedure of the Association of Official Analytical Chemists (23) (omitting the chromatographic step) and for total nitrogen by a boric acid modification of the Kjeldahl method (24). The diet composite in study 2 was also analyzed for pyridoxine glucoside (PNG) content by the method of Kabir et al (25). PNG was measured to provide data that could be used to compare the effect of PNG on vitamin B-6 status indicators (plasma PLP and urinary vitamin B-6) obtained in prior and future studies.

For both studies, 24-h urine collections from each day were assessed for completeness and evidence of adherence to the diet by measurement of creatinine (26) and urea nitrogen excretion (27) by automated procedures (Technicon Autoanalyzer; Technicon, Tarrytown, NY). Urinary 4-pyridoxic acid (4-PA) excretion was determined by an HPLC method (28); recovery of added crystalline 4-PA averaged 91 ± 6%.

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**Table 1. Composition of basal diet for vitamin B-6 studies**

<table>
<thead>
<tr>
<th>Food item</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td>Orange juice, frozen, reconstituted (g)</td>
</tr>
<tr>
<td></td>
<td>Shredded wheat cereal (g)</td>
</tr>
<tr>
<td></td>
<td>Blueberries, frozen (g)</td>
</tr>
<tr>
<td></td>
<td>Milk, 2% fat (g)</td>
</tr>
<tr>
<td></td>
<td>Biscuit (portion of recipe)</td>
</tr>
<tr>
<td>Lunch</td>
<td>Gelatin drink (mL)</td>
</tr>
<tr>
<td></td>
<td>Cheddar cheese (g)</td>
</tr>
<tr>
<td></td>
<td>Lettuce, iceberg (g)</td>
</tr>
<tr>
<td></td>
<td>Carrot, raw (g)</td>
</tr>
<tr>
<td></td>
<td>Red cabbage, raw (g)</td>
</tr>
<tr>
<td></td>
<td>Celery, raw (g)</td>
</tr>
<tr>
<td></td>
<td>French dressing (g)</td>
</tr>
<tr>
<td></td>
<td>Pears, canned, light syrup (g)</td>
</tr>
<tr>
<td></td>
<td>Apple, raw (g)</td>
</tr>
<tr>
<td></td>
<td>Biscuit (portion of recipe)</td>
</tr>
<tr>
<td>Dinner</td>
<td>Turkey, precooked frozen (g)</td>
</tr>
<tr>
<td></td>
<td>Rice, white, uncooked weight (g)</td>
</tr>
<tr>
<td></td>
<td>Green beans, canned (g)</td>
</tr>
<tr>
<td></td>
<td>Cottage cheese (g)</td>
</tr>
<tr>
<td></td>
<td>Peaches, canned, light syrup (g)</td>
</tr>
<tr>
<td></td>
<td>Milk, 2% fat (g)</td>
</tr>
<tr>
<td></td>
<td>Biscuit (portion of recipe)</td>
</tr>
<tr>
<td>Snack</td>
<td>Graham crackers</td>
</tr>
</tbody>
</table>

*1 The diet for study 2 substituted a toasted-oat cereal (Quaker Oats, Chicago) for shredded wheat (Kraft Foods, White Plains, NY). *

*2 Biscuit recipe: 75 g flour, 20 g corn oil, 10 g sugar, 15 g vitamin-free casein, 5 g baking powder, 2 g salt, and 80 mL water; divided into three portions and baked at 350 °F (177 °C) for 10 min.

*3 14 g gelatin plus 240 mL prepared drink mix.

*4 Rice was combined with 120 mL water and baked in a covered glass casserole at 350 °F (177 °C) for 25 min.*
urinary vitamin B-6 was assayed by a microbiological procedure (29) using *S. uvarum*. If the 24-h collections were judged complete on the basis of creatinine excretion, urinary 4-PA and urinary vitamin B-6 excretion were averaged over the last 3 d of each period before doing the statistical analyses. Urine samples from the day before and the day of the tryptophan load were analyzed for xanthurenic acid (XA) and kynurenic acid (KA) by the method of Price et al (30), and for volatile amines (VA) (kynurenine plus acetylkynurenine) (31). The VA assay was performed by Raymond R Brown, University of Wisconsin, Madison. Measurement of KA and VA provide a more complete picture of tryptophan metabolism than measurement of XA alone. Recoveries of XA and KA were 96 ± 5%. For the VA assay the recoveries were 95 ± 6%.

In study 1, fasting blood was drawn on days 1, 4, and 8 of each period; on day 12 of period 1; and on the morning after day 12 of period 4. In study 2, fasting blood was drawn on days 1 and 5 of each period, day 9 of period 1, and on the morning after the final day of period 3. After whole blood was removed for determination of hematocrit and hemoglobin concentration, samples were centrifuged at 1700 × g at 4°C for 15 min. Plasma and RBCs were frozen at −30°C until analyzed.

Plasma and RBC PLP concentrations were determined by a tyrosine decarboxylase apoenzyme/isotopic procedure (32). Samples were assayed in duplicate and any duplicates that varied > 5% from their mean were repeated. Interassay CVs of control samples were 5.7% and 2.3% for the plasma PLP assays in study 1 and 2, respectively, and 8.0% for the RBC PLP assay in study 2. Recovery of PLP added to plasma ranged from 82% to 109% and that added to RBCs was 54–74%. Values were not corrected for recovery.

Plasma alkaline phosphatase activity was determined by a colorimetric procedure (33). Erythrocyte aspartic aminotransferase (AST) activity and erythrocyte alanine aminotransferase (ALT) activity, with and without PLP added in vitro, were determined by the method of Woodring and Storvick (34), using a 0.033 mol-Tris/L buffer (pH 7.4) instead of 0.1 mol KPO4/L. Percentage stimulation was calculated [(activity with added PLP − activity without added PLP) + activity without added PLP]. Plasma total vitamin B-6 was determined by a microbiological procedure (29) using *S. uvarum*. For all blood vitamin B-6 status indicators, the value for the final sample in each period was used in the statistical analysis. The CV for the urinary vitamin B-6 method was 4%.

Near the end of each experimental period in study 1, subjects were given a fecal marker (50 mg FD&C Blue no. 1 mixed with 200 mg methylcellulose in a gelatin capsule) with breakfast on the first and last day of a 5-d period. Complete 5-d fecal collections were analyzed for total vitamin B-6 by a microbiological procedure (29).

Means and individual values for several of the status indicators measured were compared with values suggested for adequate vitamin B-6 status (15, 35). Statistical analyses were done with the SAS computer program (SAS Institute, Cary, NC). Analysis of variance (ANOVA) for repeated measures was used to compare the means of status indicators at the end of the experimental periods, and Scheffé post hoc test analyses were performed to determine statistical differences between the respective periods. Differences were considered statistically significant if *P* < 0.05. Simple-regression analysis of vitamin B-6 intake and the various status indicators using linear, multiplicative, exponential and reciprocal models was performed, and Pearson correlation coefficients were determined by using STATGRAPHICS (Statistical Graphics Corporation, Rockville, MD).

RESULTS

Characteristics of the subjects, including weight at the beginning and end of each study, are given in Table 2. Mean urinary excretion of creatinine and urea nitrogen, hematocrit and hemoglobin concentration did not differ among experimental periods in either study and are not reported here.

The PNG content of the diet in study 2 was 0.145 mg or 6–17% of the total vitamin B-6 intake. The PNG content of the diet in study 1 was not measured, but the only difference from the diet in study 2 was the cereal eaten at breakfast (Table 1). Based on the PNG content of shredded wheat (36) and assuming negligible PNG in toasted-oat cereal the estimated PNG content of the diet in study 1 was 0.140 mg or 6–14% of the total vitamin B-6 intake.

Direct measures of vitamin B-6 status

The mean urinary 4-PA excretion over the last 3 d of each period in study 1 and study 2 is shown in Table 3. In both studies there were significant increases (*P* < 0.05) in the means at the end of each successive experimental period. The suggested value for urinary 4-PA excretion indicating adequate status is > 3.0 μmol/d (15, 35) and the means exceeded this value at the end of all four periods of study 1 and the final two periods of study 2. However, in study 1, 3 of the 10 subjects had values < 3.0 μmol/d with a vitamin B-6 intake of 1.03 mg/d and one subject was still excreting less than adequate amounts with an intake of 1.33 mg vitamin B-6/d (Table 4). In study 2, one subject had a 4-PA excretion of < 3.0 μmol/d with a vitamin B-6 intake of 1.14 mg/d (Table 4). Mean urinary 4-PA excretion represented 60%, 52%, 54%, and 69% of total vitamin B-6 intake in the four respective periods of study 1 and 58%, 50%, and 57% of intake, respectively, in the three periods of study 2.

Mean total urinary vitamin B-6 over the last 3 d of each period was significantly higher in each successive period in study 1 (*P* < 0.05). In study 2, the mean at the end of the second and third periods were significantly higher (*P* < 0.05) than that at the end of the previous period (Table 3). The suggested value for adequate status is > 0.5 μmol/d (13, 34), and the means exceeded this value at the end of all four periods of study 1 and at the end of the final two periods in study 2. In both study 1 and study 2, however, two subjects still had values

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Height (cm)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>Beginning</td>
</tr>
<tr>
<td>End</td>
</tr>
</tbody>
</table>

*7 ± SD.*
Erythrocyte and urine excretion of vitamin B-6 was different from the mean intake, aspartic acid; vitamin B-6, VA, showed significantly increased excretion, with the mean intake of 2.34 mg/d (Table 4).

In study 2, the mean plasma total vitamin B-6 concentration at the end of period 4 was 1.14 mg/d, which was significantly lower than the mean intake of 1.33 mg/d, even though no subjects still had less than adequate plasma PLP concentrations when the vitamin B-6 intake was 1.14 mg/d (Table 4).

Although there was a trend for RBC PLP concentration to increase as vitamin B-6 intake increased, no significant differences were found in mean RBC PLP concentrations among the three periods of study 2 (Table 3). Suggested values for adequate status have not been established for RBC PLP. Fecal vitamin B-6 excretion was not significantly different among the four experimental periods in study 1 (Table 3) and was not measured in study 2.

**TABLE 4** Percentage of subjects not reaching indicator values (in parentheses) suggested by Leklem (15) for adequate status at each intake:

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Study 1 vitamin B-6 intake (n = 10)</th>
<th>Study 2 vitamin B-6 intake (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period 1 1.03 mg</td>
<td>Period 2 1.33 mg</td>
</tr>
<tr>
<td>Urine 4-PA (&gt; 30 μmol/L/d)</td>
<td>30% 10% 0% 0% 0%</td>
<td>50% 17% 0%</td>
</tr>
<tr>
<td>Urine vitamin B-6 (&gt; 0.5 μmol/L/d)</td>
<td>20% 20% 0% 0% 0%</td>
<td>67% 33% 0%</td>
</tr>
<tr>
<td>Plasma PLP (&gt; 30 nmol/L)</td>
<td>70% 40% 30% 0% 0%</td>
<td>67% 67% 0%</td>
</tr>
<tr>
<td>Plasma TB-6 (&gt; 40 nmol/L)</td>
<td>67% 33% 0%</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte ALT stimulation (&lt; 25%)</td>
<td>0% 0% 0%</td>
<td>0% 0% 0%</td>
</tr>
<tr>
<td>Erythrocyte AST stimulation (&lt; 80%)</td>
<td>0% 0%</td>
<td>0% 0% 0%</td>
</tr>
<tr>
<td>VA (&lt; 65 μmol/d)</td>
<td>30% 0% 0% 0% 0%</td>
<td></td>
</tr>
</tbody>
</table>

1 All subjects received 85 g crude protein per day during both studies. To convert milligrams vitamin B-6 to micromoles, multiply by 5.9. ALT, alanine aminotransferase; AST, aspartic aminotransferase; PLP, pyridoxal 5'-phosphate; TB-6, total vitamin B-6; 4-PA, 4-pyridoxic acid; VA, xanthurenic acid excretion after an oral 2-g L-tryptophan load.

**TABLE 3** Mean values of vitamin B-6 status indicators at the end of each experimental period of study 1 and study 2:

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Study 1 vitamin B-6 intake (n = 10)</th>
<th>Study 2 vitamin B-6 intake (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period 1: 1.03 mg</td>
<td>Period 2: 1.33 mg</td>
</tr>
<tr>
<td>Erythrocyte ALT stimulation (%)</td>
<td>26 ± 8b 34 ± 13b 17 ± 4a 17 ± 9a</td>
<td>13 ± 8 15 ± 3</td>
</tr>
<tr>
<td>Erythrocyte AST stimulation (%)</td>
<td>37 ± 11 39 ± 11 41 ± 15 39 ± 7</td>
<td>52 ± 6 53 ± 7</td>
</tr>
<tr>
<td>Fecal vitamin B-6 (&lt; 15 mg)</td>
<td>10.2 ± 2.9 11.4 ± 2.8 11.4 ± 3.0 13.0 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>KA (&lt; μmol/d)</td>
<td>102.1 ± 36.1 86.8 ± 32.3 80.1 ± 26.6 72.9 ± 36.3</td>
<td></td>
</tr>
<tr>
<td>Plasma PLP (&lt; 30 nmol/L)</td>
<td>23.2 ± 5.5a 20.5 ± 4.3a 25.3 ± 4.1a 28.4 ± 5.1a</td>
<td></td>
</tr>
<tr>
<td>RBC PLP (&lt; 300 nmol/L)</td>
<td>40.2 ± 9.8 45.5 ± 10.1 46.2 ± 14.0</td>
<td></td>
</tr>
<tr>
<td>Plasma B-6 (&lt; 15 mg)</td>
<td>440.0 ± 12.6a 451.0 ± 14.6a 452.0 ± 15.6a 453.0 ± 15.6a</td>
<td></td>
</tr>
<tr>
<td>Plasma TB-6 (&lt; 15 mg)</td>
<td>52.6 ± 13.4a 43.9 ± 10.2a 67.6 ± 17.9b</td>
<td></td>
</tr>
<tr>
<td>Period</td>
<td>0.84 mg 1.14 mg 2.34 mg</td>
<td></td>
</tr>
</tbody>
</table>

1 ± SD. All subjects received 85 g crude protein per day during both studies. To convert milligrams vitamin B-6 to micromoles, multiply by 5.9. ALT, alanine aminotransferase; AST, aspartic aminotransferase; PLP, pyridoxal 5'-phosphate; TB-6, total vitamin B-6; 4-PA, 4-pyridoxic acid; VA, xanthurenic acid excretion after an oral 2-g L-tryptophan load.
Indirect measures of vitamin B-6 status

There were no significant differences in mean percentage stimulation of erythrocyte AST activity at the end of the four periods of study 1, but the mean percentage stimulation in erythrocyte ALT activity was significantly lower at the end of periods 3 and 4 than at the end of period 2 (Table 3). In study 2, mean erythrocyte AST and erythrocyte ALT percentage stimulation did not differ significantly between the beginning of the first period and the end of the final period (Table 3). The suggested value for erythrocyte ALT percentage stimulation indicating adequate vitamin B-6 status is < 25% (15), which was achieved by the end of period 3 (1.73 mg vitamin B-6/d) in study 1. For erythrocyte AST, the suggested value is < 80% (15) and all subjects in all periods of study 1 had % stimulation values > 80%. In study 2, all subjects had acceptable values for erythrocyte AST and erythrocyte ALT percentage stimulation at the beginning of period 1 and at the end of the study.

There were no significant differences in KA, XA or VA excretion on the days before the tryptophan load among all four periods in study 1. The day before the tryptophan load, urinary KA excretion averaged 13.0 ± 1.9 μmol/d, VA excretion averaged 12.3 ± 3.8 μmol/d, and VA excretion averaged 4.4 ± 1.2 μmol/d. Table 3 shows the mean urinary excretion of tryptophan metabolites after an oral 2-g L-tryptophan load in the four experimental periods of study 1. KA excretion was significantly higher after the tryptophan load in period 1 than in the subsequent three periods. Excretion of KA and VA showed a downward trend as vitamin B-6 intake increased. For KA excretion, the variation among subjects was so high that the differences between periods were not significant. The mean VA excretion was significantly lower for periods 2, 3 and 4 compared with that of period 1, but there were no significant differences between the mean values for the final three periods. For adequate status, the suggested value for KA excretion after a 2-g tryptophan load is < 65 μmol/d (15); three subjects had posttryptophan excretions higher than this when consuming a vitamin B-6 intake of 1.03 mg/d (Table 4), although the means in all four periods were less than the suggested value.

One subject’s tryptophan metabolites data were excluded from the statistical analysis because after the experimental periods ended, she was found to have an infection. Her postload tryptophan metabolite excretion was several-fold higher than the means of the other subjects, and the infection may have contributed to this increased excretion (37). Her vitamin B-6 data were included in the statistical analysis because her other vitamin B-6 status indexes responded to changes in vitamin B-6 intake similarly to the other subjects, and her blood chemistry values did not change from the beginning to the end of the study.

Plasma alkaline phosphatase activity has been found to have an effect on plasma PLP concentrations (38), although it is not a true indirect measure of vitamin B-6 status. Mean plasma alkaline phosphatase activity in our subjects was 25.5 ± 4.9 μmol min⁻¹ L⁻¹ (range: 15.4–35.7 μmol min⁻¹ L⁻¹) in study 1 and 24.2 ± 6.1 μmol min⁻¹ L⁻¹ (range: 18.2–37.7 μmol min⁻¹ L⁻¹) in study 2 and did not differ significantly among the four periods of study 1 or the three periods of study 2. Although no significant correlation was found between alkaline phosphatase activity and plasma PLP concentrations at any vitamin B-6 intake, a significant positive correlation was found between plasma alkaline phosphatase activity and RBC PLP concentrations in periods 2 and 3 of study 2 (r = 0.938, 0.817, respectively; P < 0.05).

Correlations among vitamin B-6 status indicators

Table 5 lists the correlation coefficients between vitamin B-6 intake and the various vitamin B-6 status indicators, calculated by using the combined data from both studies. The

TABLE 5
Correlation coefficients (r) among vitamin B-6 status indicators for the two studies combined

<table>
<thead>
<tr>
<th></th>
<th>Urine 4-PA (n = 16)</th>
<th>Urine B-6 (n = 16)</th>
<th>Plasma TB-6 (n = 6)</th>
<th>Plasma PLP (n = 6)</th>
<th>RBC PLP (n = 6)</th>
<th>VA (n = 9)</th>
<th>KA (n = 9)</th>
<th>Erythrocyte AST stimulation (n = 10)</th>
<th>Erythrocyte ALT stimulation (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B-6 intake</td>
<td>0.946 ± 0.056</td>
<td>0.858 ± 0.077</td>
<td>0.747 ± 0.048</td>
<td>0.643 ± 0.075</td>
<td>0.224 ± 0.451</td>
<td>0.351 ± 0.06</td>
<td>0.583 ± 0.06</td>
<td>0.326 ± 0.06</td>
<td>0.069 ± 0.06</td>
</tr>
<tr>
<td>Urine 4-PA</td>
<td>0.875 ± 0.077</td>
<td>0.730 ± 0.048</td>
<td>0.540 ± 0.075</td>
<td>-0.338 ± 0.058</td>
<td>0.526 ± 0.06</td>
<td>0.453 ± 0.05</td>
<td>-0.205 ± 0.05</td>
<td>0.015 ± 0.05</td>
<td>0.023 ± 0.05</td>
</tr>
<tr>
<td>Urine vitamin B-6</td>
<td>0.676 ± 0.094</td>
<td>0.587 ± 0.075</td>
<td>0.400 ± 0.077</td>
<td>-0.168 ± 0.047</td>
<td>0.634 ± 0.06</td>
<td>-0.431 ± 0.05</td>
<td>0.318 ± 0.05</td>
<td>-0.094 ± 0.05</td>
<td>-0.336 ± 0.05</td>
</tr>
<tr>
<td>Plasma TB-6</td>
<td>0.271 ± 0.097</td>
<td>0.562 ± 0.088</td>
<td>0.172 ± 0.075</td>
<td>-0.702 ± 0.045</td>
<td>-0.348 ± 0.06</td>
<td>-0.278 ± 0.06</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Plasma PLP</td>
<td>0.289 ± 0.076</td>
<td>0.768 ± 0.055</td>
<td>-0.223 ± 0.05</td>
<td>0.308 ± 0.04</td>
<td>0.107 ± 0.04</td>
<td>0.308 ± 0.04</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VA</td>
<td>0.302 ± 0.078</td>
<td>-0.091 ± 0.05</td>
<td>-0.327 ± 0.04</td>
<td>0.234 ± 0.04</td>
<td>0.308 ± 0.04</td>
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<tr>
<td>XA</td>
<td>0.302 ± 0.078</td>
<td>-0.091 ± 0.05</td>
<td>-0.327 ± 0.04</td>
<td>0.234 ± 0.04</td>
<td>0.308 ± 0.04</td>
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</tr>
<tr>
<td>KA</td>
<td>0.302 ± 0.078</td>
<td>-0.091 ± 0.05</td>
<td>-0.327 ± 0.04</td>
<td>0.234 ± 0.04</td>
<td>0.308 ± 0.04</td>
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<tr>
<td>Erythrocyte AST</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.308 ± 0.04</td>
<td>0.107 ± 0.04</td>
<td>0.308 ± 0.04</td>
</tr>
</tbody>
</table>

1 Excluding one subject’s volatile amines (VA), kynurenic acid (KA), and xanthurenic acid (XA) values (see text). AST, aspartic aminotransferase; ALT, alanine aminotransferase; KA, kynurenic acid excretion after a 2-g L-tryptophan load; PLP, pyridoxal 5'-phosphate; TB-6, total vitamin B-6; 4-PA, 4-pyridoxic acid; UB-6, urinary total vitamin B-6; VA, volatile amines; XA, xanthurenic acid after an oral 2-g L-tryptophan load; RBC, red blood cell.
2 P < 0.0001.
3 Linear model: y = a + bx.
4 P < 0.001.
5 Exponential model: ln y = a + bx.
6 P < 0.05.
7 Multiplicative model: y = ax^b.
8 Reciprocal model: 1/y = a + bx.
9 P < 0.01.
model (linear, multiplicative, reciprocal, or exponential) used for each indicator was the one for which the correlation coefficient was highest. All of the status indicators except RBC PLP, KA excretion and erythrocyte AST percentage stimulation were significantly correlated with vitamin B-6 intake. A majority of the significant correlations with vitamin B-6 intake fit a linear model; however, the correlation of vitamin B-6 intake with plasma PLP was most significant when using an exponential model. Plasma PLP, plasma total vitamin B-6 and urinary 4-PA were the three indexes that were most consistently significantly correlated with other indexes. In contrast, RBC PLP was not significantly correlated with any of the variables; however, there were only six subjects in which RBC PLP was measured. Of the two amino transferases, erythrocyte ALT percentage stimulation was the one that most consistently correlated significantly with other indexes of vitamin B-6 status. Of the three urinary tryptophan metabolites measured, excretion of 4-PA (kynurenine plus acetyl kynurenine) was most consistently significantly correlated with the other indexes measured.

DISCUSSION

Our purpose was to evaluate the changes in several direct and indirect vitamin B-6 status indicators in women given a constant protein intake and vitamin B-6 intakes varying over the normal range found in this study population. Data from the Nationwide Food Consumption Survey conducted in 1985 show that average vitamin B-6 intakes for the majority of women aged 19–50 y, excluding those below the 10th percentile and those above the 90th percentile of intake, range from 0.62 to 1.81 mg/d (39). The studies reported here provide extensive data on vitamin B-6 status indicator values in women fed controlled diets at several intakes within the normal range of vitamin B-6 intakes and ratios of dietary vitamin B-6 to protein.

The PNG content of the diet was within the range of intakes reported in other studies (40, 41) and is reported here for completeness and comparison with prior and future studies. The amount of PNG in the diet can affect excretion of 4-PA and total vitamin B-6 (42, 43).

Direct measures of vitamin B-6 status

Because 4-PA is the major end product of vitamin B-6 metabolism, urinary 4-PA excretion has often been used as a direct indicator of vitamin B-6 status and is considered to be indicative of recent vitamin B-6 intake (15). In three previous studies in vitamin B-6-depleted young women (7, 10, 12), it took a vitamin B-6 intake of 1.5–1.8 mg/d (0.015–0.026 mg vitamin B-6/g protein) to restore 4-PA excretion to predepletion values. In a study of elderly men and women (44), it took 1.90–1.95 mg vitamin B-6/d to bring 4-PA excretion back to predepletion values in women consuming 78 or 54 g protein/d (0.024–0.036 mg vitamin B-6/g protein).

In the studies reported here, intakes of 1.33 mg vitamin B-6/d (0.016 mg vitamin B-6/g protein) in study 1 and 1.14 mg vitamin B-6/d (0.013 mg vitamin B-6/g protein) in study 2 were sufficient to raise urinary 4-PA excretion above adequate levels in all but one subject in each study. Urinary 4-PA excretion reached values indicative of adequate status in all subjects at a vitamin B-6 intake of 1.73 mg/d (0.020 mg vitamin B-6/g protein).

Urinary total vitamin B-6 is also considered a short-term indicator of vitamin B-6 status. In a depletion-repletion study by Donald et al (10), urinary total vitamin B-6 excretion nearly reached predepletion levels after 3 d of an intake of 1.54 mg (0.026 mg vitamin B-6/g protein), but in a recent study by Kretsch et al (12), even 2.0 mg vitamin B-6/d (0.020 mg vitamin B-6/g protein) was not sufficient to restore baseline urinary total vitamin B-6 in the vitamin B-6-depleted subjects.

In our studies, a vitamin B-6 intake of 1.03 mg/d (0.012 mg vitamin B-6/g protein) was sufficient to raise the mean urinary vitamin B-6 excretion above 0.50 μmol/d, but even at an intake of 1.33 mg/d (0.016 mg vitamin B-6/g protein), 2 of the 10 subjects still were excreting less than adequate amounts. At an intake of 1.73 mg/d (0.020 mg vitamin B-6/g protein), all the subjects excreted urinary vitamin B-6 in excess of the value indicative of adequate status. The difference between our results and those of Kretsch et al (12) may be because their subjects were depleted of vitamin B-6, or because their baseline value was unusually high (0.92 μmol/d).

Although the use of plasma PLP concentration as a status indicator has been questioned (45), plasma PLP is probably the most frequently used direct indicator of vitamin B-6 status (15). In two previous studies (7, 12), plasma PLP concentrations were restored to their predepletion values with vitamin B-6 intakes of 1.0–1.84 mg/d (0.010–0.023 mg vitamin B-6/g protein). In elderly women (44), an intake of 1.90 mg vitamin B-6/d (0.024 mg vitamin B-6/g protein) did not quite restore their plasma PLP concentrations to predepletion values; but in a second group consuming less protein, 1.33 mg vitamin B-6/d normalized their plasma PLP values (0.025 mg vitamin B-6/g protein).

At daily vitamin B-6 intakes of 0.84, 1.03 and 1.14 mg (0.10, 0.012 and 0.013 mg vitamin B-6/g protein), the mean plasma PLP concentrations of the subjects in the present studies were less than the value suggested for adequate status. Although the mean plasma PLP concentrations at these intakes may not be significantly less than the recommended cutoff of 30 nmol/L, a majority of the subjects (66–70%) had PLP concentrations < 30 nmol/L. Use of a cutoff value such as this is based on experimental data (15), but it is important to recognize that cutoff values are best used for individual data and are less useful for comparisons of mean values. For example, when the vitamin B-6 intake was 1.33 mg/d (0.016 mg vitamin B-6/g protein), the mean plasma PLP concentration was > 30 nmol/L, but 4 of the 10 subjects still had concentrations less than this. Three subjects still had PLP concentrations < 30 nmol/L with a vitamin B-6 intake of 1.73 mg/d (0.020 mg vitamin B-6/g protein), but all had plasma PLP values indicative of adequate status at 2.34 or 2.39 mg/d (0.028 mg vitamin B-6/g protein).

Shultz and Leklem (35) used 3-d dietary intake records to estimate vitamin B-6 intake in 41 females, 25–79 y old, and developed equations relating fasting plasma PLP concentrations, urinary 4-PA, and total vitamin B-6 excretion to vitamin B-6 intake and a ratio of dietary vitamin B-6 to protein. When using vitamin B-6 intakes from these studies, the equations closely predicted the respective values for plasma PLP concentration and urinary 4-PA and urinary vitamin B-6 excretion. This suggests that these indexes can be used in a free-living
population to estimate vitamin B-6 intake when it falls within the normal range of intakes.

RBC PLP concentration has been suggested as a useful indicator of vitamin B-6 status at marginal and adequate intakes (46), but no significant correlation between RBC PLP concentration and vitamin B-6 intake or any of the other vitamin B-6 status indicators was found in study 2. Limited data are available on RBC PLP concentrations, but in men consuming a daily vitamin B-6 intake of 2.1 ± 0.1 mg/d RBC PLP, concentrations averaged 85 ± 6 nmol/L (47), which is higher than the 47.2 ± 14.0 nmol/L we measured in women with an intake of 2.34 mg vitamin B-6/d in study 2. The difference in concentration may be due, in part, to the different methods used to measure RBC PLP. We had poor recovery of added PLP in the RBC PLP assay, suggesting incomplete extraction. Our results suggest that RBC PLP concentration is not useful as a status indicator in healthy subjects consuming intakes within the normal range, at least over short time periods as in study 2.

We found no significant correlation between plasma PLP concentration and alkaline phosphatase activity in these studies, but a significant direct correlation was found between RBC PLP concentration and plasma alkaline phosphatase activity. Because pyridoxal is the form of vitamin B-6 that most readily crosses membranes, it may be that a higher activity of plasma alkaline phosphatase is reflective of cellular membrane alkaline phosphatase activity, which converts PLP to pyridoxal, allowing more pyridoxal to enter the RBC where it may then be converted to PLP (48).

Mean fecal vitamin B-6 excretion did not vary among the periods of study 1 and was similar to values found by Kabir et al (18) in men consuming a diet providing 1.6 mg vitamin B-6/d. The amount excreted represents 18–33% of the total vitamin B-6 intake. From this data we cannot distinguish between the amount of unabsorbed vitamin B-6 from the diet and the amount of vitamin B-6 produced by synthesis by intestinal microflora.

Indirect measures of vitamin B-6 status

Erythrocyte aminotransferase activity percentage stimulation is considered a long-term measure of vitamin B-6 status because of the length of the life span of erythrocytes (15). In two previous depletion-repletion studies (7, 12), intakes of 1.5–1.84 mg vitamin B-6/d (0.015–0.023 mg vitamin B-6/g protein) restored erythrocyte ALT percentage stimulation or activity coefficient values to their predepletion values, and the erythrocyte AST activity coefficient was restored at 2.0 mg vitamin B-6/d (0.020 mg vitamin B-6/g protein). Ribaya-Mercado et al (44) found that the erythrocyte AST percentage stimulation in their vitamin B-6–depleted elderly subjects displayed a lag time in response to an increase in vitamin B-6 intake. For their women subjects, 1.90–1.95 mg vitamin B-6/d (0.024–0.036 mg vitamin B-6/g protein) was required to restore this indicator to baseline values.

The subjects in the studies reported here were consuming each vitamin B-6 intake for 10–15 d and only the erythrocyte ALT percentage stimulation showed significant changes. Mean values were above the < 25% value suggested for adequate status at vitamin B-6 intakes of 1.03 and 1.33 mg/d (0.012 and 0.016 mg vitamin B-6/g protein) and achieved a better than adequate value at a vitamin B-6 intake of 1.73 mg/d (0.020 mg vitamin B-6/g protein). No significant changes were seen in erythrocyte AST among the four experimental periods of study 1. These results suggest that erythrocyte ALT responds more readily to changes in vitamin B-6 intake than erythrocyte AST. Of the two measures, erythrocyte ALT is considered to be the more sensitive to changes in vitamin B-6 intake (15).

The primary urinary tryptophan metabolite that has been used to assess vitamin B-6 status is the urinary excretion of XA following a 2-g L-tryptophan load (15). Leklem et al (49) reported that a vitamin B-6 intake of 0.83 mg/d for 4 w by vitamin B-6-depleted subjects was sufficient to return mean XA excretion after a 2-g L-tryptophan load to its predepletion value, which was better than the < 65 μmol/d suggested for adequate status. Kretsch et al (12) found that normalization of postload XA excretion occurred at 1.5 mg vitamin B-6/d (0.015 mg vitamin B-6/g protein). In the study by Ribaya-Mercado et al (44), elderly women consuming 78 g protein required 1.90 mg vitamin B-6/d (0.024 mg vitamin B-6/g protein) to restore predepletion XA excretion after a 5-g L-tryptophan load.

In study 1, mean XA excretion after a 2-g L-tryptophan load was below the suggested value for adequate status (< 65 μmol/d) at all four vitamin B-6 intakes (Table 4); but with an intake of 1.03 mg/d, 30% of the subjects (3 of 10) had postload excretions of XA > 65 μmol/d, indicating inadequate status. Mean postload urinary excretion of KA was not significantly different at any of the four intakes in study 1, but showed a progressively downward trend as intake increased (Table 3). Mean VA excretion, however, was significantly lower with vitamin B-6 intakes ≥ 1.33 mg/d. Both KA and VA excretion were inversely correlated with vitamin B-6 intake and urinary 4-PA and urinary vitamin B-6 excretion (Table 5), suggesting that in healthy women, either is a good, noninvasive, functional measure of vitamin B-6 status. The one subject whose postload excretion of KA and VA was elevated, possibly due to infection, had normal values for XA excretion. This suggests postload XA excretion may be the preferred measure when infection may be present, as long as the infection is not serious enough to increase stress hormones.

Correlations among vitamin B-6 status indicators

Significant correlations between vitamin B-6 intake and urinary 4-PA, urinary vitamin B-6 and plasma PLP concentration have been reported in male and female free-living subjects whose intake was estimated from dietary records (36). In this latter study (36), there were also significant correlations found between urinary 4-PA and urinary vitamin B-6, urinary 4-PA and plasma PLP, and urinary vitamin B-6 and plasma PLP, in both male and female subjects. As shown in Table 5, the status indicators showing the strongest correlation with vitamin B-6 intake were urinary 4-PA excretion, urinary vitamin B-6 excretion, plasma total vitamin B-6 concentration, plasma PLP concentration, and XA excretion after an oral 2-g L-tryptophan load. Of interest was the finding that the correlation of plasma PLP with vitamin B-6 intake was best represented by an exponential model. This suggests that at lower intakes there is not a proportional increase in plasma PLP concentration. A possible explanation for this could be that tissue pools are being filled before there is a linear increase in plasma PLP concentration. Urinary 4-PA excretion was strongly correlated with urinary vitamin B-6 excretion, and plasma total vitamin B-6 and PLP
concentrations. The strong correlation between plasma total vitamin B-6 and PLP concentrations was expected because 66–81% of the plasma total vitamin B-6 was PLP. There was also a strong inverse correlation between plasma PLP concentration and post-tryptophan-load urinary KA excretion. The aminotransferase that converts kynurenine to KA and requires PLP as a coenzyme is a cytosolic enzyme that is more responsive to changes in PLP concentration than the mitochondrial aminotransferase, which converts 3-hydroxykynurenine to KA (50). These data indicate that urinary 4-PA and urinary vitamin B-6 excretion, plasma total vitamin B-6 and PLP concentrations, and KA and VA excretion after a tryptophan load are all reflective of vitamin B-6 intake and can be used as reliable indicators of vitamin B-6 status in healthy women. Based on the correlation data, it appears that one measure is sufficient to assess vitamin B-6 status. However, this approach is tempered because of the factors that can influence vitamin B-6 status indicators (many of which were controlled or constant in this study) as well as differences in analytic methods. Thus, as previously recommended (51), we believe that at least three measures should be used, especially when subjects are free-living.

Vitamin B-6 requirements

The vitamin B-6 requirement suggested by the studies of Donald et al (10) and Brown et al (7) may underestimate the vitamin B-6 intake necessary to achieve adequate vitamin B-6 status considering most of the vitamin B-6 intake in those studies was as crystalline pyridoxine hydrochloride, a form considered to be highly bioavailable. The suggested requirement does not reflect the bioavailability of vitamin B-6 in a typical diet composed of natural foods (18, 25, 36, 40, 52).

The diets in the studies reported here were composed mostly of natural foods with 60–100% of the total vitamin B-6 intake derived from the diet at the five lowest intakes (43% and 36% at the two highest intakes). In study 2, the percentage of the total vitamin B-6 intake as PNG, a form found to have lower bioavailability compared with pyridoxine (42, 43), was 6–17%, which may be comparable with intakes in a typical diet (40, 41). PNG is not present in animal products, but constitutes 5–80% of the total vitamin B-6 in various fruit and vegetables (25, 41). Therefore, individuals who consume few or no animal products may have a higher percentage of the less bioavailable PNG in their diets and may need a higher intake of vitamin B-6 to meet their requirement. Although Shultz and Leklem (53) found no significant differences in plasma PLP, urinary 4-PA, or urinary vitamin B-6 between vegetarian (n = 7) and non-vegetarian women (n = 13) with similar vitamin B-6 intakes, the means of all the indicators were lower in the vegetarians. It is possible that with a larger sample size the differences could become significant.

Although some status indicators reached values indicative of adequate status at lower intakes, it took a vitamin B-6 intake > 1.33 mg/d (ratio of vitamin B-6 to protein > 0.016 mg/g) for all status indicators measured to achieve the values suggested for adequate status. This suggests that the minimal vitamin B-6 requirement for young adult women is in the range of 1.33–1.73 mg/d with a protein intake of 85 g/day (0.016–0.020 mg vitamin B-6/g protein). Kretsch et al (12) concluded that the requirement for vitamin B-6 is between 0.015 and 0.020 mg/g protein, and suggested that adding a margin of safety to account for bioavailability differences and variance in the requirement of individuals would raise the vitamin B-6 RDA above the currently recommended amount of 0.016 mg/g protein. Our results confirm and reinforce their conclusions.

We thank Karin Hardin and Jim Ridlington for their technical assistance.

REFERENCES

21. Lee CM, Leklem JE. Differences in vitamin B₆ status indicator re-