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Short Communication

Separation and quantification of the B_6 vitamers in plasma and 4-pyridoxic acid in urine of adolescent girls by reversed-phase high-performance liquid chromatography

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ABSTRACT

The vitamin B_6 status of seemingly healthy adolescent girls was determined using several accepted and proposed parameters in an effort to establish guidelines for status evaluation. High-performance liquid chromatography-derived plasma B_6 vitamers (pyridoxal phosphate, PLP; pyridoxine phosphate, PNP; pyridoxamine phosphate, PMP; pyridoxal, PL; pyridoxine, PN; and pyridoxamine, PM) and 4-pyridoxic acid (4-PA) concentrations and urinary 4-PA levels of 28 white adolescent females, 12–15 years, having radiomonitored plasma PLP concentrations and coenzyme stimulation of erythrocyte alanine aminotransferase activities indicative of adequate status were determined. Mean vitamin B_6 and protein intakes were 1.48 mg and 78.3 g. Ranges for plasma B_6 vitamer and 4-PA concentrations (nmol/l) were: PLP, 40.9–122.2; PNP, non-detectable (ND)–16.1; PMP, ND–8.1; PL, ND–15; PN, ND–21.9; PM, ND–17.8; and 4-PA, ND–55.7. PLP was the only vitamer found in plasma of all subjects. Urinary 4-PA concentrations ranged from 0.11 to 2.50 μmol/mmol of creatinine. B_6 vitamer values of these girls should be of use in the establishment of normal ranges for vitamin B_6 status parameters.

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INTRODUCTION

Vitamin B_6 exists in three interconvertible forms: pyridoxine (PN), also known as pyridoxol, pyridoxal (PL) and pyridoxamine (PM), each of which has a corresponding 5′-phosphate (P). 4-Pyridoxic acid (4-PA) is the major excretory catabolite [1].

Methodologies that have been utilized to determine vitamin B_6 status include enzymatic procedures, *e.g.*, xanthurenic acid excretion, coenzyme stimulation of aspartate and alanine aminotransferase (ALAT) activities in whole blood or erythrocytes (E), and plasma PLP concentrations via the radioisotopically monitored $^{14}CO_2$ formation from [^{14}C]L-tyrosine. Some researchers have utilized microbiological techniques to determine vitamin B_6 concentrations of plasma. Urinary 4-PA concentration has been evaluated by both physical and chemical methodologies. Currently most vitamin B_6 researchers believe that the radiomonitored PLP method is the most acceptable procedure for the determination of status; however, these researchers also have indicated that more than one parameter should be used in the assessment of vitamin B_6 status [2,3].

Recent interest has focused on the use of high-performance liquid chromatography (HPLC) as a means of determining vitamin B_6 levels in plasma. The physico-chemical and ionic properties of the B_6 vitamers facilitate their assay by HPLC methodologies. HPLC methods have been extended for use on biological materials from humans [4–11]. Chrisley *et al.* [8] recently reported plasma B_6 vitamer and 4-PA concentrations of men fed controlled diets as determined by a newly developed HPLC method. Gregory and Kirk [12] reported a simple method for measuring 4-PA levels in urine by HPLC which may be useful as a means of determining vitamin B_6 status.

Few studies have examined the vitamin B_6 status of female adolescents [13–17]. These girls are recognized as being at high nutritional risk due to many factors including the stresses of growth and sexual maturation and the effects of peer influence on dietary habits. Research is needed in order to establish "norms" for the evaluation of vitamin B_6 status of adolescent females using accepted as well as proposed status indicators which have the potential of being better indicators.

Therefore, the objectives of this study were to use the HPLC methodology recently developed in our laboratory [8] to analyze the B_6 vitamer and 4-PA concentrations in the plasma and 4-PA levels in the urine of adolescent females, 12–15 years, who had been shown to have adequate vitamin B_6 status. The dietary intakes of the subjects were compared to their radioisotopically monitored PLP concentrations as well as HPLC-derived concentrations of B_6 vitamers and 4-PA in plasma and 4-PA values in urine. Since guidelines have been established for vitamin B_6 status in particular by EALAT assays, and have been suggested for plasma PLP concentrations, criteria for status need to be extended to B_6 vitamer concentrations as determined by other methodologies such as HPLC for various population groups.

EXPERIMENTAL

Subjects

Twenty-eight white adolescent females were recruited as subjects from Southwest Virginia following approval of the study by the Institutional Review Board for Research Involving Human Subjects. The girls were 12–15 years of age. The subjects and their parents were given a written explanation of the study and asked to sign a consent of participation form. The subjects and their parents reported that the girls were in good health. Demographic characteristics of the subjects were obtained via use of questionnaires similar to those described by Christakis [18].

Blood and urine collections

Approximately 15 ml of blood from fasting subjects were obtained in vacutainers containing EDTA by a Registered Medical Technologist between 7:30 and 10:00 a.m. The samples were kept in crushed ice and protected from light. Blood samples were centrifuged at 3000 *g* and 5°C for 10 min using a refrigerated rotor. Plasma was frozen at −20°C for future radiomonitored plasma PLP and HPLC analyses. Erythrocytes were treated as described by Heddle *et al.* [19] and frozen at −20°C for EALAT analysis.

Premeasured specimen cups were given to the girls to acquire random, freshly voided urine samples. The specimen was then transferred into a brown bottle which contained 1 ml of toluene which was used as a preservative. The bottles of urine were frozen at −20°C for future 4-PA and creatinine analyses.

Dietary intake procedures

Food consumption records were obtained from the subjects. A 24-h recall was obtained using the interview technique; food models and cross-checking were used. Consecutive two-day food records were obtained; thus three consecutive days of dietary intakes were obtained. Questionnaires were fashioned after those described in Nutritional Assessment in Health Programs [18]. The reported kilocalorie, protein, and vitamin B_6 intakes of the subjects were estimated using handbook values [20–22]. The values were compared with the 1989 RDAs [23] for the various age groups. Information concerning use of nutrient supplementation was also obtained from the girls.

EALAT, plasma PLP, and plasma B_6 vitamer and 4-PA determinations

Plasma PLP concentrations were measured enzymatically by stimulation of tyrosine decarboxylase apoenzyme [24]. Recoveries of 92–94% were obtained when plasma samples were spiked with PLP before analyses. EALAT activities were determined according to the method of Heddle *et al.* [19]. PLP was utilized as described by Raica and Sauberlich [25] in measuring the coenzyme stimulation of EALAT activities. Plasma B_6 vitamer and 4-PA concentrations were deter-

mined by the HPLC method developed by Chrisley *et al.* [8]. Representative chromatograms of B_6 vitamers and 4-PA in combined standard solutions and in human plasma extract have been published [8]. Quantifiable peaks were calculated when peak heights were two times noise levels.

Urinary 4-PA and creatinine determinations

Urinary 4-PA analyses were performed using the HPLC methodology developed in our laboratory [8] and the extraction method of Gregory and Kirk [12]. The urinary creatinine method was a modification of the Jaffe method utilizing a Stanbio kit (Fisher Scientific, Raleigh, NC, U.S.A.).

Statistical analyses

All data were evaluated by general linear model procedures [26]. Means and standard deviations (S.D.) were calculated. There were no significant differences found between values obtained from the 24-h and two-day intakes; thus, these data were combined. Pearson r coefficients were determined between data obtained by the various assay parameters.

RESULTS AND DISCUSSION

Twenty-eight white girls volunteered as subjects. These included 15–12, 3–13, 4–14, and 6–15 year olds. The height and weight values of the girls in the four age groups are presented in Table I. The 12 year olds were significantly shorter ($p < 0.05$) than the 14 and 15 year olds, and the 12 and 13 year olds weighed significantly less ($p < 0.05$) than the 15 year but not the 14 year groups.

Hemoglobin and hematocrit measurements

No age differences in hemoglobin and hematocrit levels of the girls were observed. The hemoglobin and hematocrit values of the subjects were as follows (mean ± S.D.): 136 ± 9 g/l and 0.39 ± 0.02, respectively.

TABLE I

AGES, HEIGHTS, AND WEIGHTS OF ADOLESCENT FEMALE SUBJECTS

Values represent means ± S.D.

Age (years)	n	Height (cm)	Weight (kg)
12	15	157.4 ± 4.5[a]	49.3 ± 8.2[b]
13	3	161.7 ± 5.9	49.1 ± 1.6[b]
14	4	166.4 ± 3.3	57.4 ± 5.7
15	6	165.9 ± 5.0	59.7 ± 2.5

[a] Significantly lower ($p < 0.05$) than values of 14 and 15 year groups.

[b] Significantly lower ($p < 0.05$) than values of 15 year but not 14 year groups.

TABLE II

ESTIMATED DAILY KILOCALORIE, PROTEIN, AND VITAMIN B_6 INTAKES AND VITAMIN B_6/PROTEIN RATIOS OF ADOLESCENT FEMALE SUBJECTS

Values represent means ± S.D.

Age (years)	*n*	kcal	Protein (g)	Vitamin B_6 (mg)	Vitamin B_6/ protein ratio
12–14[a]	22	2138 ± 505	80.1 ± 18.0	1.59 ± 0.70	0.020 ± 0.009
15	6	1984 ± 539	71.8 ± 17.0	1.11 ± 0.30	0.016 ± 0.004

[a] Age grouping used in 1989 RDA [23].

Height and weight measurements

The mean heights and weights of the 28 girls who volunteered as subjects were similar to those reported for the 50th percentiles for girls of similar ages [27]. The subjects and their parents reported that the girls were in good health. In addition, all of the girls exhibited acceptable hemoglobin and hematocrit values when compared to recommended levels [28]; therefore, these girls were deemed to be generally healthy.

Kilocalorie, protein and vitamin B_6 intakes

There were no significant differences in the estimated kilocalorie (kcal), protein, or vitamin B_6 intakes or the ratio of vitamin B_6 to protein intakes for the girls in the different age groups. The estimated kcal, protein, and vitamin B_6 intakes and vitamin B_6/protein ratios of the girls over the three consecutive days are given in Table II; these data are presented by the age groupings utilized in the 1989 RDA [23] publication.

The subjects had estimated energy and protein intakes similar to those in this same age range which have been published by others [13–17]. The estimated vitamin B_6 intakes of the girls in the current study (Table II) were similar to published values [13–17]. Approximately 14% of the 12–14 year (three of twenty-two girls) and 33% of the 15 year (two of six girls) groups consumed $<2/3$ 1989 RDA for the vitamin. Because the vitamin B_6 1989 RDA for the 15 year group is 1.5 mg *versus* 1.4 mg for the younger (12–14 year) girls, many of the 15 year girls consequently fell in the $<2/3$ 1989 RDA even though their intakes were similar to those of the younger subjects. A ratio of 0.02 mg vitamin B_6/g protein was suggested for use in estimating the vitamin B_6 allowance in the 1980 RDA publication [29]; however, a 0.016 ratio appears to ensure acceptable vitamin B_6 values in adults according to the 1989 RDA publication [23]. Ten of the 28 girls in the current study reported consuming <0.016 mg vitamin B_6 per g protein.

Vitamin B_6 status determinations

EALAT activity has been reported to be more sensitive in the detection of

TABLE III

VALUES FOR VITAMIN B_6 STATUS PARAMETERS OF ADOLESCENT FEMALE SUBJECTS

Parameter abbreviations: EALAT-AC, coenzyme stimulation of erythrocyte alanine aminotransferase activity; R, radiomonitored; F, HPLC–fluorometric; and U, HPLC–UV. Non-detectable levels were calculated as zeros.

Parameter	n^a	Mean ± S.D.	Range
EALAT-AC (%)	28	8.8 ± 4.6	0–15.4
Plasma RPLP (nmol/l)	28	78.4 ± 19.3	43.3–116.9
Plasma FPLP (nmol/l)	28	78.1 ± 19.3	40.9–122.2
Plasma UPLP (nmol/l)	28	78.4 ± 20.3	40.5–125.4
Plasma FPNP (nmol/l)	10	2.2 ± 3.7	ND–16.1
Plasma UPNP (nmol/l)	5	1.1 ± 3.1	ND–15.2
Plasma PMP (nmol/l)	5	0.8 ± 1.9	ND–8.1
Plasma PL (nmol/l)	3	1.1 ± 3.4	ND–15.0
Plasma PN (nmol/l)	3	1.2 ± 4.3	ND–21.9
Plasma PM (nmol/l)	16	4.2 ± 5.2	ND–17.8
Plasma 4-PA (nmol/l)	12	7.8 ± 12.8	ND–55.7
Urinary 4-PA/creatinine[b] (μmol/mmol/l)	28	0.76 ± 0.60	0.11–2.50

[a] Number subjects having detectable levels.
[b] Random urine samples.

vitamin B_6 deficiency states than aspartate aminotransferase activity [30]; coenzyme stimulation (with PLP) of EALAT activity is a more sensitive status indicator than the activity determined without additional cofactor [31]. Coenzyme stimulation of EALAT activities, radiomonitored plasma PLP (RPLP), HPLC-derived B_6 vitamer concentrations, and urinary 4-PA/creatinine ratios of the subjects are given in Table III. No differences in values obtained for any of these status parameters were observed between the age groups.

Coenzyme stimulation of EALAT activities $>25\%$ are indicative of vitamin B_6 deficiency [32]; values $>16\%$ are indicative of some inadequacy of the vitamin [13,15]. All of the subjects had coenzyme stimulation values $<16\%$ and hence would be considered to have adequate B_6 status. Other researchers have reported finding that teenage female subjects frequently had coenzyme stimulation values $>16\%$ as well as $>25\%$ [13–17].

The girls' vitamin B_6 status was also determined by the widely used plasma RPLP assay utilizing apotyrosine decarboxylase. This method is currently considered to be the most acceptable criteria for status determination [2,3]. Several researchers [16,33–36] have proposed guidelines for plasma RPLP levels indicative of inadequate, marginal, or low vitamin B_6 status. All of the subjects in the current study had plasma RPLP concentrations higher than any of the levels suggested as being indicative of vitamin B_6 inadequacy. In contrast, Driskell and

Moak [16] found that black and white teenage female subjects frequently had RPLP levels indicative of inadequate vitamin B_6 status.

The HPLC-derived PLP concentrations of the subjects were in the ranges reported by others who used similar HPLC methodologies [4,5,7–10]. The mean PLP values for the girls (Table III) were somewhat lower than the mean PLP values for males in a study conducted by Chrisley *et al.* [8] utilizing the same HPLC methodology. However, plasma PLP values for all the adolescent females in the present study were indicative of an adequate vitamin B_6 status regardless of which set of guidelines were utilized.

The subjects' plasma PNP concentrations were determined by utilizing both UV and fluorometric HPLC determinations; a high correlation ($r = 0.81$, $p < 0.0001$) was also observed between plasma PNP levels of the girls as obtained using the two HPLC detectors. Ten of the subjects had detectable plasma levels utilizing the HPLC fluorometric detector and five of these subjects had detectable levels utilizing the HPLC–UV detector. The PNP values for these girls were lower than values reported recently for males in a study by Chrisley *et al.* [8] using both UV and fluorometric detection. Hollins and Henderson [5] reported finding quantifiable plasma PNP values in a healthy volunteer but not in ten other adults. Other researchers [4,7,9,10] did not report finding detectable plasma PNP values.

Plasma PMP was detected in five of the girls in the current study; the values were in the ranges of those reported by Shephard *et al.* [7], Lumeng *et al.* [37], and Liu *et al.* [6]. The girls exhibited lower plasma PMP values than those reported in adult men by Chrisley *et al.* [8], Coburn and Mahuren [4], Chauhan and Dakshinamurti [38], and Vanderslice *et al.* [10]. The mean plasma PL values were lower than reported values [4,5,7–9]. Plasma PN values were in the ranges of those reported by Shephard *et al.* [7] and Liu *et al.* [6]. The plasma PM values detected in the subjects were in the ranges of values reported by Coburn and Mahuren [4] and Lumeng *et al.* [37]. Plasma 4-PA values detected in twelve girls were lower than the values reported by others for adults in studies by Chrisley *et al.* [8], Shephard and coworkers [7,9], Hollins and Henderson [5], Coburn and Mahuren [4], Lumeng *et al.* [37], and Liu *et al.* [6]. The plasma B_6 vitamer levels which have been reported previously and to which the girls' values were compared were those of adults, primarily men.

The predominant plasma B_6 vitamer was PLP which constituted approximately 89% of the distribution of the vitamers in plasma from girls in this study. Other researchers [4–9,33,37,38] have also reported PLP to be the predominant plasma B_6 vitamer. Subjects in the current study had practically equal distributions of PL and PN. Coburn and Mahuren [4] also reported finding nearly equal distributions of PL and PN in plasma from 38 adults although their values were higher than those found in the current study.

Shultz and Leklem [33] suggested guidelines for evaluating the vitamin B_6 status for women with urinary 4-PA values < 4.6–5.2 μmol per 24 h being indicative of marginal vitamin B_6 status. All of the girls in this study had urinary 4-PA

values indicative of adequate vitamin B_6 status. Donald *et al.* [39] found that repletion of eight young women with 8.9 μmol pyridoxine satisfied their vitamin B_6 requirements; the girls in this study had a mean daily vitamin B_6 intake of 8.8 μmol.

Comparisons of values obtained by different status parameters

The coenzyme stimulation of EALAT activities of the subjects were significantly correlated with plasma PLP values as determined radiometrically ($r = -0.80, p < 0.0001$) and by HPLC utilizing the UV ($r = -0.78, p < 0.0001$) and fluorometric ($r = -0.77, p < 0.0001$) detectors. Plasma PLP concentrations of the subjects quantitated using the radiometric method were highly correlated with those obtained by HPLC utilizing both the UV ($r = 0.96, p < 0.0001$) and the fluorometric ($r = 0.98, p < 0.0001$) detectors; in addition, high correlation was also observed between values obtained using the two HPLC detectors ($r = 0.97, p < 0.0001$). Thus, similar PLP data were obtained utilizing all three of these techniques. This is in agreement with previous findings in which PLP was measured radiometrically [16]. The urinary 4-PA/creatinine values of the girls correlated significantly with their estimated vitamin B_6 intakes ($r = 0.45, p < 0.05$) and their vitamin B_6/protein intake ratios ($r = 0.41, p < 0.05$) thus indicating that urinary 4-PA/creatinine values seemed to be reflective of recent dietary vitamin B_6 levels. No significant correlations were observed between other parameters. No relationships were observed between per capita income and various parameter values. Parameter values of premenarcheal and postmenarcheal girls were similar; seven of the fifteen girls in the 12 year group had not experienced menarche. In agreement with findings in the present study, premenarcheal and postmenarcheal girls have been reported to have similar values for vitamin B_6 status parameters [15–17].

Although the calculated mean vitamin B_6 intakes for the 15 year but not the 14 year girls were below the 1989 RDA [23] (1.4 mg daily for 11 to 14 years and 1.5 mg daily for those 15 to 18 years), vitamin B_6 requirements of the subjects appeared to be satisfied as demonstrated by the values obtained for the various vitamin B_6 status parameters which were all indicative of adequate status. Shultz and Leklem [33] using vitamin B_6 and protein intake data obtained from three-day dietary records for men who reported consuming 1.25–1.50 mg vitamin B_6 per day and/or a dietary vitamin B_6/protein ratio of 0.0125–0.015 established lower and upper marginal range limits for status guidelines for plasma PLP concentrations and urinary 4-pyridoxic acid and total vitamin B_6 excretion levels. These researchers reported that these intake levels (1.25–1.50 mg per day) of vitamin B_6 appeared to satisfy the vitamin B_6 requirement of adults. Horwitt [40] indicated that 1.8 mg vitamin B_6 per day was more than adequate for a diet containing 100 g protein per day. The vitamin B_6 requirements of young men were satisfied with diets calculated to contain means of 0.75–0.98 mg per day of the vitamin daily for eight weeks and 80.8–84.5 g protein as demonstrated by

their having plasma RPLP levels indicative of adequate status. These men were fed diets calculated to contain mean vitamin B_6 intakes of 1.22–1.67 mg per day [41]. Hence, the analyzed vitamin B_6 content of the diets consumed by these men was 56–62% of the calculated values. The girls in the present study were estimated to have consumed a calculated 1.48 ± 0.66 mg vitamin B_6 (mean ± S.D.) and 78.3 ± 17.8 g protein daily utilizing data obtained from 24-h recalls followed by two-day food records. The 28 adolescent female subjects in this study exhibited adequate vitamin B_6 status as indicated by accepted as well as proposed parameters while consuming diets estimated to contain a calculated 1.48 ± 0.66 mg (mean ± S.D.) of the vitamin daily. The plasma B_6 vitamer and urinary 4-PA/creatinine values of these white adolescent girls should be of use in the establishment of normal ranges for vitamin B_6 status parameters.

REFERENCES

1 E. Snell, B. Guisard and R. Williams, *J. Biol. Chem.*, 143 (1942) 519.
2 J. E. Leklem and R. D. Reynolds (Editors), *Methods in Vitamin B-6 Nutrition: Analysis and Status Assessment*, Plenum Press, New York, 1981, pp. 389–392.
3 R. D. Reynolds and J. E. Leklem (Editors), *Current Topics in Nutrition and Disease, Vol. 13, Vitamin B-6: its Role in Health and Disease*, Alan R. Liss, New York, 1985, pp. 482–487.
4 S. P. Colburn and J. D. Mahuren, *Anal. Biochem.*, 129 (1983) 310.
5 B. Hollins and J. M. Henderson, *J. Chromatogr.*, 380 (1986) 67.
6 A. Liu, L. Lumeng and T. K. Li, *Am. J. Clin. Nutr.*, 41 (1985) 1236.
7 G. S. Shephard, M. E. J. Louw and D. Labadarios, *J. Chromatogr.*, 416 (1987) 138.
8 B. Mc. Chrisley, F. W. Thye, H. M. McNair and J. A. Driskell, *J. Chromatogr.*, 428 (1988) 35.
9 G. S. Shephard, L. Van Der Westhuizen and D. Labadarios, *J. Chromatogr.*, 491 (1989) 226.
10 J. T. Vanderslice, C. E. Maire and G. R. Beecher, *Am. J. Clin. Nutr.*, 34 (1981) 947.
11 J. T. Vanderslice and C. E. Maire, *J. Chromatogr.*, 196 (1980) 176.
12 J. F. Gregory and J. R. Kirk, *Am. J. Clin. Nutr.*, 32 (1979) 879.
13 A. Kirksey, K. Keaton, R. P. Abernathy and J. L. Greger, *Am. J. Clin. Nutr.*, 31 (1978) 946.
14 L. R. Sutker, B. M. Chrisley and J. A. Driskell, *Nutr. Rep. Int.*, 29 (1984) 655.
15 J. A. Driskell, A. J. Clark, T. L. Bazzarre, L. F. Chopin, H. McCoy, M. A. Kenney and S. W. Moak, *J. Am. Diet. Assoc.*, 85 (1985) 46.
16 J. A. Driskell and S. W. Moak, *Am. J. Clin. Nutr.*, 43 (1986) 599.
17 J. A. Driskell, A. J. Clark and S. W. Moak, *J. Am. Diet. Assoc.*, 87 (1987) 307.
18 G. Christakis (Editor) *Nutritional Assessment in Health Programs*, American Public Health Association, Washington, DC, 1973.
19 J. C. Heddle, E. W. McHenry and G. H. Beaton, *Am. J. Biochem. Physiol.*, 41 (1963) 215.
20 B. K. Watt and A. L. Merrill, *Composition of Foods: Raw, Processed, Prepared*, U.S. Department of Agriculture Handbook, No. 8, U.S. Government Printing Office, Washington, DC, 1963.
21 U.S. Department of Agriculture, *Composition of Foods: Raw, Processed, Prepared*, Agriculture Handbook No. 8-1 to 8-17, 8-20, 8-21, U.S. Government Printing Office, Washington, DC, 1976–1989.
22 M. L. Orr, *Pantothenic Acid, Vitamin B_6, and Vitamin B_{12} in Foods*, USDA Home Economics Research Report No. 36, U.S. Government Printing Office, Washington, DC, 1969.
23 Subcommittee on the Tenth Edition of the RDAs, Food and Nutrition Board, National Research Council, *Recommended Dietary Allowances*, National Academy Press, Washington, DC, 10th ed., 1989.
24 M. E. Fries, B. M. Chrisley and J. A. Driskell *Am. J. Clin. Nutr.*, 34 (1981) 2706.
25 N. Raica and H. E. Sauberlich, *Am. J. Clin. Nutr.*, 15 (1964) 67.
26 R. F. Sokal and F. J. Rohlf (Editors), *Biometry*, W. H. Freeman, San Francisco, CA, 1969.

27 National Center for Health Statistics, *NCHS Percentiles —Stature and Weight: Boys and Girls: 2 to 18 Years,* National Center for Health Statistics, Rockville, MD, 1977.
28 A. S. Lowenstein and F. W. Johnson, *Preliminary Findings of the First Health and Nutrition Examination Survey, United States, 1971–1972: Dietary Intake and Biochemical Findings,* DHEW Publication (HRA) 74-1219-1, U.S. Department of Health, Education and Welfare, Washington, DC, 1974.
29 National Research Council, *Recommended Dietary Allowances,* National Academy Press, Washington, DC, 9th ed., 1980.
30 M. Brin and V. F. Thiele, *J. Nutr.,* 93 (1967) 213.
31 A. D. Cinnamon and J. R. Beaton, *Am. J. Clin. Nutr.,* 23 (1970) 696.
32 H. E. Sauberlich, R. P. Dowdy and J. H. Skala, *Laboratory Tests for the Assessment of Nutritional Status,* CRC Press, Cleveland, OH, 1974.
33 T. D. Shultz and J. E. Leklem, in J. E. Leklem and R. D. Reynolds (Editors), *Methods in Vitamin B-6 Nutrition: Analysis and Status Assessment,* Plenum Press, New York, 1981, pp. 297–317.
34 C. S. Rose, P. György, M. Butler, R. Andres, A. H. Norris, N. W. Shock, J. Tolin, M. Brin and H. Spiegel, *Am. J. Clin. Nutr.,* 29 (1976) 847.
35 I. F. Hunt, N. J. Murphy, P. M. Martner-Hewes, B. Faraji, M. E. Swendseid, R. D. Reynolds, A. Sanchez and A. Mejia, *Am. J. Clin. Nutr.,* 46 (1987) 563.
36 R. E. Cleary. L. Lumeng and T. K. Li, *Am. J. Obstet. Gynecol.,* 121 (1975) 25.
37 L. Lumeng, A. Liu and T. K. Li, *J. Clin Invest.,* 66 (1980) 688.
38 M. S. Chauhan and K. Dakshinamurti, *Clin. Chim. Acta,* 109 (1981) 159.
39 E. A. Donald, L. D. McBean, M. H. W. Simpson, M. T. Sun and H. E. Aly, *Am. J. Clin. Nutr.,* 24 (1971) 1028.
40 M. K. Horwitt, *Am. J. Clin. Nutr.,* 44 (1986) 973.
41 J. A. Driskell, B. M. Chrisley, F. W. Thye and L. K. Reynolds, *Am. J. Clin. Nutr.,* 48 (1988) 122.