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Title: VITAMIN B-6 METABOLISM AND STATUS IN YOUNG AND MIDDLE-AGED WOMEN

Abstract Approved: Dr. James E. Leklem

To investigate the responses of healthy adult women to two levels of vitamin B-6, a seven week two-part experiment was conducted with five young adults and eight middle-aged subjects. During the four unsupplemented weeks, dietary vitamin B-6 was 2.3-2.4 mg/day. In the final three weeks, the same diet was supplemented with 8.0 mg pyridoxine to give an intake of 10.3-10.4 mg/day. Plasma pyridoxal 5'-phosphate (PLP), plasma and urinary vitamin B-6 (PB6, UB6), and urinary 4-pyridoxic acid (4PA) were monitored throughout the study as indicators of status. Tryptophan load tests (TLT) were administered twice at each vitamin B-6 intake. The older women had significantly lower PLP, PB6 and UB6 and slightly higher 4PA values on normal vitamin B-6 intakes. With supplementation, in both age groups, the circulating status indicators increased 3-4 fold and excreted indicators increased 5-6 fold. During the supplemented weeks, only the difference in UB6 remained significant between age groups. The TLT revealed no between-group differences in xanthurenic acid or

kynurenic acid excretion. Erythrocyte pyridoxine kinase activity differences ($p < 0.05$) between the groups were documented, with the older women being 50% higher throughout. Supplementation did not alter kinase activity. Plasma and erythrocyte magnesium were likewise unchanged with supplementation and did not differ between groups. These results demonstrate an age related difference in vitamin B-6 status in women under controlled conditions of dietary intake with adequate vitamin B-6. They support results of studies done under less controlled conditions.

The decrease in circulating levels and increase in excretory product may be a reflection of age-related metabolic changes suggesting increased catabolism. The disappearance of significant age differences in three of four status indicators with vitamin B-6 supplementation suggests there may be age differences in the requirement for this vitamin. Constant TLT results, however, suggest that 2.3 mg/day is adequate for both groups of women.

VITAMIN B-6 METABOLISM AND STATUS IN YOUNG AND MIDDLE-AGED WOMEN

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VITAMIN B-6 METABOLISM AND STATUS IN YOUNG AND MIDDLE-AGED WOMEN

CHAPTER I

INTRODUCTION

Rationale

Aging is related to many functional and structural changes in the human body. Vitamin B-6 metabolism is an example of an important complex of reactions which exhibits age-related changes. Although reports of deficiency of vitamin B-6 by clinical assessment are rare in this country in healthy adults, concern may be justified because of a number of reports of lowered vitamin B-6 status of older versus younger adults based on intake studies or biochemical evaluation. These studies have been varied in design and purpose, but there has been, with few exceptions, general agreement of an age-related decrement in vitamin B-6 status based on studies of elderly men or mixed populations. However, none of these studies controlled for some important variables that might exist between the age groups and which could have influenced the interpretation of the results.

The primary unanswered question is whether the age-related decrements which have been observed in older individuals were in fact due to age effects per se or to other variables in the older subjects which influence vitamin B-6 metabolism. These variables include dietary intake, activity level, lean body mass and intestinal absorption.

Should an age-related difference in vitamin B-6 metabolism exist, the underlying cause still needs to be identified. One possible cause may be changes in the levels or activities of the enzymes which convert the vitamers from the absorbed free forms of pyridoxine, pyridoxal and pyridoxamine to the coenzyme form, pyridoxal 5'-phosphate. Since plasma pyridoxal 5'-phosphate has been observed by various workers to be lower in older subjects, pyridoxine kinase, the enzyme responsible for the initial phosphorylation of the vitamers, seems a likely source of variation. Several of the vitamin B-6 enzymes require divalent cations as cofactors. Levels of these minerals were seen as possibly being related to enzyme activity.

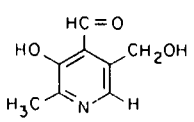
This study of vitamin B-6 metabolism and status in young and middle-aged women was based on the hypothesis that aging results in decreased vitamin B-6 status which reflects metabolic changes. The first objective was to investigate the responses of vitamin B-6 status indicators in young and older women when all subjects were fed identical constant diets. The second objective was to study in young and older women one specific possible cause for a metabolic change, i.e. the activity of pyridoxine kinase. A third objective was to compare in young and older women the plasma and erythrocyte levels of one divalent cation, magnesium, which has been reported to be related to vitamin B-6 blood levels and can serve as a cofactor for pyridoxine kinase.

History

Although in 1932 Japanese researchers isolated from rice polishings a substance with the structure we now accept as vitamin B-6, most historical accounts begin in 1934 with Gyorgy's naming of the vitamin (Gyorgy, 1934). Within several years, vitamin B-6 was determined to be essential for the rat (Gyorgy and Eckhard, 1939). At least five laboratories had isolated the crystalline vitamin by 1938. In the following year, pyridoxine was characterized and synthesized (Rosenberg, 1945). But it was not until an unfortunate processing error in 1954 destroyed the vitamin B-6 in an infant formula that the essentiality of this vitamin was confirmed (Molony and Parmalie, 1954; Coursin, 1954) for humans.

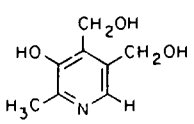
Occurrence and Bioavailability

Vitamin B-6 is the collective term for six biologically active forms of the vitamin (Snell and Haskell, 1971).



PYRIDOXAL

(PL)



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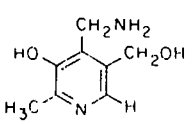
(PN)



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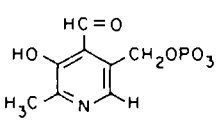
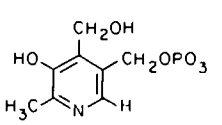
(PM)



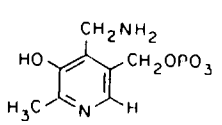
PYRIDOXAL PHOSPHATE

(PLP)



PYRIOOXINE PHOSPHATE

(PNP)



PYRIDOXAMINE PHOSPHATE

(PMP)

These vitamers are widely distributed in nature, with animal tissue and other foods high in protein being good sources of vitamin B-6 (Orr, 1969). As reviewed by Sauberlich (1981), about 40% of our dietary intake comes from meat, in which PL/PLP predominate. Dairy products, again primarily containing PL/PLP, provide about 12%. Cereal products and legumes, which contain vitamin B-6 mainly as PN, provide another 15%. Vegetables, in which vitamin B-6 is mainly as PN, contribute 22%. Fish contain primarily PM, but this category is not a significant contribution in American diets. With the exception of bananas and avocados, fruits are not good sources of the vitamin. The Recommended Dietary Allowances of 2.2 mg for men and 2.0 mg (RDA 1980) for women are not difficult to achieve for omnivores eating a good mixture of meats, seeds and other vegetables products. One hundred grams of the following foods each contains more than 20% of the RDA: liver, chicken dark meat, tuna, pinto beans, peanuts, bananas, avocados, and soybeans. Ten to twenty percent of the RDA is contained in 100 grams of chicken white meat, beef, pork, salmon, spinach, potatoes, raisins and whole wheat flour. Although numerous foods contain significant quantities of vitamin B-6, this is not all available for absorption. Many factors influence vitamin B-6 bio-availability (the ratio of the amount of a particular nutrient which can be absorbed from a food to the total amount of the nutrient in that food). These include physical characteristics of the food, such as piece size, degree of mastication, other constituents of the food, such as fiber and dietary protein, the form of the vitamin in a food

(whether it is free or bound), and the effects of processing (Leklem et al., 1980; Gregory and Kirk, 1981; Tarr et al., 1981; Kabir et al., 1983).

Functions

Vitamin B-6 is best known for its role as a coenzyme in amino acid metabolism. Over 60 enzymes, predominately transaminases, have been confirmed to require PLP as a cofactor (Sauberlich, 1968). In most of these reactions, PLP functions as a Schiff base, as in the removal of the α-amino group forming α-keto analogues by transamination. These reactions are essential in gluconeogenesis and in the synthesis of non-essential amino acids, among others. Many decarboxylases, which form amines, also use PLP as a Schiff-base-forming coenzyme. PMP can function as the cofactor for some enzymes, especially those involving loss of nitrogen.

In carbohydrate metabolism, PLP plays an essential conformational role in glycogen phosphorylase (Krebs and Fischer, 1964). PLP also forms a Schiff-base in these reactions but this interaction is with the enzyme itself rather than with the substrate, as occurs in transamination. PLP acts as an electrophile, interacting with the phosphate group of ATP and transferring it to glucose, forming glucose 1-phosphate. PLP does not relinquish its phosphate group in these reactions (Withers et al., 1981).

Vitamin B-6 is also required for the one-carbon transfers in nucleic acid synthesis. Its importance in the functioning of the

immune system may be a result of involvement in nucleic acid synthesis (Axelrod, 1971). Vitamin B-6 plays a significant role in hormone action affecting the binding of glucocorticoid hormones to their receptors (Cake et al., 1978). Again, PLP acts here through Schiff-base formation via the positively charged glucocorticoid-receptor complex which had become activated by losing inorganic phosphorus. In the presence of PLP, the availability of the positive charged complex to associate with DNA is reduced so the sequence to snythesizing protein is disrupted. Thus, in vitamin B-6 deficiency, less of the complex would be inactivated. There would then be less glucocorticoids needed to produce the same number of activated receptors. DiSorbo et al. (1980) did, in fact, find in their vitamin B-6 deficient animals an enhanced metabolic rate of the steroids as evidenced by both an increase in the number of activated receptors and their more rapid translocation into the nucleus.

Finally, vitamin B-6 is believed to play a role in lipid metabolism, since animals deficient in vitamin B-6 exhibit abnormal lipid metabolism. A group of Japanese workers (Iwami and Okada, 1982; Suzuki and Okada, 1982; Okada and Ochi, 1971; and Suzuki et al., 1976) have, based on evidence from rats made deficient in vitamin B-6, suggested a broad interaction of vitamin B-6 in lipid metabolism. In deficient animals, liver lipids accumulated, primarily triglycerides and cholesterol esters. There was a significant inverse relationship between liver triglycerides and pyridoxal, which they hypothesized might be due to decreased concentration of

phosphatidylcholine in the liver and an impairment in liver triglyceride export. They also observed a decrease in lipogenesis in the deficient animals. Additionally, cholesterol metabolism was altered, with increasing levels of bile acids, cholesterol and phospholipids in bile being noted in animals receiving PN-deficient diets. From their results they were not able to discern whether the overall alteration in cholesterol metabolism was due primarily to altered cholesterogenesis or to altered cholesterol catabolism.

Absorption

The B-6 vitamers are absorbed in the small intestine, primarily in the jejunum and secondarily in the ileum, by passive diffusion (Booth and Brain, 1962; Brain and Booth, 1964). It is mainly the free forms which are absorbed since an active phosphatase hydrolyzes most of the phosphorylated forms in the intestinal lumen (Lumeng and Li, 1975). Small amounts of the phosphorylated forms can be slowly transported into the mucosa then dephosphorylated, especially when present in high concentrations. Vitamin B-6 absorption is rapid, with half the dose disappearing in 10 minutes and 95% within 2 hours (Yamada and Tsuji, 1980). Some of the evidence which exists for a difference in absorption of the specific vitamers includes Wozenski and coworkers' (1980) report of smaller plasma and urinary responses to PM than either PN or PL and Yamada and Tsuji's (1980) report that PL was absorbed faster than PN and both PL and PN faster than PM.

Metabolism

Interconversion and Transportation. The biotransformation of vitamin B-6 has been included in reviews by at least 15 researchers since Snell and Haskell (1971) published their detailed metabolic review (Ebadi and Govitrapong, 1980). The highlights are summarized below.

Following absorption, the free forms of the vitamer are transported into portal circulation without interconversion occurring. Although some PN may enter the erythrocytes directly, the majority of the free forms enter the liver. Here PN, along with PM and PL, are phosphorylated by PN kinase (EC 2.7.1.35) to PNP, PMP, and PLP. This enzyme requires ATP and a divalent cation such as Zn^{+2} (or Mg^{+2}). The kinase enzyme has been found in most mammalian tissues (including liver, kidney, brain, skeletal muscle, spleen, large and small intestine, and blood cells) (McCormick et al., 1961) indicating that most tissues can phosphorylate the vitamers. The next step is one in which PNP and PMP are oxidized to PLP. This is accomplished by PNP (PMP) oxidase (EC 1.4.3.5), an enzyme which requires FMN as a cofactor (Wada and Snell, 1961). The liver has a very high concentration of the oxidase, but other tissues (such as kidney, brain, and erythrocytes) have much less and some tissues have none or extremely small amounts (lung, heart, pancreas, skeletal muscle) (Mehansho and Henderson, 1980). These latter tissues would likely not be able to oxidize the primary vitamers to PLP and thus would require prior conversion of PN and PM to PL by other tissues (Mehansho and Henderson,

1980). Hydrolysis of PLP (as well as PMP and PNP) is accomplished by non-specific phosphatases. Either free PL or PLP bound to albumin can be exported from the liver via plasma to other tissues (Lumeng et al., 1974). The liver is the main (or perhaps sole) source of newly synthesized plasma PLP (Lumeng et al., 1974). Erythrocytes can take up PN and convert it to PLP, but they lack a mechanism to release the PLP as such into the plasma or other tissues (Anderson et al., 1971). It would be necessary for the PLP to be hydrolyzed to PL prior to release. In fact, cell membrane transport of the vitamers in all tissues (except the liver) is considered to be as unphosphorylated forms because the charged phosphate group would hinder passage through any membrane (Snell and Haskell, 1971).

Regulation. In human tissues, PLP and PMP are the primary forms of vitamin B-6; only small amounts of the free vitamers are present. Because of the high reactivity of PLP towards proteins, including inactivation of enzymes in vitro, intracellular regulation is critical. Lumeng and Li (1980) reviewed the following four possible control mechanisms. Pyridoxal kinase, because of its widespread distribution in mammalian tissue and its essentiality in the formation of PLP, seems a likely candidate for a control point. However, comparison of PLP levels of various tissues with the kinase activity in tissues of rats and mice did not reveal any significant correlation (Bukin, 1976). These authors concluded that steady-state tissue PLP is controlled by factors other than pyridoxal kinase activity.

Another potential control point for tissue PLP is pyridoxine phosphate oxidase, also widely distributed in certain mammalian tissues. Snell and Haskell (1971) hypothesized this control, in conjunction with the other factors, would be effective when PN or PM is the substrate because PN-oxidase is subject to feedback inhibition by the product, PLP. Li and coworkers (1974) found that although the oxidase did affect hepatic PLP synthesis, it alone was not sufficient to account for the high degree of regulation of PLP concentration in vivo. Merrill and coworkers have verified this in rabbits (1978).

Hydrolysis of free or loosely-bound coenzyme may play a role in regulating the cellular concentration of PLP. This possibility was first explored by Anderson and coworkers (1971) in human erythrocytes. They inhibited the phosphatase with inorganic phosphate and found that PLP accumulated in erythrocytes. Lumeng and Li (1974) confirmed this in erythrocytes as well as in liver (Li et al., 1974). Since the phosphate affected only the phosphatase and not the kinase or oxidase enzymes, they concluded that hydrolysis of excess PLP appeared to be a significant factor in the regulation of cellular PLP concentration.

A fourth possible control mechanism is the binding of PLP by proteins. Li and coworkers (1974) demonstrated the protective effect of protein-bound PLP against hydrolysis by phosphatase in the liver and the plasma. However, when the PLP concentration exceeded the protein-binding capacity of the cells, it was rapidly hydrolyzed.

The authors suggest that this mechanism may function jointly with the previous one to maintain the steady state of tissue PLP.

Storage. Although liver is the primary site of PLP synthesis, the main storage site is skeletal muscle. About half of the body's vitamin B-6 is contained in muscle (Krebs and Fisher, 1964) mostly bound to glycogen phosphorylase (Black et al., 1977). Liver (Eisenstein, 1962) and the adrenals (Tiglao and Eisenstein, 1964) also have phosphorylase storage capacity in rats. During starvation or deficiency, phosphorylase content decreases, releasing vitamin B-6 for necessary transaminase reactions. In liver, PLP also binds to transaminases and some unidentified proteins (Bosron et al., 1978).

Albumin-bound PLP in the plasma may also serve as a short term storage for the vitamin. In this form it is largely protected from phosphorylase hydrolysis as well as other Schiff-base reactions.

Hemoglobin-bound PLP in the red blood cell could function as a further reservoir of vitamin B-6 storage (Lumeng and Li, 1980; Anderson, 1980). This may, however, be a trap since the removal of PLP from hemoglobin is difficult and requires rigorous conditions in vitro for freeing the vitamer for microbial assay (Miller and Edwards, 1981).

Degradation and Excretion. The major metabolite of the B-6 vitamers is 4-pyridoxic acid which is excreted in the urine. Its formation from pyridoxal is catalyzed by aldehyde oxidase (FAD-dependent) and aldehyde dehydrogenase (NAD-dependent) (Stanulovic 1980, Snell and Haskell, 1971). Lesser amounts of the free vitamers and

very small amounts of the phosphorylated forms are also excreted (Rabinowitz and Snell, 1949).

Kabir (1984) summarized the results of ten human metabolic studies in which the vitamin B-6 intake was known and urinary excretion of total vitamin B-6 and/or 4-pyridoxic acid was measured. In three studies, fecal excretion was also reported. There is a wide variation in factors which could affect the results. Dietary vitamin B-6 varied from 0.16 to 15.76 mg/day. Dietary protein varied from 20 to 150 g/day. The studies themselves varied in length from one to forty-three days. The percent of dietary vitamin B-6 excreted as urinary vitamin B-6 ranged from 3-25%. The percent of intake excreted as urinary 4PA ranged from 15-51% with several exceptions (one report each of 313%, 128%, 97%, 65% and 0%). Two studies reported comparable fecal excretion (30% and 28-46%) while the third reported a wide range (5-93%). Because of the variation of experimental conditions employed, it is difficult to make generalizations except that the excretion of 4PA and total urinary vitamin B-6 both reflect recent dietary intakes of vitamin B-6 (Sauberlich, 1981; Shultz and Leklem, 1981). It also seems that the level of dietary protein influences excretion of 4PA and the B-6 vitamers, since lowered excretions were found with higher protein diets (Canham et al., 1969; Miller and Leklem, 1978; Sauberlich et al., 1974).

Status Assessment

Clinical Methods. The diagnosis of vitamin B-6 deficiency with certainty by strictly clinical means is very difficult due to the non-specific symptoms. Sauberlich and Canham (1980) recently summarized these symptoms of vitamin B-6 deficiency in man, most of which have emerged from depletion studies. Personality changes characterized by irritability, depression, apathy and the loss of sense of responsibility, and somnolence have been noted. Oral lesions (cheilosis, glossitis and stomatitis) occurred but could not be distinguished from deficiency symptoms of other B vitamins. Acneform papular rash developed on the forehead. Seborrhea-like lesions also formed, especially around the eyes and in nasolabial folds. Pellegra-like dermatitis developed on the neck, arms, and thighs. Under the breasts and in other moist areas, intertrigo developed. Occasionally sensory peripheral neuropathy was noted, later impairing motor function. Weight loss accompanied the deficiency in all subjects. Anemia and changes in electroencephalograms have also been noted (Sauberlich, 1981; Ebadi, 1978).

Vitamin B-6 deficiency has not been documented in Americans free of pathological conditions except in newborns and infants maintained on low vitamin B-6 commercial liquid formula (Coursin, 1954). However, more than 20 instances of vitamin B-6 deficiency have been documented in various abnormal conditions including chronic alcoholism, sideroblastic anemias, some peripheral neuropathies and in the use of certain drugs (reviewed by Sauberlich, 1981). These

have been verified mostly by the relief of symptoms with vitamin B-6 administration. More commonly, however, assessment is made by biochemical means. There appear to be no published studies which document a significant relationship between hypovitaminemia and clinical signs of vitamin B-6 deficiency. Fleming (1982) reviewed vitamin deficiency studies on children. These studies indicate an absence of correlation between clinical symptoms and biochemical status evaluation in healthy populations as well as hospital in-patients.

Biochemical Methods. The biochemical assessment of vitamin B-6 nutriture generally involves the direct measurement of the vitamers in plasma or blood cells or of the urinary excretion of the vitamers or their principal metabolite, 4-pyridoxic acid. Indirect measures are also used. These involve assessing deviations from normal metabolism or enzymatic processes in which vitamin B-6 is required. At least five vitamin B-6 reviews by different research groups include details of status assessment in humans (Linkswiler, 1967a; Sauberlich et al., 1972; Shane and Contractor, 1975; Jerance and Stanulovic, 1982; Leklem and Reynolds, 1981). In this discussion, only currently used methods will be highlighted.

(1) Direct measurement by blood or plasma vitamers. Plasma pyridoxal 5'-phosphate is widely used and highly recommended as a sensitive and reliable indicator of vitamin B-6 status. It is a direct measure of the active coenzyme and, at least in rats, reflects tissue levels (Lumeng et al., 1978). Li and Lumeng (1981) report

that in humans, plasma PLP remains relatively constant over time on regular, unsupplemented diets or when vitamin B-6 is restricted or supplemented. However, plasma PLP concentration changes with alteration in dietary intake and has been observed to plateau within one month in women receiving 1.7 or 18 mg of pyridoxine (Brown et al., 1975). Thus, when using plasma PLP as an indicator of vitamin B-6 status, consideration must be given to the vitamin B-6 intake and whether or not the subjects are in metabolic equilibrium (Li and Lumeng, 1981).

(2) Direct measurement by urinary excretion of vitamers or 4-pyridoxic acid. This is one of few non-invasive procedures available and could prove useful with population groups. However, the excretion levels, like the circulating levels, do tend to reflect recent dietary intake (Canham et al., 1969). In cases of developing deficiency, these urinary excretion forms will become negative in advance of the appearance of a true deficiency; therefore, they alone cannot be used to diagnose a deficiency. But false positives are rare, so using these results to confirm plasma PLP or other tests could be helpful. In fact, Brown et al. (1975) found that changes in plasma PLP closely paralleled those of urinary 4-PA in depletion-repletion studies with young adult women. The ion-exchange chromatographic separation of 4-pyridoxic acid followed by fluorometric quantitation is precise and accurate (Reddy et al., 1958). HPLC may further improve these determinations (Gregory and Kirk, 1979).

(3) Indirect measurement by tryptophan metabolism. Because PLP is active at several sites in the metabolism of tryptophan (Brown, 1981), metabolites preceding these sites appear in the urine when inadequate vitamin B-6 is available. An example of this is xanthurenic acid excretion. However, the reason for the increase in excretion of this metabolite with vitamin B-6 deficiency seems anomalous (Brown, 1981). Since xanthurenic acid is formed by the action of PLP-requiring kynurenine aminotransferase, one might expect its excretion to be decreased with decreasing PLP availability. However, the alternative pathways for metabolizing kynurenine and 3-hydroxykynurine (by kynureninase) are also PLP dependent and are preferentially blocked in a vitamin B-6 deficiency state. Also the distribution of the transaminases between the cytosol and mitochondria plays a role, with the mitochondrial enzymes being slower to respond to a vitamin B-6 deficiency. Thus, the substrates for the aminotransferases build up and urinary excretion of xanthurenic acid and kynurenic acid are noted (Brown, 1981).

Measurement of xanthurenic excretion following the tryptophan load was one of the earliest measures of vitamin B-6 status (Council on Pharmacy and Chemistry 1951). The tryptophan load test is considered by one recent reviewer (Brown, 1981) to be one of the most sensitive and responsive indices of the functional adequacy of vitamin B-6 nutrition in healthy subjects. This test more nearly indicates coenzyme functional level, as opposed to dietary intake reflected by the previous direct methods. This method is non-inva-

sive and requires collcecting 24-hour urines. The ion exchange chromatographic separations and subsequent fluorometric quantitation are precise and accurate (Price et al., 1965).

(4) Indirect measurement by methionine metabolism. PLP is also required for several steps in the conversion of methionine to taurine. In cases of inadequate vitamin B-6, urinary excretion of cystathionine (an intermediate product) is increased. Stressing the pathway with a methionine load further increases the cystathionine excretion. Linkswiler (1981) reviewed several studies in healthy subjects and commented that dietary protein and/or methionine, as well as dietary B-6, affects the rate and extent of the altered methionine metabolism. This test is positive only in severe deficiency (Leklem et al., 1977).

(5) Indirect measurement by blood transaminase activity. PLP is a cofactor for many transaminase reactions. In human research, blood is the most easily available biopsy tissue for these enzymes. The isolation technique for leukocytes, coupled with the low level in blood necessitating large samples, makes vitamin B-6 status assessment by leukocyte transaminase level impractical. The level of plasma aminotransferases depends on cell death in various tissues of the body. Since a mechamism for regulating plasma aminotransferases is lacking, assessment of B-6 status by these enzymes is likewise unsatisfactory. Erythrocyte transaminase activity level and in vitro stimulation with PLP are other methods used to assess vitamin B-6 status. Although they probably do reflect the long-term dietary B-6

intake, the relationship to other B-6 status indicators has not been fully evaluated. Additionally differences in erythrocyte turnover time affect the results (Leklem and Reynolds, 1981). Therefore, the use of erythrocyte aminotransferases is not recommended as a primary B-6 status indicator (Leklem and Reynolds, 1981).

(6) Current position on biochemical status assessment. Leklem and Reynolds (1981) summarized the presentations of many scientists attending a recent vitamin B-6 methods workshop. They recommended the following triad of measurements to give a complete picture of the body's vitamin B-6 at one point in time: plasma pyridoxal 5'-phosphate, urinary 4-pyridoxic acid, and urinary tryptophan metabolite excretion after a tryptophan load test. They emphasize that including a measure of dietary intake of both vitamin B-6 and protein is a further improvement for overall vitamin B-6 status assessments.

Dietary Intake Methods. There are three major methods to determine dietary intake, each with its own special characteristics. Dietary recall methods are considered adequate for assessing intakes of large groups of subjects (Young et al., 1952), but the reliability is lower for elderly subjects when compared to younger groups (Campbell and Dodds, 1967). The elderly have difficulty estimating amounts of food eaten and remembering all foods. They commonly underestimate calories (Young et al., 1952; Madden et al., 1976; Campbell and Dodds, 1967) and coincidentally, but not necessarily proportionally, other nutrients. Also, being very short-term, 24-

hour recalls may not reflect regular long-term food consumption patterns. Because they are short-term, they are less expensive to carry out.

Diet histories cover longer periods of time and attempt to obtain information about general food consumption patterns. They eliminate the short-term variability of recalls, but they are expensive to supervise. As with the recall, inaccuracy of recalling food patterns results in errors (Young et al., 1952). Protein can be overestimated (Marr, 1971).

Food records or diaries, if obtained from measured or weighed food portions, can be the most accurate but may influence what the subjects choose to eat. This method has been successfully used in an institutional setting (Mitchell and Leklem, 1985).

Irrespective of the method of obtaining the dietary intake information, errors will still result from analyzing and interpreting the data. Food composition tables (Orr, 1969; Watt and Merrill, 1975), especially for nutrients like vitamin B-6, are incomplete, leading to underestimates of vitamin. Processing and food preparation techniques have a variable influence on retention of vitamin B-6 (Gregory and Kirk, 1981; Kabir et al., 1983), which are often not accounted for, potentially leading to overestimates of vitamin B-6 intakes.

Evaluations of the intake data are most commonly based on the Recommended Dietary Allowances (RDA 1980). These have been set for young adults as the amount required for optimal health plus two

standard deviations to cover 97.5% of the population. Requirements for the age group 51 years and older is based on extrapolation of the needs from the younger adults. In 1980, the Committee on Dietary Allowances concluded that RDA's for the older group could not be adequately established based on the limited studies available (RDA, 1980). With this in mind, one can still conclude that if a group intake mean is below the RDA, some individuals in that group will be at risk. For most accurate assessment of vitamin B-6 status, nutrient intake data should be combined with clinical and biochemical measures.

Vitamin B-6 Status in Aging Americans

The nutritional status of aging Americans is becoming an increasingly important concern for many researchers. The main reason is that this older group is increasing proportionately more rapidly in the U.S. than is any other age group (Bureau of Census 1976). However, they use a disproportionate amount of the health care funds (Bureau of Census 1976). Nutrition can be an important tool in sustaining optimum health. In order to use this tool effectively, there must be a base of information on which to build the applications. A number of studies have been carried out to assess vitamin B-6 status based on biochemical methods and intake evaluations. This section will review the evidence available on the vitamin B-6 status in aging Americans based on clinical, biochemical and intake assessments. Age-associated physiological changes which may account for the status

findings will be reviewed. This section will be concluded by information pertaining to the requirement for vitamin B-6.

Dietary Intake Evidence. With increasing age, people tend to decrease their caloric intake and coincidentally the intake of many nutrients (Barrows and Roeder, 1977). Some groups of people (e.g. low income elderly) have been singled out as being at risk because intakes do not meet RDA's for a significant proportion of them for certain key nutrients including vitamin B-6 (Vir and Love, 1977, 1979; Garry et al., 1982; Jacobs et al., 1968). Since the manifestation of these deficiencies as clinical changes is very rare, the significance of these findings is not yet fully understood. The following discussion is a summary of some of the results of surveys conducted to estimate dietary intake of vitamin B-6. Most are flawed in two respects. First, the reliability of the methods for acquiring food intake information from older subjects has not been determined. Secondly, the incompleteness of the food composition tables (Sauberlich, 1981) from which the intake values were calculated results in significant underestimate of vitamin B-6 intake. Additionally there is a problem with accurately reflecting food processing and preparation losses. Food composition tables do provide values for some products which have undergone some processing, such as canning and freezing (e.g. Orr, 1969). Jacobs et al. (1968) reported a group of elderly (>65 yr.) men and women consumed 1.45 mg vitamin B-6 per day. This intake is very nearly the same as Hampton et al. (1977) and Chrisley and Driskell (1979) reported for men, but

higher than their values for women. Hampton et al. (1977) found 17 healthy men over age 60 consuming an average of 1.46± 0.64 mg/day, but approximately one-fifth consumed less than 50% of the RDA. The men over age 60 in the study of Chrisley and Driskell (1979) consumed an average of 1.5 mg B-6/day, while the intake of the women averaged 1.1 mg/day. Both studies were based on 24-hour recalls and 2-day records from healthy volunteers. One recent study of Oregonians (Extension Service 1983) revealed low intakes of vitamin B-6 for all three age categories, ranging from 37% of those under 30 falling below the RDA to 43% of those over 65 also consuming less than the RDA.

It must be remembered that low intake data alone are not equivalent to low status, although they may be useful in understanding the potential for inadquate B-6 status (Leklem and Reynolds, 1981). There is no evidence that an inadequate diet itself has led to vitamin B-6 deficiencies as determined by biochemical parameters within the group of otherwise healthy aging adults. Before such a relationship can be proven, more reliable information needs to be obtained on the actual amount of vitamin B-6 being consumed, the effect of food processing and preparation on nutrient retention, and also the bioavailability of the vitamin from various sources (Sauberlich, 1981).

Biochemical Evidence. Numerous groups of researchers have attempted to assess vitamin B-6 status in the aging population using all of the methods previously discussed. There seems to be overall agreement that vitamin B-6 biochemical inadequacy is a problem among

the elderly, but the significance of this finding is unknown. To date, the biochemical inadequacy has not been correlated with clinical findings, although widespread occurrence of non-specific physical and mental dysfunction among the elderly contribute to the confusion in this area (Fleming, 1982). Summarized here are the findings grouped by the type of biochemical parameter measured.

(1) Plasma Pyridoxal 5'-phosphate. Lower plasma PLP values have been documented in older subjects (Hamfelt, 1964; Walsh, 1966; Chabner and Livingston, 1970; Lumeng and Li, 1974; Rose et al., 1976). It should be pointed out that since women have lower PLP values than men (Chabner and Livingston, 1970; Shultz and Leklem, 1981), these results are not clear cut because of the combination of sexes at different ratios in the studies. The study of Rose and coworkers (1976) divided men by age into decades and found a progressive decrease from mid-life. For 414 subjects, they found 14.6 ng/ml for 18-29 yrs; 14.8 for the 30's, 13.2, 40's; 11.6, 50's; 12.2, 60's; 11.8, 70's; and 7.7, > 80. This is a decline of 0.9 ng/ml/decade. The decreases found by Hamfelt (1964) are even more dramatic. At age 20-29, plasma PLP's were 11.3 ng/ml; 30-59, 7.1; and >60, 3.4. It is interesting that some subjects in the >60 year group had values of "0" ng/ml. Rose et al. (1976) studied healthy business and professional men living in comfortable circumstances for whom money presumably did not limit their food intake. Hamfelt did not identify the sex of his subjects and since they were composed of a cross-section of blood donors, it seems reasonable to assume that

at least some would have had restricted food budgets. Lumeng and Li (1974) included a group of 94 healthy male volunteers in an alcohol abuse investigation and found age-related decrements in plasma PLP for both healthy and alcoholic subjects. Their 20-year old group averaged 13 ng PLP/ml; 30's, 12; 40's, 11; 50's, 9; and 60's, 8 ng/ml. The alcoholics, by comparison, dropped from an average of 7 ng/ml for those in their 20's to 4 ng/ml for those 60-70 years of age. Both subject numbers and range decreased with age in this study. Also, the minimum values in the healthy group, approximately 5-6 ng/ml, occurred with about equal frequency in each decade, suggesting a constant "low" group. The decreasing means seemed to reflect a loss of the high values over time. Nearly 50% of the values for the 20-year olds exceeded 15 ng/ml whereas by age 50, none of the values even reached 12 ng/ml. Shultz and Leklem (1981) did not find significant differences in plasma PLP which could be attributed to subject age although there was a trend for PLP to decrease with age.

(2) Plasma Total Vitamin B-6. This indicator of status does not appear to have been previously reported for assessment of vitamin B-6 status in aging populations.

(3) Blood Transaminases. This category of functional tests includes several different PLP-dependent enzymes which have been used in an attempt to assess vitamin B-6 nutriture. Unfortunately, some researchers focused on plasma/serum transaminases, which do not likely play a metabolic role in vitamin B-6 metabolism or provide a

useful picture of overall status. They owe their presence in plasma to the degradation of cells in other tissues and can reflect numerous pathological conditions. Ranke and coworkers (1960) found elderly subjects had a much lower serum GOT than younger subjects, but the activities were equalized after vitamin B-6 supplementation. They state that their data suggest the depressed SGOT activity is not due to an agewise decrement in vitamin B-6 intake. Hamfelt (1964) also found a greater increase in SGOT in the presence of PLP among older hospitalized patients. He mentioned that three patients with the highest stimulation values also had low plasma PLP and pathological tryptophan load tests. Values of one person did not return to normal after vitamin B-6 supplementation. Rose and coworkers (1976) found no significant decrease in plasma GOT with age.

Erythrocyte transaminases have been widely used in studies focusing on vitamin B-6 nutriture. These enzymes do reflect long-term B-6 intake but they are not as sensitive, rapid, reliable or helpful as other indices (Shane, 1978; Brown, 1981). It is not currently recommended that either the enzyme activity nor its stimulation in the presence of excess PLP be used as primary indicators of vitamin B-6 status (Leklem and Reynolds, 1981). Chen and Fan-Chiang (1981) found a significant increase in the activity coefficient of EGPT with age, on the basis of which they claimed that aging is associated with a decrease in vitamin B-6 status. They found no sex difference between 42 men and 17 women. In their study based on 17 men and 10 women over age 60, Hampton and coworkers

(1977) found no significant difference in EGPT activity related to sex, age, use of vitamin supplements, money spent on food, or meals away from home. They stated that there was a relationship between percent stimulation of EGOT activity and dietary intake, but when correlations were calculated from their published data, the results did not appear to support their conclusion. In one-third of Driskell's subjects above age 60, stimulated EGPT activity indicated inadequate vitamin B-6 status (1978). In Chrisley and Driskell's (1979) study, less than 3% of the subjects under age 59 could be classified as biochemically deficient whereas 24% of their men and 30% of their women over age 60 had enzyme stimulation values indicating subclinical deficiency. Rose and coworkers (1976) found that EGOT was not lower in their older subjects. Vir and Love (1978) found biochemical evidence for vitamin B-6 deficiency based on EGOT stimulation values. Nearly half of their subjects over age 65 showed >15% stimulation. It is interesting to note that 9% of those receiving vitamin B-6 supplements of 2.5 mg/day were also in this deficient category. These findings were not accompanied by any clinical or subjective symptoms of deficiency. Smith and coworkers (1984) assessed vitamin B-6 status in 146 institutionalized elderly by EGOT stimulation and found 28% to be of low status. Lemoine and coworkers (1980) found no correlation between EGOT stimulation and clinical symptoms in 656 hospital in-patients. In fact, their "controls" had a higher incidence of deficiency (nearly double) than any of the

patients evaluated, including alcoholics and some with other liver diseases.

(4) Urinary Total Vitamin B-6. This indicator has been infrequently used to assess vitamin B-6 status. It is greatly influenced by recent dietary intake without reflecting size of body storage pools or state of deficiency (Shultz and Leklem, 1981; Sauberlich, 1981). Shultz and Leklem (1981) found a trend (but not significant relationship) for urinary total B-6 to decrease with age in men but not women. They also reported a consistent sex difference, with women's values being approximately 75% of the men's (0.92 $\pm$ 0.49 and 0.76 $\pm$ 0.24 umol/24 hr). Because of the size of the standard deviations, both groups contained some individuals falling below the 0.59 umol/24 hr thought to suggest inadequate intake (Linkswiler, 1967b). Sauberlich and coworkers (1972) recommended similar cutoffs with less than 0.33-0.53 umol/24 hr being considered reflective of inadequate vitamin B-6 consumption.

(5) Urinary 4-pyridoxic Acid. This major metabolite of vitamin B-6 has been measured by several groups investigating vitamin B-6 status. Chen and Fan-Chiang (1981) studied elderly who were institutionalized versus free living and found over 20% of their subjects to be below the critical minimum of <2.73 umol/g creatinine given by Woodring and Storvick (1970). However, they did not separate results by sex or age and it is known that there are consistent sex differences. Shultz and Leklem (1981) found men excreted 7.5 $\pm$ 4.3 umol/24 hr while women excreted 5.6 $\pm$ 3.1 umol/24 hr. There are

conflicting reports of the change of 4-PA excretion with age. Simon and coworkers (1982) reported an age-related increase for both sexes. However, Shultz and Leklem (1981) found a non-significant trend in the opposite direction. Because 4-PA excretion reflects immediate dietary intake (Sauberlich et al., 1974), the sex differences might be explained by the fact that women eat less than men. Likewise, the report by Shultz and Leklem (1981) of decreasing excretion with age might also be a reflection of dietary reductions accompanying the decreased caloric intake of the elderly. This is supported by the significant inverse relation they observed between age and energy intake in a group of male vegetarians (Shultz and Leklem, 1983). Sauberlich and coworkers (1974) suggested that 4-PA excretion of less than 2.7-4.4 umol/24 hr indicated subjects may be consuming inadequate dietary levels of vitamin B-6. Linkswiler's cutoff was higher at 5.5 umol/24 hr (1967). Shultz and Leklem's values, based on data relating 4-PA and dietary intake, were <5.0-5.7 for men and <4.6-5.2 for women classified as having marginal status (1981).

(6) Urinary Trypotophan Metabolites. The tryptophan load test is used to detect vitamin B-6 deficiency or clinical problems involving tryptophan metabolism. It is considered by some to be one of the most sensitive and responsive indices of functional adequacy of vitamin B-6 nutriture. However, because the urinary excretion of tryptophan metabolites does not change very much over a considerable range of adequate vitamin B-6 intakes, this method must be used with

caution in assessing vitamin B-6 nutriture in healthy subjects (Brown, 1981).

Ranke and coworkers (1960) reported that their men and women subjects (average age 75 years) excreted twice as much XA after a tryptophan load as did their young subjects (average age 25 years). The excretion was normalized after 3 weeks supplementation at 15 mg of vitamin B-6/day. The young subjects excreted 16.5 $\pm$ 3.6 mg XA/24 hours after a 15 g DL-tryptophan dose. The older subjects excreted 32.6 $\pm$ 6.3 mg before and 15.4 $\pm$ 1.9 mg/24 hr after supplementation. The young subjects were not supplemented with vitamin B-6.

In Hamfelt's early study (1964), eleven normal subjects older than 60 yr who had low mean plasma PLP values (1.8 ng/ml) also had a high mean XA excretion (15.8 umol/kg/24 hr) after a tryptophan load of 100 mg/kg body weight. Their mean excretion of xanthurenic acid became normal (1.6 umol/kg/24 hr) after 7 days supplementation with 100 mg of vitamin B-6/day, during which time the mean plasma PLP value also rose (38.3 ng/ml).

In a table of criteria for vitamin adequacy reviewed by Fleming (1982), xanthurenic acid in urine was the only method selected for determining vitamin B-6 adequacy by the Interdepartmental Committee for National Defense in 1957. Based on observations of healthy 25-year old men, they determined "low" status to be 50-99 mg XA/24 hr and "deficient" to be anything >100 mg XA/24 hr, but Fleming did not state the load used.

Leklem and Reynolds (1981) recommended that the triad of tryptophan load test, plasma PLP and urinary 4-PA be used together to give a complete picture of vitamin B-6 status. They stated that at least two of these biochemical indices, in conjunction with dietary intake data (vitamin B-6 and protein), are necessary to properly assess vitamin B-6 status.

Clinical Evidence. There is no evidence to date of any clinical deficiencies of vitamin B-6 in an otherwise healthy (e.g, not hospitalized for alcoholism, chronic liver impairment and other pathologies) group of aging Americans. There is an increase in the number of non-specific symptoms which suggest that general morbidity and decreased appetite may be related to impaired vitamin status (Lemoine et al., 1980). Data available can neither confirm nor refute this.

Age-related Physiological Changes. Aside from decreased dietary intake, based on a number of psychological, sociological, and physical factors beyond the scope of this review, a number of age-related changes have been documented which have been hypothesized to account for reduced nutritional status. None has been correlated with a decrease in vitamin B-6 status directly, but all are possibilities.

A major age-related change is the increased incidence of certain diseases. Fleming (1982) estimated that 85% of the population over 65 suffers some chronic disease. A number of these diseases, including cancer and liver cirrhosis, have serious consequences related to

the utilization of vitamin B-6. Likewise, many drugs (such as hydralazine, an antihypertensive) are known to be antagonistic to vitamin B-6 (Baker et al., 1980). However, this review will focus on the healthy population.

Baker et al. (1979) summarized age-related changes in some factors which could affect digestion of the foods (or supplements) which provide the vitamin B-6. These factors include decreased mastication due to decreased dentition, and decreased secretion of gastric hydrochloric acid along with decreased secretion of gastric enzymes. These factors all act in the direction of making digestion less complete, thereby potentially contributing to a lower status.

Although Southgate and Durnin (1970) found no age-related impairment of absorption of carbohydrates, fats or proteins, other workers have documented findings which could decrease the absorption of vitamin B-6. Holt (1982) reviewed studies which found a decreased cell proliferation rate with age. He also reviewed evidence for a decrease in the absorptive surface of the villi. Since the total surface area is one controlling factor in the absorption of nutrients (including vitamin B-6) by passive diffusion, any decrease in surface area should result in decreased absorption of the vitamin. From a quantitative standpoint, this would not likely be important because of the large excess in intestinal absorptive capacity for the B-6 vitamers (Johansson et al., 1966). A decrease in the mucosal blood supply has been suggested (Holt, 1982) and could also be a factor here.

Subsequent to absorption, the vitamin is metabolized. Defective metabolism could account for less vitamin B-6 being activated to the coenzyme form. Altered reaction rate or altered capacity for reaction of the activating enzymes can decrease the coenzyme levels. Since most major organs, including the liver, exhibit a net cell loss with aging (Libow, 1981), the metabolizing capacity could definitely be decreased with age. Sherman and Libow (1981) point out that liver function is decreased in older persons. Hamfelt (1964) hypothesized that decreased phosphorylation could be a factor in the declining vitamin B-6 status values he observed with age. The age-related increased action of alkaline phosphatase (Gillibrand et al., 1979) could be responsible for dephosphorylating more of the PLP, making more PL available for catabolism to 4-PA.

An age-related increase in urinary loss of vitamin B-6 could account for a decrease in vitamin B-6 status. An age-related decrease in binding sites in the liver has been documented and hypothesized to reduce the number of binding sites for vitamin storage. Thus more vitamin B-6 could be excreted (Baker et al., 1979). Likewise, any increase in catabolism of vitamin B-6 could be a factor here. Age-related decreases in glomerular filtration rate and renal plasma flow, which likewise could influence vitamin B-6 excretion, have also been documented (Moment, 1982).

Vitamin B-6 Requirement. When the revisions for the recent update of the RDA's were being made, the Committee on Dietary Allowances reviewed reports of vitamin B-6 inadequacy in the elderly

but did not consider the evidence sufficient to formulate specific RDA's for the over 50 year old group (RDA 1980). Until information is available to identify reliably measured biochemical indices which reflect optimal body stores of vitamin B-6 or what indices correspond to the first metabolic, functional or morphological disturbance, it will be difficult to establish an exact requirement. In the meantime, an extrapolation of the data from younger adults must suffice. This may not be an erroneous approach, for vitamin B-6 at least, since for the rat studies reviewed by Fleming (1982) no age difference in requirements from weaning to 24 months (elderly) was found.

An increased need for vitamin B-6 in elderly humans has been suggested by results of some supplementation studies. The age-related blood decreases of vitamin B-6 and altered functional tests which could be brought into young adult range were used as evidence that the elderly have a greater need for vitamin B-6 to maintain the same status index values. But the increased need must be related to the current dietary level also, which is often lower for the elderly. Shultz and Leklem (1981) cited eight studies where intakes of 1.25 to 1.50 mg PN/day were adequate to prevent elevated levels of tryptophan metabolites following the tryptophan load test and three studies where the same supplemental level of PN corrected 4-PA and UB6 levels in young persons depleted of vitamin B-6. However, a recent study (Baker et al., 1980) involving 228 ambulatory nursing home residents (mean age = 87 years) who consumed a "good" diet under supervision

along with a daily multivitamin pill containing 2 mg PN revealed vitamin deficits in 39% of the subjects, with vitamin B-6 being most frequently cited (40% of the single vitamin deficits and 19% of multi-vitamin deficits based on blood total vitamin B-6 levels by protozoan assay). One intramuscular injection of 70 mg of PN brought 90% of the deficits into the range of the healthy 20-50 year olds when blood vitamin B-6 was measured 3 months after the PN injection. Vir and Love (1978) have recommended a minimum requirement of 2.5 mg vitamin B-6/day and have suggested a recommended allowance of 25 mg/day for the elderly. This suggested increase in the RDA has not been supported widely.

Approach

To accomplish the objectives of this study, a seven-week, two part experiment was conducted with five young women and eight middle-aged women. These subjects were healthy and normal based on physical examinations, blood biochemical analyses and intestinal absorption tests. They were all sedentary, since exercise can affect vitamin B-6 metabolism. They were not users of alcohol, nicotine, hormones or other drugs which could affect vitamin B-6 metabolism or its subsequent laboratory measurement. They were not using nutritional supplements immediately prior to or throughout the study, since all subjects had to receive identical nutrient intakes.

Throughout the study, the intake of vitamin B-6, along with all other nutrients, was controlled and constant for all subjects. Dur-

ing the first four experimental weeks, the vitamin B-6 level was approximately equal to the RDA, enabling observation of response differences under normal recommended intake levels. During the final three experimental weeks, the diet was supplemented to five times the RDA so that the responses of both groups of women could be observed at a higher, but not pharmacological, level of vitamin B-6 intake.

During the experiment, vitamin B-6 status was assessed by five different commonly used methods because of the lack of consensus of researchers as to which biochemical measurements are best and because at least two are recommended to properly assess vitamin B-6 status. There were two measures of the circulating vitamers: plasma total vitamin B-6 and plasma pyridoxal 5'-phosphate; two measures of excreted vitamers: urinary total vitamin B-6 and urinary 4-pyridoxic acid; and one functional measure: urinary tryptophan metabolites excreted following a tryptophan load. The activity of erythrocyte pyridoxine kinase was measured along with the level of plasma and erythrocyte magnesium. Those parameters were compared between the two age groups at both vitamin B-6 intake levels.

Chapter 2

DIFFERENCES IN VITAMIN B-6 STATUS INDICATOR RESPONSES BETWEEN YOUNG AND MIDDLE-AGED WOMEN FED CONSTANT DIETS WITH TWO LEVELS OF VITAMIN B-6

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ABSTRACT

To investigate the responses of healthy adult women to two levels of vitamin B-6, a seven-week two-part metabolic experiment was conducted with five young adults and eight middle-aged subjects. During the first four weeks, a constant diet containing 2.3-2.4 mg vitamin B-6 intake per day was fed. In the final three weeks, the same diet was supplemented with 8.0 mg pyridoxine to give an intake of 10.3-10.4 mg vitamin B-6 per day. Plasma pyridoxal 5'-phosphate (PLP), plasma and urinary vitamin B-6 (PB6 and UB6), and urinary 4-pyridoxic acid (4PA) excretion were monitored throughout the study. Two-gram tryptophan load tests (TLT) were administered twice at each vitamin B-6 intake. The older women had significantly lower plasma PLP, PB6 and UB6 and slightly higher urinary 4PA values with normal vitamin B-6 intakes. With supplementation in both age groups, the circulating status indicators increased 3-4 fold and urinary indicators increased 5-6 fold. During the supplemented weeks, only the difference in UB6 remained significant between the two age groups. The TLT revealed no significant between-group differences in xanthurenic acid or kynurenic acid excretion although the older group consistently excreted higher levels of these metabolites following the tryptophan load. These results demonstrate an age-related difference in vitamin B-6 status in women under controlled conditions of dietary intake with adequate vitamin B-6 and support studies done

under less controlled conditions. The decrease in circulating levels and increase in urinary 4PA may be a reflection of age-related metabolic changes suggesting increased catabolism. The disappearance of significant age differences in three of four status indicators with pyridoxine supplementation suggests there may be age differences in the requirement for this vitamin; however, the absence of differences in the TLT responses suggests that 2.3 mg vitamin B-6 per day is adequate for all of the women studied.

These results reveal an age-related difference in vitamin B-6 metabolism. In addition they provide useful information for establishing a requirement for vitamin B-6 in older women.

INTRODUCTION

Comparison of the vitamin B-6 status of older versus younger adults based on intake studies (Driskell and Chrisley, 1981; Garry et al., 1982; Hampton et al., 1977) and biochemical assessments (Hamfelt, 1964; Rose et al., 1976; Crepaldi et al., 1975; Vir and Love, 1978; Baker et al., 1979 and 1980; Chen and Fan-Chiang, 1981; Darcy, 1984) suggest there is a difference in vitamin B-6 status which is related to age. Plasma pyridoxal 5'-phosphate (PLP) has been shown to decrease with increasing age (Hamfelt, 1964; Rose et al., 1976; Lumeng and Li, 1974). Total vitamin B-6 in blood is also lower in elderly compared with younger persons (Baker et al., 1979, 1980). Older women excrete less UB6 but more 4PA than younger women (Darcy, 1984). Response to the TLT shows elderly individuals excrete

more xanthurenic acid (XA) than young adults (Crepaldi et al., 1975; Ranke et al., 1960; Leklem, 1971). Based on erythrocyte pyruvate aminotransaminase stimulation, vitamin B-6 status was found to be low in over one-quarter of non-institutionalized (Hampton et al., 1977; Chen and Fan-Chiang, 1981) and hospitalized (Vir and Love, 1978) elderly. However, none of these studies controlled for some important variables that might exist between the age groups which could have influenced the results. These variables include dietary intakes, activity levels, lean body mass and intestinal absorption. The present study was carried out to investigate the responses of status indicators in adult women to two levels of vitamin B-6 when all were being fed the same diets. Women were selected as subjects because metabolic studies have tended to neglect them and because they are at potentially greater risk of deficiency due to their smaller dietary vitamin B-6 intakes, which decrease from young adulthood (USDA-SEA 1980). We chose five different commonly used methods to assess vitamin B-6 nutriture (Li and Lumeng, 1981; Shultz and Leklem, 1981; Brown, 1981; Sauberlich, 1981) because of the lack of consensus of researchers as to which biochemical measurements are best and because at least two are recommended to properly assess vitamin B-6 status (Leklem and Reynolds, 1981). We did not include aminotransferase activity or stimulation in this seven-week study because of their reflection of long-term dietary vitamin B-6 intake (Leklem and Reynolds, 1981), which was beyond our scope.

MATERIALS AND METHODS

Subject Selection

Subjects were 13 healthy volunteers who were judged normal based on physical examinations, blood biochemical analyses, and xylose absorption tests. From the outset of the study, both groups had normal vitamin B-6 status based on plasma and urinary indicators measured. They had no history of intestinal, renal or metabolic disorders which could influence absorption, metabolism or excretion of vitamin B-6. All were sedentary and did not alter their activity levels throughout the study except four times to ride a stationary bicycle for 20 minutes as part of an exercise investigation. They did not use alcohol, nicotine, hormones or other drugs which could influence vitamin B-6 metabolism or determination. They were free from food allergies. They did not use vitamins or other nutritional supplements four weeks prior to the study. All of the middle-aged women were post-menopausal.

Before the study, all subjects were informed of the purpose and associated risks. Each signed informed consent forms. This study was approved by the Committee for Protection of Human Subjects at Oregon State University.

The experimental groups were composed of eight middle-aged women (55.3 $\pm$ 4.0 yr) and five young adults (24.4 $\pm$ 3.2 yr). The physical characteristics of the groups are summarized in Table 2.1. Although the older group was 20% heavier than the younger group, when their

lean body mass was estimated by subtracting their kg of body fat (determined by the method of Jackson et al., 1980) from their total weight, the groups were not significantly different ($p > 0.05$).

Diets

The foods comprising the diet were selected for nutritional content, appeal, common usage, cost and availability in a form which would not change appreciably over the seven experimental weeks. Table 2.2 shows the two menus which were used. The moderate carbohydrate diet (providing 2.3 mg of vitamin B-6 per day) was fed for weeks 1, 2, 4, 5 and 6. During weeks 3 and 7 a high carbohydrate diet (providing 2.4 mg of vitamin B-6 per day) was fed. The high carbohydrate diet was included to accommodate co-investigators using the same subjects for an exercise study (Manore, 1985; Walter, 1985) and did not affect these results as indicated by ANOVA. Throughout weeks 5, 6 and 7 the diet was supplemented with 8.0 mg of pyridoxine, given daily at breakfast as the hydrochloride dissolved in 1% acetic acid. Thus the diet contained 10.3 mg of vitamin B-6 during weeks 5 and 6 and 10.4 mg of vitamin B-6 during week 7. The vitamin B-6 content of the diet was determined from analyses of food composites made each week.

To minimize the day-to-day nutrient variation, sufficient quantities of all foods for the entire study (except milk) were purchased from one lot. When a food contributing significant vitamin B-6 to the diet was expected to be variable (e.g., canned tuna, frozen

turkey), the amount for the whole study was combined prior to beginning the experiment then repacked randomly into one-day portions. Sufficient pyridoxine HCl (PN) was prepared in 1% acetic acid at the start of the supplementation period and frozen as individual doses in glass vials at -20°C until used. Throughout the seven weeks, weight maintenance was achieved by adjusting quantities of vitamin B-6-free foods (salad dressing and margarine). Meals were prepared in the metabolic kitchen of the Department of Foods and Nutrition by nutritionists, assisted by trained nutrition students. Every portion of each food was individually weighed and consumed under supervision. Iron supplements were given on alternate days (32 mg ferrous sulfate).

Sample Analyses

Twenty-four hour urine collections were made throughout the study and monitored for completeness by creatinine analysis (Pino et al., 1965). A portion of each sample was frozen until analyzed. Blood and urine samples from experimental weeks 2, 3, 6 and 7 were analyzed for the vitamin B-6 status indicators. Each diet period began on a Monday. Urinary vitamin B-6 (Miller and Edwards, 1981) and urinary 4-pyridoxic acid (Reddy et al., 1958) were measured on samples collected each Tuesday, Wednesday and Friday. A food composite representing the food for one day was prepared each Tuesday and subsequently analyzed for total vitamin B-6 (AOAC 1980). Fasting blood samples were drawn from the antecubital vein prior to breakfast

each Wednesday and Saturday for analysis of plasma PLP (Chabner and Livingston, 1970) and plasma vitamin B-6 (Miller and Edwards, 1981). Hemoglobin and hematocrit values were determined on blood samples collected Saturday of each week. Inter-assay coefficients of variation were: Plasma PLP $\pm$ 6% (22 replicates), total PB6 $\pm$ 6% (14 replicates), total UB6 $\pm$ 3% (18 replicates) and 4-pyridoxic acid $\pm$ 8% (35 replicates). Two grams of L-tryptophan were given with breakfast the last two Wednesdays of each experimental week. Urine samples from the day before and the day of the load test were assayed for xanthurenic and kynurenic acid (Price et al., 1965). One non-experimental week (continuing the unsupplemented, moderate carbohydrate diet) was inserted between the two levels of vitamin B-6 to ensure that the young subjects were in the same portion of their menstrual cycles during blood sampling and tryptophan load tests for both non-supplemented and supplemented samples. Body weight was measured before breakfast each day and changes were corrected by adjustment in energy intake as previously mentioned.

Statistical Methods

The data was analyzed by standard statistical techniques of Student's t-tests, analysis of variance and correlations based on linear regression best-fit equations (Snedecor and Cochran, 1980). A p-value of ≤ 0.05 was considered significant.

RESULTS

Under conditions where dietary intakes were identical, middle-aged women showed biochemical evidence of lower vitamin B-6 status than their younger counterparts (Table 2.3). It should be stressed that all parameters were within accepted normal value ranges of adult women for both of the study groups (Shultz and Leklem, 1981). The significance of the age-related differences observed are summarized in Table 2.4

The initial values, obtained from samples collected at the beginning of the study, reflect conditions prior to the experiment. They are included for reference in Table 2.3. For both age groups, the initial mean urinary vitamin B-6 and mean urinary 4-pyridoxic acid excretion levels were above the minimum values suggested for women (Donald, 1978; Shultz and Leklem, 1981). Plasma values for the younger women also exceeded the plasma pyridoxal 5'-phosphate minimum, whereas those of the older women were slightly below the minimum acceptable level of 32 nM for this circulating vitamer (Shultz and Leklem, 1981). Both circulating indices of vitamin B-6 status increased during the first week of the study, indicating that many of the women had not been consuming diets containing 2.3 mg of vitamin B-6/day.

Under conditions where all subjects were receiving idential dietary vitamin B-6, significant age differences were noted by week 2 of the experiment. In the middle-aged women, the mean level of plasma pyridoxal 5'-phosphate was two-thirds that of the younger

group ($p < 0.05$). There was no further increase in these levels at the end of week 3 for either group. The average of the PLP values for weeks 2 and 3 are shown in Figure 2.1. Supplementation resulted in a 3-4 fold increase in plasma pyridoxal 5'-phosphate in both age groups. Although the older women's values were only 80% of the younger group, the standard deviations were correspondingly increased and the age-differences were no longer significant (Figure 2.1). Plasma pyridoxal 5'-phosphate values plateaued within one week of beginning supplementation and there was no significant difference in the values for either group between weeks 6 and 7.

Plasma total vitamin B-6, of which 75% was pyridoxal 5'-phosphate, followed a similar pattern. By week 2, significant age differences ($p < 0.05$) were observed, with the mean levels of the middle-aged women being about three-quarters of the means of the younger group. These values did not change significantly during week 3. As with plasma pyridoxal 5'-phosphate, total plasma vitamin B-6 increased 3-4 fold in both groups with supplementation and did not change further from week 6 to week 7. Age related differences in total plasma vitamin B-6 were not significant during the supplemented period.

The mean values of the urinary indices for weeks 2 and 3 also increased from the initial values, although to a lesser extent than the circulating indices (Table 2.3). Sequential analysis of daily urine samples confirmed that individuals adjusted to the 2.3 mg vitamin B-6/day within one week at the start of the experiment and

again within one week from the onset of supplementation with 8.0 mg/d pyridoxine (data not given). At every sampling period, middle-aged women excreted only about 80% as much total urinary vitamin B-6 as compared to the younger group. These age differences were significant only during the unsupplemented weeks 2 and 3. Vitamin B-6 supplementation resulted in a four-fold increase in total urinary vitamin B-6 for both groups. Again, because of the wide variation in individual responses, the age-related differences were not significant during supplemented weeks 6 and 7.

Excretion of 4-pyridoxic acid differed by less than 10% between the age groups at every sampling period (Table 2.3 and Figure 2.1). This difference was not statistically significant (Table 2.4), but the middle-aged women consistently excreted more 4-pyridoxic acid than the younger women. As with other status indicators, 4-pyridoxic acid increased five fold with supplementation. Excretion of 4-pyridoxic acid represented 55.7% of the 2.3 mg/day dietary vitamin B-6 intake and 66.9% of the 10.3 mg/day intake.

There were no significant age differences in urinary excretion of xanthurenic acid or kynurenic acid at any sampling time and no significant change in the amount excreted in either tryptophan metabolite with vitamin B-6 supplementation (Table 2.3). The mean change (yield) in excretion of both xanthurenic acid and kynurenic acid was consistently less for the middle-aged group as compared to the younger group.

When all of the data collected for the experiment were combined into one set, each of the four vitamin B-6 status indicators was found to correlate significantly with the others ($p < 0.001$). However, when considering the portion of the data collected when all women received the same intake (either 2.3 or 10.3 mg/day periods), the relationships between indicators was not as strong. At the 2.3 mg vitamin B-6 intake, only the plasma pyridoxal 5'-phosphate vs plasma total vitamin B-6 correlation remained at the same level of significance ($p < 0.001$). The only other significant relationship was plasma pyridoxal 5'-phosphate with urinary 4-pyridoxic acid ($p=0.03$). The others which showed some ($p < 0.10$) relationship were plasma and urinary total vitamin B-6 ($p=0.06$), plasma total vitamin B-6 and urinary 4-pyridoxic acid ($p=0.06$), and plasma pyridoxal 5'-phosphate and total urinary vitamin B-6 ($p=0.09$). During the supplemented weeks at 10.3 mg/day intake, only the plasma pyridoxal 5'-phosphate and total vitamin B-6 correlation was significant (p 0.001). The only other relationship which seemed to exist during supplementation was between plasma and total urinary vitamin B-6 ($p=0.10$), but it was not strong.

DISCUSSION

This study demonstrates, for the first time, that healthy, middle-aged women have significantly different vitamin B-6 metabolism than comparable younger women under conditions where dietary intake was adequate and constant for extended periods of time. The age-

related differences we observed simultaneously in three indicators of vitamin B-6 status indicators, plasma pyridoxal 5'-phosphate, total plasma vitamin B-6 and total urinary vitamin B-6 do not appear to have been previously reported in women. However, it is possible to compare the results of other studies in men or mixed groups with ours by expressing the results as a middle-aged:young ratio. For plasma pyridoxal 5'-phosphate, our ratio of 0.66 is very similar to Hamfelt's 0.63 (1964), calculated from his results of a group of 20-29 year olds compared with 30-59 year olds. Two other researchers reported less of an age difference. The values for the men in the study of Rose et al. (1976) give a ratio of 0.79 when comparing 18-29 year olds with those in their 50's. In the study of Lumeng and Li (1974), the ratio was calculated to be 0.77 when comparing subjects in their third decade with those in their fifth and sixth. It should be mentioned that Shultz and Leklem (1981) found no significant differences in plasma pyridoxal 5'-phosphate which could be attributed to age. Their 76 subjects were from age 20 to 79 and were consuming a relatively wide range of vitamin B-6.

There are a number of possibilities that may explain the differences we observed. An age-related impairment of absorption is one possibility. Holt's recent review (1982) details numerous changes in intestinal structure and function. These changes could decrease absorption of vitamin B-6. One of our screening criteria for subject selection was a normal xylose absorption. There was no significant difference in this parameter between our age groups.

Although we cannot extrapolate absorption of that carbohydrate to vitamin B-6, we have no reason to believe absorption was impaired, especially when considering that there was an age-related elevation in excretion of 4-pyridoxic acid. A second, and more likely, possibility is that a difference in metabolism of vitamin B-6 exists. The major source of pyridoxal 5'-phosphate is the liver (Lumeng et al., 1974), which decreases in function with age (Sherman and Libow, 1981). Hamfelt (1964) has suggested that the lower PLP levels could be due to an inadequate phosphorylation of the B-6 vitamers. On the other hand, excess catabolism could also be a possible explanation for the lower PLP levels observed in the older subjects. The level of alkaline phosphatase, the enzyme responsible for dephosphorylating PLP prior to its catabolism to 4-pyridoxic acid, increases with age (Gillibrand et al., 1979). Albumin, the binding protein which protects plasma pyridoxal 5'-phosphate from phosphatase hydrolysis (Li et al., 1974; Lumeng and Li, 1974), was significantly lower in our middle-aged women compared with the younger group (4.2 ± 0.3 g/dl versus 4.6 ± 0.2 g/dl, respectively, $p < 0.05$). These two phenomena, decreased protection and an increased level of a catabolic enzyme, together could explain the lowered plasma pyridoxal 5'-phosphate (and concommitant plasma total vitamin B-6) as well as the increased urinary excretion of the metabolite 4-pyridoxic acid. Of the two, the phosphatase role is likely of greater importance because of the tremendous excess in PLP-binding capacity of albumin, even at the 10% reduction in the albumin levels in the older women. Smith and co-

workers (1983), however, found no consistent correlation between pyridoxal 5'-phosphate levels and alkaline phosphatase activity in human leukocytes and postulated that pyridoxal 5'-phosphate hydrolysis *in vivo* is unlikely to be the principal function of the phosphatase. The potential role of hydrolysis of free or loosely-bound coenzyme in regulating the cellular concentration of PLP must not be overlooked. Anderson and coworkers (1971) first explored this possibility in human red cells by inhibiting the phosphatase with inorganic phosphate, which resulted in an accumulation of PLP in the red cells. Lumeng and Li (1974) confirmed this in erythrocytes as well as in liver (Li et al., 1974). Since phosphate affected only the phosphatase and not the kinase or oxidase enzymes, they concluded that hydrolysis of excess PLP appeared to be a significant factor in the regulation of cellular PLP concentration.

As mentioned above, the urinary excretion of 4-pyridoxic acid in our study tended to be greater, but not significantly so, in the middle-aged women as compared to the younger women. There are conflicting reports on the change in 4-pyridoxic acid excretion with age. Simon and co-workers (1982) reported an age-related increase for both sexes. However, Shultz and Leklem (1981) found a non-significant trend in the opposite direction. They also reported decreased urinary vitamin B-6 excretion in men; no such trend was evident in the women they studied. This latter study involved free-living individuals with differing intakes of vitamin B-6. However, there is some physiological support for the trends we observed. Baker et al.

(1979) recently reviewed studies documenting an age-related decrease in binding sites in the liver, which he hypothesized could leave an inadequate number of binding sites for vitamin storage. This would mean more vitamin B-6 could be available for excretion. Decreased glomerular filtration rate and renal plasma flow have also been documented (Moment, 1982). As mentioned previously, any increase in catabolism by increased alkaline phosphatase activity (Gillibrand et al., 1979) could also contribute to an increased 4-pyridoxic acid excretion.

The older women excreted significantly less urinary creatinine than the younger women (1.04 $\pm$ 0.04 versus 1.17 $\pm$ 0.04 g/24 hr, respectively, $p < 0.01$) over the entire study. Since creatinine excretion reflects muscle mass (Forbes and Bruining, 1976; Heymsfield et al., 1983), our data suggest that muscle mass was less in our middle-aged women. In rats, glycogen phosphorylase in skeletal muscle has been demonstrated to be a storage form of pyridoxal 5'-phosphate (Black et al., 1977, 1978). Lumeng and Li (1978) have found that plasma pyridoxal 5'-phosphate in rats is significantly correlated with muscle levels of the vitamer. If these findings hold true for humans, the higher plasma pyridoxal 5'-phosphate in our younger group could be related to their higher muscle mass. However, Black et al. (1978) suggest that pyridoxal 5'-phosphate is released from skeletal muscle only when there is a calorie deficit. There was no calorie deficit in our study since the subjects maintained constant weight over the seven weeks.

There were no statistically significant effects on levels of the vitamin B-6 status indicators measured when the weeks of moderate carbohydrate intake were compared with those of elevated carbohydrate intake. However, the design of the experiment did not allow conclusive statements due to the lack of randomization. In both cases, the increase in carbohydrate followed the moderate intake for all subjects. The results of the tryptophan load test were most sensitive to this manipulation, with basal levels of xanthurenic acid and kynurenic acid excretion tending to increase with increasing dietary carbohydrate ($p=0.06$). Additional studies are required to investigate this effect, since it was evident only when interactions of dietary carbohydrate and another factor, such as age and/or dietary vitamin B-6, were considered.

With the exception of the three metabolic studies done in oral contraceptive users and the control subjects for those studies (Donald et al., 1971; Leklem et al., 1975; Shin and Linkswiler, 1974), there is a relative lack of data available on which to base a recommended dietary allowance for vitamin B-6 in women, especially in the older age groups. The disappearance of the significant age-related differences in circulating and excreted vitamin B-6 status indicators with supplementation suggests that the requirement for older women is higher than 2.3 mg/day. However, the results of the tryptophan load test, one measure of the functional adequacy of vitamin B-6 status (Brown, 1981), are in opposition to this. The xanthurenic acid and kynurenic acid excretion was nearly identical

for the two age groups studied and for both levels of vitamin B-6 intake. This is evidence that 2.3 mg/day dietary vitamin B-6 is probably an adequate intake for the women studied.

Table 2.1. Physical characteristics of young adult and middle-aged women subject groups.

	young adult n=5	middle-aged n=8
Age (years)	24.4 ± 3.2[1]	55.3 ± 4.0
Height (cm)	164 ± 6	163 ± 6
Weight (kg)	55.8 ± 6.9	59.9 ± 6.5
Body fat (%)[2]	20.4 ± 1.5	24.7 ± 2.9
Estimated Lean Body Mass (kg)[3]	44 ± 5	44 ± 3

[1] X ± SD.
[2] Determined by the method of Jackson et al. (1980).
[3] Lean body mass was estimated by subtracting the kg body fat from the total weight of each subject.

Table 2.2 Controlled diets fed during moderate carbohydrate and high carbohydrate weeks.

Meal	Foods in Both Diets		Moderate CHO only	grams	High CHO only	grams
Breakfast	Orange juice[1]	170				
	Muffin[2]	40				
	Wheat flakes[3]	30				
	Milk		3.8% fat	200	<0.5% fat	200
	Raisins			20		30
Lunch	Tuna[4]	60				
	Lettuce	10				
	Dill pickle	15				
	Carrot sticks	50				
	Peaches[5]	100				
	Bread		Whole wheat	50	White	50
	Spread		Mayonnaise	24	Salad dressing	14
	Vanilla wafers[6]			16		32
			Egg white[7]	45		
					Apple juice[8]	200
Dinner	Turkey breast[9]	60				
	Lettuce	50				
	Red cabbage	15				
	French dressing	20				
	Carrots[10]	15				
	Margarine	15				
	Green vegetable		Beans[10]	100	Peas[9]	30
	Rice		Brown	45	Mixed[11]	60
	Milk		3.8% fat	200	<0.5% fat	200
	Bread		white	25		50
	Pears[12]			100		125
	Dessert		Ice cream	70	Ice milk	70
					Honey	25

Note:
Moderate carbohydrate diet was fed on weeks 1, 2, 4, 5, and 6. It provided 49% of the calories as CHO, 16% as protein and 35% as fat. The total vitamin B-6 content was 2.3 ± 0.1 mg/day.

High carbohydrate diet was fed on weeks 3 and 7. It provided 63% of the calories as CHO, 16% as protein and 21% as fat. The total vitamin B-6 content was 2.4 ± 0.1 mg/day.

1 Reconstituted orange juice made daily from frozen concentrate.
2 Muffins were baked on the premises weekly.
3 Wheaties, General Mills, Minneapolis, MN.
4 Water pack.
5 Canned in light syrup.
6 Nabisco brand.
7 Egg white cooked in microwave, minced and combined with tuna sandwich filling.
8 Canned.
9 Frozen.
10 Canned in brine.
11 White and brown rice in 1:2 weight ratio.
12 Canned in light sirup.

Table 2.3 Plasma and urine values for selected vitamin B-6 status indicators in young and middle-aged women fed constant diets.[1]

Status Indicator	Age Group[2]	Initial Valuea (Days 1-2)	Vitamin B-6=2.3 mg/day Week 2	Vitamin B-6=2.3 mg/day Week 3	Vitamin B-6 = 10.3 mg PN/day Week 6	Vitamin B-6 = 10.3 mg PN/day Week 7
Plasma pyridoxal 5'-phoaphate (nM)	Y	35.5±14.8[3]	62.4±20.2	61.7±25.6	210±52	202±45
	MA	31.3±13.3[4]	42.3±11.3	40.5±12.2	175±32	168±38
Plasma total vitamin B-6 (nM)	Y	49.4± 9.7[4]	81.4±19.7	84.1±24.0	288±68	284±75
	MA	46.0±13.0[4]	59.6± 8.4	57.5±13.2	239±23	237±25
Urinary total vitamin B-6 (μmol/24)	Y	0.958±0.279[4]	1.11± 0.22	1.12± 0.29	6.89±1.14	6.89±0.86
	MA	0.813±0.161[4]	0.858±0.133	0.894±0.188	5.54±1.28	5.48±1.26
Urinary 4-pyridoxic Acid (μmol/24 hr)	Y	4.69±1.76[4]	6.51±0.71	6.89±0.55	37.1±2.6	36.6±1.9
	MA	6.61±1.41[4]	7.21±0.85	7.35±0.80	38.3±3.5	38.5±4.7
Tryptophan Load Test Xanthurenic Acid (μmol/24 hr)						
Basal day	Y	5	14.8±1.6	15.7±1.5	15.4±2.0	16.3±4.9
	MA		17.2±5.2	20.7±5.4	18.4±5.3	17.3±2.9
Load day	Y		33.2±3.8	32.9±3.2[6]	33.0±3.0	35.6±6.0
	MA		33.8±5.0	35.7±6.4	32.3±3.3	32.3±4.8
Change (Load - Basal)	Y		18.4±4.5	17.1±3.4[6]	17.6±3.6	19.3±3.7
	MA		16.6±6.0	14.3±6.7	13.8±5.0	15.0±4.3
Kynurenic Acid (μmol/24hr)						
Basal Day	Y		12.4±1.8	13.3±1.6	12.1±1.9	14.7±3.8
	MA		12.6±3.1	12.4±2.5	13.3±3.4	12.8±2.3
Load Day	Y		76.7± 9.0	78.2±10.5[6]	76.8±10.3	87.8±25.5
	MA		62.6±21.8	60.3±18.4	68.2±18.7	67.6±19.1
Change (Load - Basal)	Y		64.3± 9.8	67.7±10.9[6]	64.7± 9.0	73.8±21.7
	MA		50.0±20.5	47.9±17.2	55.0±17.9	54.8±17.9

[1] Moderate carbohydrate diet provided 49% of calories as carbohydrate (weeks 2 and 6); high carbohydrate diet, 63% (weeks 3 and 7).
[2] Y = 5 young adult women. MA = 8 middle-aged women.
[3] X ± SD.
[4] n = 7. Initial samples for one subject not available.
[5] Tryptophan load tests were not run initially.
[6] n = 4.

Table 2.4. Significance of age-related differences in responses to four status indicators in young and middle-aged women fed constant diets at two levels of vitamin B-6.

	Vitamin B-6 = 2.3 mg/day (weeks 2 + 3)[1]		Vitamin B-6 = 10.3 mg/day (weeks 6 + 7)	
	F[2]	p[3]	F[2]	p[3]
Plasma pyridoxal-5′-phosphate	4.85	.05	2.44	.15
Total plasma vitamin B-6	7.58	.02	3.19	.10
Total urinary vitamin B-6	5.23	.04	4.50	.06
Urinary 4-pyridoxic acid	2.13	.17	0.65	.44

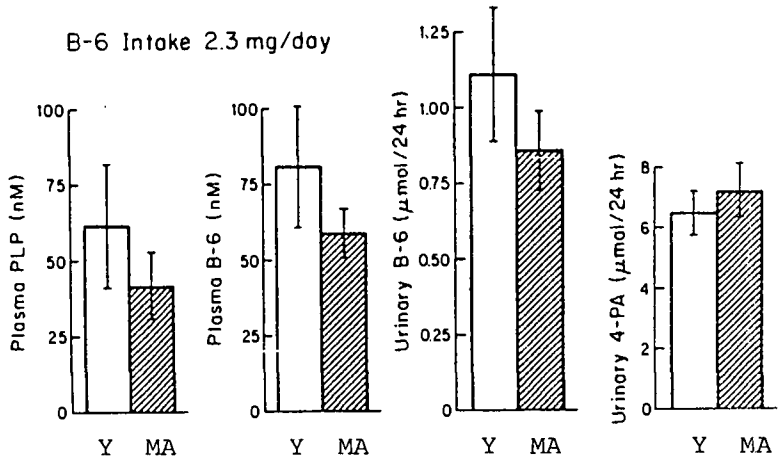
[1] There were no significant carbohydrate effects for any status indicator at either level of vitamin B-6 (p′s >0.2) nor age/carbohydrate interactions (p′s >0.3).

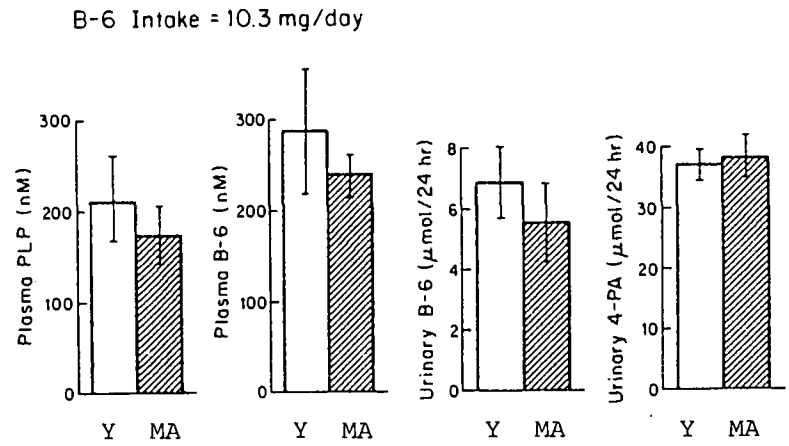
[2] F = age effect mean square/error mean square.

[3] p = 2-tail probability.

Figure 2.1. Circulating and excreted forms of vitamin B-6 for young and middle-aged women fed constant diets.

Note: Each bar graph represents average of values for weeks 2 and 3 and for weeks 6 and 7, respectively. The open bar represents the young women (Y) and the hatched bar, the middle-aged women (MA)





Chapter 3

ERYTHROCYTE PYRIDOXINE KINASE ACTIVITY DIFFERENCES IN YOUNG AND MIDDLE-AGED WOMEN FED CONTROLLED INTAKES OF VITAMIN B-6

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ABSTRACT

The purpose of this 7-week study was to investigate the activity of erythrocyte pyridoxine kinase and its relationship to circulating and excreted forms of vitamin B-6 in 5 young (21-29 yr) and 8 middle-aged (48-61 yr) women fed controlled diets. For the 4-week unsupplemented period, the women consumed a diet providing 2.3 mg vitamin B-6 per day. For the 3-week supplemented period, the women consumed the same diet with an additional 8.0 mg of pyridoxine. Supplementation did not alter the kinase activity during the experimental period. The middle-aged women had significantly ($p < 0.05$) higher kinase activity over the 7 weeks (1.4 ± 0.2 vs. 0.9 ± 0.2 nmol pyridoxine phosphorylated per minute per gram hemoglobin). Pyridoxine kinase activity correlated negatively ($p < 0.05$) with total urinary vitamin B-6 throughout the experiment. The hypothesized interrelationship between age and vitamin B-6 metabolism is supported by our findings of significant differences in erythrocyte pyridoxine kinase activity and several measures of vitamin B-6 nutriture.

INTRODUCTION

In 1964, Hamfelt suggested that defective phosphorylation due to decreased activity of pyridoxal phosphokinase (E C 2.7.1.35) might be one of the factors which could explain the fall in circulating pyridoxal 5'-phosphate with age (Hamfelt, 1964). In the intervening 20 years, an age-related change in vitamin B-6 nutriture has been

documented by others (Rose et al., 1976; Chabner and Livingston, 1970; Ranke et al., 1960; Walsh, 1966; Chen and Fan Chiang, 1981; Lumeng and Li, 1974; Jacobs et al., 1968; Hines and Love, 1969). Researchers have also attempted to define physiologic and pharmacologic factors which affect the activity of pyridoxine kinase (Lumeng and Li, 1974; Solomon, 1982; Solomon and Hillman 1976, 1977, 1979a; Kark et al., 1976; Hines and Cowan, 1970, 1974; Anderson, 1980; Chillar et al., 1976).

Our research was designed to integrate these two areas under carefully controlled dietary conditions. If the pyridoxine kinase activity is, in fact, related to circulating plasma pyridoxal 5'-phosphate (Hamfelt, 1964), or to total circulating or excreted forms of vitamin B-6, there should be a significant correlation between the eyzyme activity and the vitamin levels. We chose to study healthy women; whereas, others focused on men of unspecified groups, often with alcohol-related pathologies (Hines, 1969, 1970, 1975; Hines and Cowan, 1970, 1974; Solomon and Hillman, 1979a). We carefully controlled the diet, which contained adequate B-6 intake, in otherwise free-living adults. For supplementation, we selected a physiological level which brought the diet of 2.3 mg pyridoxine equivalents/day to 10.3 mg/day. Other researchers induced synthesis of the kinase or increased its activity in human erythrocytes with pharmacological supplements ranging from 50-450 mg/day (Solomon and Hillman, 1977, 1978, 1979a; Kark et al., 1976). Methodological differences in measurement of pyridoxine kinase activity itself

further complicate the comparison of results between laboratories. Some groups used methods based on phosphorylation of pyridoxal, which is not completely recovered from samples containing hemoglobin due to formation of a Schiff base (Hamfelt, 1967; Hines and Love, 1969, Lumeng and Li, 1974). Two groups avoided this difficulty by measuring the pyridoxine which was phosphorylated (Solomon and Hillman, 1976; Chern and Beutler, 1975a). Finally, there is not a consensus as to which indicator method best reflects vitamin B-6 metabolic changes (Leklem and Reynolds, 1981). We chose Chern and Beutler's kinase method (1975a) for its precision, coupled with four different vitamin B-6 indicator assays to measure response to dietary vitamin B-6 levels.

Our findings support the hypothesis of an interrelationship between age and vitamin B-6 metabolism as reflected by significant differences in erythrocyte pyridoxine kinase and several measures of vitamin B-6 nutriture.

METHODS

Subjects

Thirteen healthy, sedentary, independent-living adult women were studied for 7 weeks. The characteristics of the group of 5 young women (24.4 $\pm$ 3.2 yr) and the 8 middle-aged women (55.3 $\pm$ 4.0 yr) are given in Table 3.1. All had normal blood and urine biochemistry (including glucose, creatinine, urea nitrogen, uric acid, total protein, albumin, cholesterol, sodium, potassium, chloride, calcium,

phosphorus, bilirubin, serum glutamate oxaloacetate transaminase, lactic dehydrogenase, alkaline phosphatase, globulin) and normal intestinal absorption as measured by xylose absorption (Harris, 1969). Medical histories and physical examinations revealed no present or past conditions which could interfere with the digestion, absorption and metabolism of vitamin B-6. The middle-aged women were post-menopausal. None used hormones, alcohol, nicotine or other drugs. None used vitamins or other nutritional supplements 4 weeks prior to and throughout the study. They consumed all weighed meals under supervision. Ten subjects were Caucasion. One of the middle-aged women was Filipino. One of the young women was Latin American and another was North American Indian.

Sample Collection

Daily complete urine samples and twice weekly (Wednesday/Saturday) fasting blood samples were collected. Erythrocyte kinase activity was determined on Saturday samples from weeks 2 and 3 (unsupplemented) and weeks 6 and 7 (supplemented). Values for the circulating and excreted vitamin levels are averages from the Wednesday and the Saturday collections of weeks 2 and 3 and weeks 6 and 7. Sampling was timed so that the younger women would be at the same point in their menstrual cycles for both unsupplemented and supplemented collections. Values designated "initial" were determined on blood or urine collected at the beginning of the study.

Experimental Design

The 7-week study consisted of an initial one week adjustment period on the unsupplemented diet (vitamin B-6 = 2.3 mg per day) followed by two weeks on the same diet during which samples were collected. A fourth week was inserted prior to the supplemented period so that young subjects would be in the same phase of their menstrual cycle during samples collected in both periods. Supplementation with 8.0 mg pyridoxine per day was begun at week 5. Samples were collected for the supplemented period during weeks 6 and 7.

Diets

For all except weeks 3 and 7, the diet given in Table 3.2 was fed each day. Based on the analysis of food composites, this diet provided 2.3 mg of vitamin B-6 per day and at 2000 kcal consisted of 16% protein, 35% fat and 49% carbohydrate by calculation. During weeks 3 and 7, minor alterations were made to increase the carbohydrate level (correspondingly decreasing the fat level) with constant protein and vitamin B-6 levels to facilitate a simultaneous research project by other investigators. The diet for week 3 and week 7 contained 2.4 mg of vitamin B-6. For the last three weeks the diet was supplemented with 8.0 mg of pyridoxine, given daily at breakfast as the hydrochloride dissolved in 1% acetic acid. Iron supplements were given on alternate days (32 mg ferrous sulfate). These changes did not alter the parameters measured for this research ($p > 0.2$) as determined by analysis of variance.

Assays

Pyridoxine kinase activity was measured by a modification of the method of Chern and Beutler (1979b). Freshly drawn heparinized blood was washed 3 times with isotonic saline and the buffy coat layer was discarded. Red cells were frozen at -40°C until analyzed. A 2% hemolysate was prepared by suspending red cells in twice-distilled water. Ghosts were pelletized by centrifugation at 16,000 x g for 10 min at 4°C. Fifty ul of a 2% hemolysate were added to 200 ul of a 2:1:1 mixture of 1M KH_2PO_4/K_2HPO_4 buffer (pH 7.0), 10 mM freshly prepared ATP and 10 mM $MgCl_2$. The mixture was pre-incubated 10 minutes at 37°C in a shaking water bath at 40 oscillations/minute. The reacton was started by adding 500 ul 10^{-5}M (^{14}C) pyridoxine (Amersham, Arlington Heights, IL) to each sample. After incubation for 2 hr at 37°C in a shaking water bath at 80 oscillations/min, the reaction was terminated by removing samples to an ice bath and immediately adding 700 ul of 12% perchloric acid. After mixing, samples stood 5 min in the ice bath and were then filtered through Whatman No. 2 filter paper. To neutralize the mixture, 600 ul of filtrate was combined with 225 ul of 1M K_2CO_3, mixed and allowed to stand at room temperature 5 minutes. Unreacted substrate was removed by combining 500 ul of the clear supernatant with 200 ul of a 1:2 Dowex 50 resin in 0.05 M potassium formate buffer (pH 4.3). The final sample pH was adjusted to 4.3 with 0.5 M formic acid to optimize association of pyridoxine with the resin. After mixing 30 minutes on a platform shaker at 200 oscillations/min, samples were

filtered through glass wool to remove the resin beads. Five hundred ul of clear filtrate were counted in 10 ml of Bray's solution. Sample blanks were prepared for each hemolysate in an identical manner except the incubation was carried out prior to substrate addition. The labelled pyridoxine was added to the sample blanks in the ice bath immediately prior to adding the perchloric acid. For each assay, an isotope dilution factor was determined by preparing another blank, this one with twice-distilled water in place of the hemolysate and potassium formate buffer in place of the resin:buffer slurry. The radioactivity of the blank, which had gone through all the manipulations of the assay along with the samples, was compared with the radioactivity in a 500 ul aliquot of the 10^{-5} M labelled pyridoxine and this dilution ratio used to adjust results from each day's assay. all samples and blanks were done in triplicate. Within assay precision, measured on two people in 5 repeat determinations was $\pm$ 5.0% and $\pm$ 7.5% for kinase activities of 1.6 and 1.2 nmol. $min^{-1}.gHb^{-1}$, respectively. Between-assay coefficient of variation was $\pm$ 8.2% when measured on erythrocytes which had been stored at $-40^{o}C$ and included in 10 assays over several months.

The circulating and excreted forms of vitamin B-6 were measured by methods commonly used in our laboratory. Plasma pyridoxal 5'-phosphate was assayed by the tyrosine decarboxylase method of Chabner and Livingston (1970). Total vitamin B-6 in plasma and urine (as pyridoxine equivalents) was measured by the Miller and Edwards (1981) microbiological assay. Urinary 4-pyridoxic acid was determined by

the procedure of Reddy and coworkers (1958). Inter-assay coefficients of variation were: plasma pyridoxal 5'-phosphate ± 6% (22 replicates), total plasma vitamin B-6 ± 6% (14 replicates), total urinary vitamin B-6 ± 3% (18 replicates), and 4-pyridoxic acid ± 8% (35 replicates). Hematocrits and hemoglobins were measured in duplicate by standard methods at the time of each blood sampling. Triplicate hemoglobins were also measured for each hemolysate.

Statistical Analyses

To test for age and supplementation differences in the parameters measured, an analysis of variance was performed. To test for relationship between the pyridoxine kinase activity and the circulating and excreted forms of vitamin B-6, simple correlations were calculated (Snedecor and Cochran, 1980). A p-value of ≤ 0.05 was considered significant.

RESULTS

Pyridoxine Kinase Activity

The mean pyridoxine kinase activity values for middle-aged and young women are listed in Table 3.3. The older women's values were 50% above those of the younger women ($p \leq 0.05$). The values of weeks 3 and 4 as well as those at weeks 6 and 7 were not significantly differenc and were within 2% of each other. Vitamin B-6 supplementation had no significant effect on the kinase activity in either age group (Figure 3.1).

Correlation of Pyridoxine Kinase Activity With Measures of Vitamin B-6 Nutriture

The summary values of the circulating (Table 3.4) and excreted (Table 3.5) forms are included for reference, since status assessment has been discussed elsewhere (Lee and Leklem, 1984). Data from weeks 3 and 4 as well as that for weeks 6 and 7 showed that pyridoxine kinase activity correlated negatively with urinary vitamin B-6 when all subjects were included as a group (n = 13) (Table 3.6). One young subject, a North American Indian, had some outlying values, and when her results were excluded from the calculations, three additional correlations with kinase activity were significant at $p < 0.05$. Specifically, for the unsupplemented weeks (i.e. 2 and 3), kinase activity correlated negatively with circulating plasma pyridoxal 5'-phosphate as well as total plasma vitamin B-6 (n = 12). For the supplemented weeks (i.e. 6 and 7), the negative correlation between plasma B-6 and kinase was also significant (n = 12).

Correlation of Pyridoxine Kinase Activity with Age

Combining all the kinase activity values for all women studied resulted in a positive correlation between the activities and age ($r = 0.76$, $p < 0.05$). Figure 3.1 illustrates this relationship as well as the low values for three non-Caucasian subjects. The middle-aged Filipino woman had kinase activity values of 0.99 and 0.91 nmol. $min^{-1}.gHb^{-1}$ for weeks 2+3 and 6+7, respectively. The values for the young North American Indian and Latin American were 0.65 and 0.87 for

weeks 2+3 and 0.74 and 0.85 for weeks 6+7, respectively. The individual values for kinase activity are found in Table 3.8.

Erythrocyte Indices

Neither hematocrit nor hemoglobin values varied significantly during the experimental periods (Table 3.8). For these indices, there were no significant differences between the middle-aged and young women at any point in the study.

DISCUSSION

This study revealed an age-related difference in the activity of erythrocyte pyridoxine kinase. More than 20 years ago, Hamfelt (1964) first hypothesized that defective phosphorylation of the vitamin to its active form might account for the lower levels of circulating vitamin B-6 in elderly subjects, in conjunction with lowered nutritional intake, defective intestinal absorption, or increased urinary loss. Our middle-aged women had approximately 50% more kinase activity than the younger women, which could support Hamfelt's hypothesis if one accepts the postulate of Ebadi et al. (1970) that tissue availability of pyridoxal 5'-phosphate controls the activity of the kinase. Their research in rabbits revealed an inverse relationship between the concentration of pyridoxal 5'-phosphate in the brain tissue and the activity of the kinase from the same tissue. Some of Solomon and Hillman's studies with alcoholics (1978, 1982) suggest a direct relationship between circulating vitamin B-6 forms

and the activity of the pyridoxine kinase, with alcoholics being abnormally low in both factors. However, these studies were not carefully controlled with regard to dietary intake of vitamin B-6, among other factors, and it is difficult to extrapolate their findings to a healthy population. They conclude, additionally, there may be species and tissue differences in production and degradation which could be important in regulating intracellular levels of the B-6 vitamers (Solomon and Hillman, 1979b).

One shortcoming of our research was the mixture of races included in a predominately Caucasian study. Several authors have reported on lowered pyridoxine kinase activity in blacks (Chern and Beutler, 1975a-c, 1976; Chillar et al., 1976; Solomon and Hillman, 1978). We, therefore, excluded blacks. The information regarding the Orientals is not as clear. Solomon and Hillman's data (1978) revealed a mean for Orientals that was only 7% below the Caucasians and had a standard deviation equal to 50% of the mean. Chern and Beutler (1975c) found no significant difference between white Americans, Chinese, Japanese and Asian Indians living in Los Angeles. They did, however, find that Filipinos were significantly lower than whites (1.07 $\pm$ 0.22 nmol pyridoxine phosphate formed per minute per gram of hemoglobin vs 1.34 $\pm$ 0.29 nmole for whites). We could not locate any data on Latin Americans nor the North American Indian. Hence, when one Filipino, one Latin American, and one North American Indian met all of our other criteria, we included them with the 10 Caucasian women. When considering all of the status indicator data,

their values are not apparently unusual and were included in all analysis of variance tests for age-related differences. However, the North American Indian had such a low kinase activity that excluding her data in the correlation calculations involving kinase increased the number of significant correlations from just urinary total vitamin B-6 (n = 13) to include the circulating forms also (n = 12).

Although pyridoxine kinase has not been studied in aging mammals, many other enzymes have, and results have been reviewed critically (Wilson, 1973; Finch, 1972). The results of the present study are not in disagreement with their findings. For example, in Finch's collection of human erythrocyte enzymes studied, all enzymes which changed increased 30% in subjects over age 60. Wilson, on the other hand, reports most erythrocyte enzymes remain unchanged in aging man, although she also included some which decreased and one which increased. Wilson stressed the difficulty in comparing enzyme levels reported by different workers due to choice of different workers due to choice of different baselines (e.g., protein, nitrogen, cell number) coupled with inadequate assessment of baseline changes during the experiment or with aging. In research of this type, it is also important to choose a reliable technique. Chern and Beutler's assay method was selected for our study because it gave reproducible results which were in line with others who have used it (Chern and Beutler, 1975a-c). Our Caucasian total group average (n = 10) was 1.37 $\pm$ 0.25 nmole pyridoxine phosphate formed per minute per gram of hemoglobin, which is not different from Chern and

Beutler's 1.338 ± 0.286 nmol using the same method for erythrocytes of 25 healthy Caucasian adults (1975c), or 1.46 ± 0.087 nmol for outdated human blood (1976) but lower than Solomon and Hillman's 1.94 ± 0.75 for 27 Caucasians, a study in which a different assay was used (1978). We did obtain values as high as Solomon and Hillman for whites who were healthy controls for another study in our laboratory (Leklem, Kingsley and Lee, unpublished). However, since this was not an enzyme study per se, many of the variables known to affect the level of kinase activity were not controlled (such as dietary intake of vitamin B-6) so the usefulness of these data are limited.

We did not find significant loss (< 5%) of radioactivity through conversion to labeled pyridoxal 5'-phosphate, which Solomon and Hillman (1976) had suggested as a possible reason for lower activities using the assay of Chern and Beutler (1975b). We reported results in activity per gram of hemoglobin, since hemoglobin does not change in women over the age range studied (Helman and Rubenstein, 1975). Likewise, there is no indication that erythrocyte lifespan changes with age (Lamb, 1977). This is important when considering results of researchers who studied erythrocytes of different ages from the same individuals and found kinase activity differences (Chern and Beutler, 1976; Solomon and Hillman, 1979b). We studied the total erythrocyte population and based our age-related differences on that sample. Solomon and Hillman (1979a) observed a rise in pyridoxine kinase activity after supplementation with 450 mg pyridoxine/day in one subject with anaemia of inflammation. Although

the first post-supplement measurement of kinase activity was not until 6 weeks, it appears from their data that by the third week the pyridoxine kinase activity was elevated by more than one standard deviation from the mean of the regression line found in normal individuals. The first post-supplemental measurement, made at the eighth week without additional vitamin B-6, showed pyridoxine kinase activity remaining 1 SD above the mean. These authors had previously reported significant increases in erythrocyte pyridoxine activity in five normal Caucasions who were given at least 200 mg/day pyridoxine for 10-14 weeks (Solomon and Hillman, 1978). It is difficult to compare these results with the present study in which much smaller pyridoxine doses were used as well as shortened supplementation times. On the other hand, in a rabbit study, increasing the intramuscular injections of pyridoxine decreased the kinase activity and the level of pyridoxal 5'-phosphate in brain tissue (Ebadi et al., 1970). In a rat study, increasing dietary pyridoxine had no significant effect on liver and brain levels of pyridoxal 5'-phosphate (Li et al., 1974). In the rat study, kinase activity was not measured. There appear to be several confounding factors involved in the relationship between kinase activity and dietary pyridoxine supplementation and more work is required before a definitive statement can be made.

The present study indicates that erythrocyte pyridoxine kinase activity increases with age and is inversely related to excreted forms of vitamin B-6. Supplementation with vitamin B-6 did not alter the activity of this enzyme although it increased circulating and

excreted forms of the vitamin. The principal source of circulating pyridoxal 5'-phosphate in rested mammals is the liver (Lumeng et al., 1974). It is also known that erythrocytes contain low but significant amounts of enzyme activity for synthesizing this active cofactor (Lumeng and Li, 1974). The function of pyridoxine phosphorylation in the erythrocytes is still not known. How accurately it reflects vitamin B-6 metabolism in other tissues likewise is still not clear. Anderson et al. (1975) concluded that it would be unreasonable to think that such an active metabolism of vitamin B-6 in erythrocytes is without metabolic significance. The inverse relationship between pyridoxine kinase and plasma pyridoxal 5'-phosphate might be explained by the theory that tissue availability of pyridoxal 5'-phosphate regulates the enzyme activity (Ebadi et al., 1970). With lower circulating pyridoxal 5'-phosphate levels, as is the case with our middle-aged subjects, the kinase could become more active. If this difference in activity were due to differences in levels of the enzyme itself (rather than due to differences in activation of existing kinase), one might not expect to see a change in the total erythrocyte population during only three weeks supplementation with 8.0 mg of pyridoxine.

The most consistent correlation seen in our study is the inverse relationship between the kinase activity and the level of urinary vitamin B-6. This relationship suggests that either red cell metabolism of vitamin B-6 is very important overall or that the kinase activity in the red cell reflects kinase activity in other

tissues. The distinction cannot be made from these results. The relationship between kinase activity and the circulating levels of the vitamin is not clear from this study. The constancy of erythrocyte pyridoxine kinase activity in the presence of vastly different levels of pyridoxal phosphate concentrations (more than 300% change in plasma levels from unsupplemented weeks 2-3 to supplemented weeks 6-7) might be evidence for a lack of enzyme inhibition by the product. This supports Solomon and Hillman's report (1976) of no significant inhibition of the red cell kinase in opposition to the earlier report of White and Dempsey (1970) of inhibition of the pyridoxine kinase from E. coli. Although it is not absolutely clear, Karawya and Fonda (1978) imply inhibition of sheep liver pyridoxal kinase by "product inhibitors and vitamin B-6 analogs."

Karawya et al. (1981) reported inhibition of sheep liver pyridoxal kinase with tryptophan metabolites, including xanthurenic acid. Our results (Lee and Leklem, 1984) showed an inverse correlation between the kinase activity and the increase in xanthurenic acid excreted following a 2 gram L-tryptophan load ($p \leq 0.01$), which supprots their finding.

Racial differences may have confounded our results, masking a relationship between the enzyme and circulating of vitamer levels. If the one North American Indian subject with the very low kinase activity is, in fact, representative of her race and different from the Caucasian group, then there is a significant inverse relationship between the circulating levels of the vitamin and the enzyme activity

among the Caucasian subjects when the vitamin B-6 intake approximated the RDA. This supports Ebadi's hypothesis of tissue levels controlling enzyme activity (1970). Additionally, the somewhat higher 4-pyridoxic acid excretion in the older subjects may be due to increased catabolism, which coincidentally would lower the circulating levels of the vitamin. The activities of the other two major enzymes involved, the oxidase and the phosphatase, need to be compared in younger and older subjects to fully explain these findings.

Table 3.1. Subject group characteristics

	young women n=5	middle aged women n=8
age (years)	24.4±3.2 [1]	55.3±4.0
height (cm)	164 ±6	161 ±6
weight (kg)	55.8±6.9	59.9±6.5

1 mean ± SD

Table 3.2 Daily diet for 2000 kcal

Item	grams
Breakfast	
Wheat flakes, fortified	30
Whole milk	200
Raisins	20
Muffin	40
Orange juice	170
Lunch	
Tuna sandwich	
whole wheat bread	50
water pack tuna	60
egg white	45
lettuce	10
dill pickle	15
mayonnaise	24 *
Raw carrots	50
Peaches in light syrup	100
Vanilla wafers	16
Dinner	
Brown rice	45
Whole milk	200
White bread	25
Turkey breast	60
Lettuce	50
Red cabbage	15
French dressing	20 *
Canned carrots	15
Canned green beans	100
Ice cream	70
Margarine	15 *

* Foods essentially free of vitamin B-6 were adjusted to maintain weight for each subject.

Table 3.3 Erythrocyte pyridoxine kinase activity [1]

Period	Weeks	Young Women n=5	Middle-aged Women n=8
		$nmols \cdot min^{-1} \cdot gHb^{-1}$	
Unsupplemented	3-4	0.934±0.199	1.44±0.21[2]
Supplemented	6-7	0.953±0.153	1.43±0.23[2]

[1] Mean ± standard deviation.

[2] The means for the middle-aged women were significantly different from those of the young women at $p < 0.01$ (Students' t-test).

Table 3.4. Circulating plasma levels of vitamin B-6.

Period	Weeks	Total vitamin B-6 (nM)		Pyridoxal 5′-phosphate (nM)	
		Young Women n=5	Middle-aged Women n=8	Young Women n=5	Middle-aged Women n=8
Initial	1	49.4 ± 9.7[1]	46.0 ± 13.4[2]	35.5 ± 14.8	31.3 ± 13.3[2]
Unsupplemented	2-3	82.7 ± 20.6[3]	58.5 ± 11.7	62.1 ± 21.5	40.9 ± 13.0
Supplemented	6-7	286 ± 71	239 ± 26	203 ± 44	174 ± 32

[1] $\bar{X}$ ± SD. Initial samples drawn days 1 and 2 of study.
[2] n = 7. Initial sample from one subject was not available.
[3] Average of four samples from each subject for each period.

Table 3.5 Urinary excretion of vitamin B-6

Period	Weeks	Total vitamin B-6 μmol/24 hr		4-pyridoxic acid μmol/24 hr	
		Young Women n=5	Middle-aged Women n=8	Young Women n=5	Middle-aged Women n=8
Initial	0-1	0.958±0.279 [1]	0.813±0.161 [2]	4.69±1.76	6.61±1.41 [2]
Unsupplemented	2-3	1.12±0.27 [3]	0.874±0.129	6.70±0.77	7.28±0.94
Supplemented	6-7	6.92±1.03	5.48±1.26	36.7±2.3	38.4±4.4

[1] $\bar{X}$ ± SD. Initial samples drawn days 1 and 2 of study.
[2] n = 7. Initial sample from one subject was not available.
[3] Average of four samples from each subject for each period.

Table 3.6 Correlation between erythrocyte pyridoxine kinase activity and circulating or excreted forms of vitamin B-6

	Unsupplemented Period (B-6=2.3mg)		Supplemented Period (B-6=10.3mg)	
	r	sig.	r	sig.
Circulating forms:				
Plasma PLP	- 0.48[1]	NS[2]	- 0.44	NS
Plasma Total Vitamin B-6	- 0.54	NS	- 0.46	NS
Excreted forms:				
Urinary Total Vitamin B-6	- 0.67	$p<0.05$	- 0.82	$p<0.05$
Urinary 4-PA	+ 0.50	NS	+ 0.29	NS

[1] n = 13 for all comparisons.

[2] NS = not significant.

Table 3.7 Individual Subject Characteristics (weeks 0-1)

	Subject No.	age (yr)	race[1]	height (cm)	weight (kg)	fat[2] (%)	xylose excr[3] (%)	SGOT[4] U/l	SGPT[4] U/l
Middle-aged	2	53	F	153	51.8	27.6	26.8	29	19
	4	54	C	168	70.3	30.3	24.9	32	25
	6	59	C	164	58.0	22.2	27.0	27	19
	7	61	C	157	56.4	25.0	22.2	27	19
	9	55	C	168	61.8	27.6	27.0	26	22
	12	55	C	161	53.9	26.2	30.0	25	24
	15	48	C	161	59.4	28.9	22.8	33	28
	16	57	C	155	68.2	31.0	34.2	33	50
Young	1	24	I	168	65.0	21.9	58.2	29	18
	11	26	C	173	60.9	21.2	22.3	23	13
	14	21	C	164	53.1	18.1	29.2	26	16
	17	29	C	161	52.2	20.0	35.7	22	13
	18	22	L	156	48.2	20.9	26.0	22	23

[1] Race F = Filipino, C = Caucasian, L = Latin American, I = North American Indian.
[2] % fat by method of Jackson et al (1980).
[3] % xylose excretion according to method of Harris (1969).
[4] Analyzed by Good Samaritan Hospital, Corvallis, OR. SGOT = serum glutamic oxalacetate transaminase; SGPT = serum glutamic pyruvate transaminase.

Table 3.8

Pyridoxine kinase activities, hemoglobins and hematocrits of individual subjects during the controlled feeding study.

	Subject No.	PnK Activity n mol · min^{-1} · gHb^{-1} B-6=2.3 mg	PnK Activity n mol · min^{-1} · gHb^{-1} B-6=10.3 mg	Hemoglobin g/100 ml Initial	Hemoglobin g/100 ml End	Hematocrit % packed cells Initial	Hematocrit % packed cells End
Middle-aged	2	0.99	0.91	14.3	14.4	38.8	40.2
	4	1.67	1.65	16.8	17.3	44.7	46.8
	6	1.38	1.40	14.8	13.8	40.4	39.6
	7	1.56	1.52	14.4	14.4	39.3	38.6
	9	1.37	1.35	13.1	12.9	35.3	35.7
	12	1.61	1.56	13.2	13.8	36.1	38.3
	15	1.53	1.49	15.4	14.8	40.7	40.2
	16	1.45	1.58	15.0	15.4	38.1	39.0
Young	1	0.65	0.74	13.7	12.6	37.8	36.9
	11	1.04	1.03	14.3	14.4	38.0	36.8
	14	1.17	1.11	14.9	14.1	40.4	39.6
	17	0.95	1.05	14.4	15.8	37.7	38.0
	18	0.87	0.85	13.4	13.4	36.4	37.9

Table 3.9

Circulating and excreted forms of vitmain B-6 for individual subjects during the controlled feeding study

	Subject No.	Vitamin B-6 Intake = 2.3 mg				Vitamin B-6 Intake = 10.3 mg			
		PLP[1] nM	PB6[2] nM	UB6[2] μmol/24 hr	4PA[3] μmol/24 hr	PLP[1] nM	PB6[2] nM	UB6[2] μmol/24 hr	4PA[3] μmol/24 hr
Middle-aged	2	35.1	53.2	0.885	6.39	182	266	8.53	33.5
	4	28.8	46.4	0.987	8.00	117	204	5.17	35.4
	6	53.6	65.8	0.888	6.92	217	255	4.81	44.4
	7	33.3	55.4	0.653	7.53	159	225	4.77	39.1
	9	43.6	56.8	0.802	6.95	197	255	4.98	37.6
	12	32.4	45.1	0.902	8.43	146	213	5.64	41.7
	15	37.0	59.3	0.822	7.78	159	229	4.86	42.1
	16	63.2	79.5	1.057	6.26	199	264	5.05	33.7
Young	1	48.9	73.8	1.383	7.56	153	209	6.62	38.3
	11	58.4	80.4	0.964	6.38	195	266	6.31	39.0
	14	63.8	85.0	0.792	6.32	225	323	5.94	34.6
	17	40.1	57.6	1.060	6.92	182	242	7.27	36.1
	18	99.1	116.9	1.350	6.34	227	390	8.34	35.9

1 Plasma pyridoxal 5'-phosphate (PLP) by method of Chabner and Livingston (1970).
2 Plasma (PB6) and urinary (UB6) vitamin B-6 by method of Miller and Edwards (1981).
3 Urinary 4-pyridoxic acid (4PA) by method of Reddy et al. (1958).

Table 3.10

Circulating and excreted forms of vitamin B-6 for indibidual subjects at the beginning of the controlled feeding study.

	Subject No.	Circulating PLP[1] nM	Circulating PB6[2] nM	Excreted UB6[2] μmol/24 hr	Excreted 4PA[3] μmol/24 hr
Middle-aged	2	15.8	27.9	0.84	5.71
	4	missing	missing	missing	missing
	6	34.9	48.8	0.96	5.98
	7	18.9	31.0	0.51	4.63
	9	49.6	43.8	0.82	5.88
	12	21.5	51.3	0.73	8.33
	15	32.4	52.5	0.83	7.71
	16	46.1	66.9	1.00	8.03
Young	1	56.5	43.3	1.38	6.97
	11	35.7	61.9	0.98	6.15
	14	26.1	50.1	0.70	3.28
	17	17.9	37.0	0.71	3.98
	18	41.1	54.9	1.02	3.09

1 Plasma pyridoxal 5'-phosphate (PLP) by method of Chabner and Livingston (1970).

2 Plasma (PB6) and urinary (UB6) vitamin B-6 by method of Miller and Edwards (1981).

3 Urinary 4-pyridoxic acid (4PA) by method of Reddy et al. (1958).

Figure 3.1 Correlation between pyridoxine kinase activity and subject age in women fed constant diets

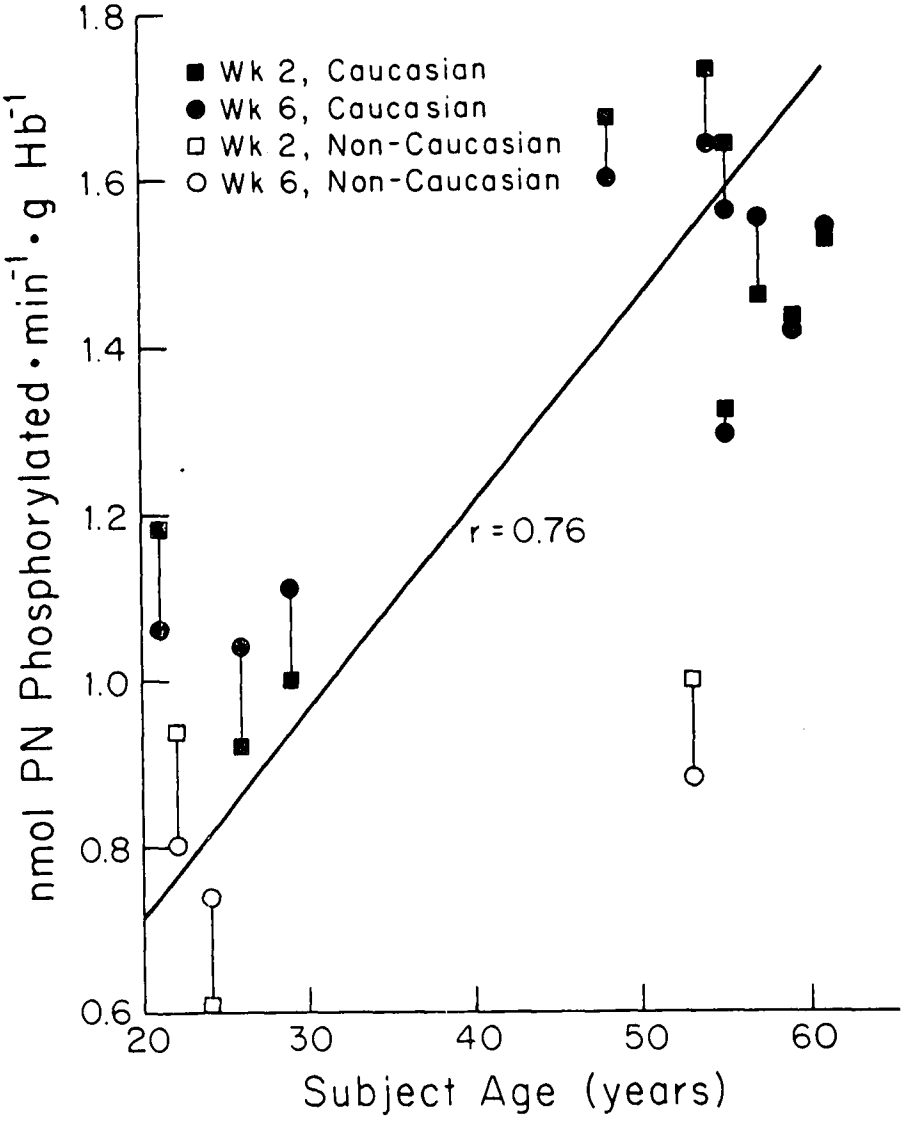
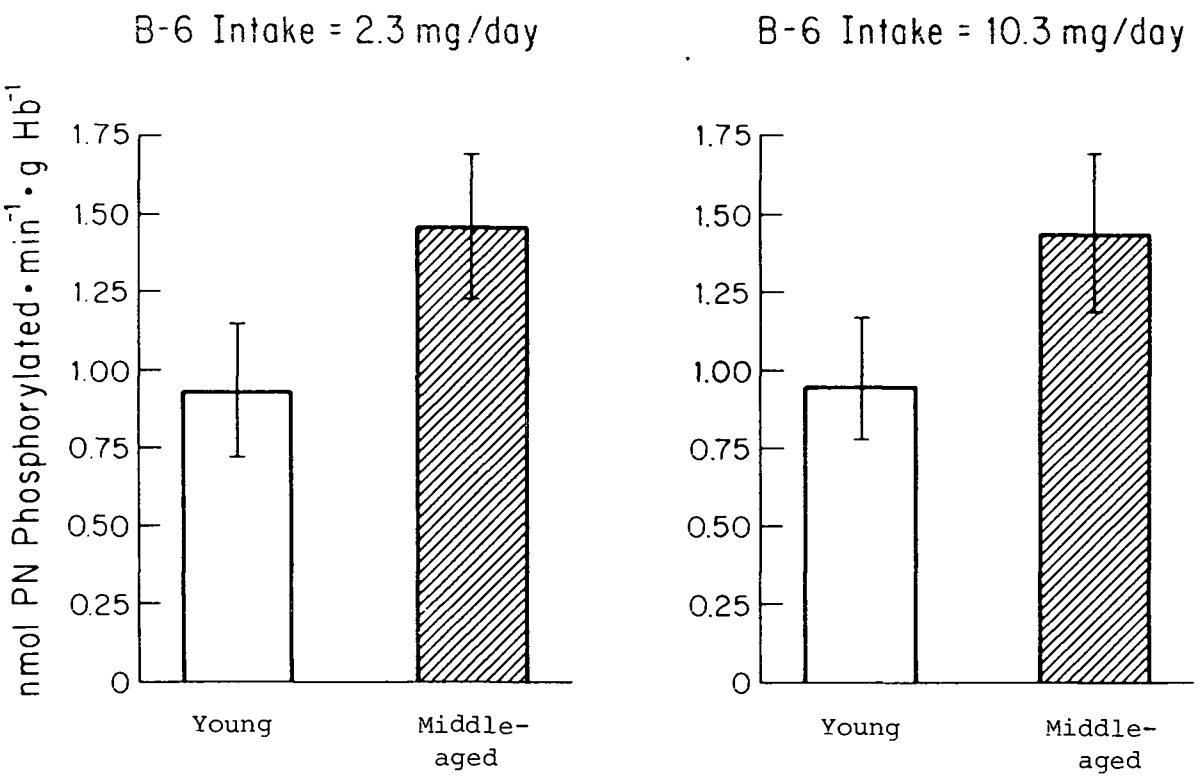


Figure 3.2 Constancy of pyridoxine kinase activity in young and middle-aged women fed constant diets at two levels of vitamin B-6 intake



Chapter 4

BLOOD MAGNESIUM CONSTANCY WITH VITAMIN B-6 SUPPLEMENTATION IN PRE- AND POST-MENOPAUSAL WOMEN

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ABSTRACT

Plasma and erythrocyte (RBC) magnesium was unchanged in ten pre-menopausal and seven post-menopausal subjects during the 48-day controlled study where vitamin B-6 intake was either 2.3 mg or supplemented to 10.4 mg per day. Dietary magnesium was kept at 240 to 270 mg per day throughout. All subjects were within reported normal ranges during all experimental periods for plasma and RBC magnesium. These results suggest that at usual intakes, vitamin B-6 does not influence the levels of plasma and RBC magnesium in normal women.

INTRODUCTION

Vitamin B-6 and magnesium have been studied together in various disease states, including autism (Barthelemy et al., 1981; Lelord et al., 1981; Lelord et al., 1982) and kidney stone formation (Faragalla and Gershoff, 1963). Their interaction in normal metabolism was not documented until recently when Abraham et al. (1981) postulated a fundamental role for vitamin B-6 in the active transport of minerals across cell membranes. This was based on their evidence of apparently elevating RBC magnesium concentrations by feeding 100 times the RDA of vitamin B-6 to nine pre-menopausal women low in RBC magnesium. Their postulate is in opposition to the position held for at least 50 years (Greenberg et al., 1933; Searcy, 1969) that red cell membranes are impermeable to magnesium. However, errors in original interpretations of metabolic processes have been discovered by subsequent

researchers, and the present authors attempted to repeat the magnesium exchanges in our laboratory. In order to test this postulate under more usual circumstances, magnesium levels were followed in normal pre- and post-menopausal women four weeks on a diet containing an adequate amount of vitamin B-6 and three weeks on the same diet supplemented to five times the RDA (Food and Nutrition Board, 1980). Dietary magnesium was kept at 240 to 270 mg per day, approximately the average intake for women in the Western U.S. (USDA 1980).

MATERIALS AND METHODS

Subjects

Ten pre-menopausal women aged 20 to 30 and seven post-menopausal women aged 48 to 61 volunteered to participate in the study. Each subject signed an informed consent form before being accepted into the study, which was approved by the Committee for Protection of Human Subjects at Oregon State University. All subjects were judged healthy by blood chemistry screens, physical examinations and xylose absorption. Fasting blood samples were drawn from the antecubital vein on days 1, 10, 13, 17, 20, 31, 38, 41, 45 and 48 into Vacutainer brand tubes for trace element determination (No. 6527, Becton, Dickinson & Co., Rutherford, NJ).

Treatment

During the first four weeks of the study, subjects consumed a controlled diet providing 2.3 mg of vitamin B-6 per day. Foods free

of vitamin B-6 were used to adjust calories from 2000 to 2500 to meet individual requirements. The diet provided adequate intakes of all macro- and micronutrients for which there are Recommended Dietary Allowances (RDA 1980) except iron. Iron supplements were given on alternate days (32 mg ferrous sulfate, Bi-Mart Drugs, Eugene, OR). During the final three weeks, 8.1 mg of pyridoxine (as pyridoxine-mono-hydro-chloride, Calbiochem, San Diego, CA) were administered orally in 5 ml of one percent acetic acid in addition to the same foods consumed during the first phase. The dietary magnesium was controlled throughout at 240 to 270 mg per day. The low level (< 2 ppm) of magnesium in the local water supply contributed less than 5 mg per day.

Methods

Dietary vitamin B-6 was assayed by the Association of Official Analytical Chemists microbiological method (AOAC 1980).

Dietary magnesium was assayed by atomic absorption spectrophotometry on perchloric acid digested diet composites (Perkin-Elmer, 1976).

Plasma magnesium, whole blood magnesium and hematocrits were assayed by the method of Abraham et al. (1981) with two minor alterations. Hemolysis was carried out in Mg-free glass vials by alternately freezing at -20°C and thawing at room temperature three times to produce a cell-free liquid. Samples were diluted 1:50 in one percent HCl for direct aspiration. All samples for plasma magnesium

for all subjects were assayed on the same day. Two weeks later, all whole blood magnesium samples were assayed. Standards were prepared from Banco Magnesium Reference Standard for atomic absorption (Anderson Laboratories, Inc., Ft. Worth, TX) over the range of 0.1 to 0.5 ppm Mg, which gave a linear response and covered the range of samples analyzed.

Red blood cell magnesium was calculated from the formula of Abraham et al. (1981), which takes into account the hematocrit of each sample in conjunction with the plasma and whole blood magnesium levels. No correction was made for the small amount of trapped plasma in the red cells nor for the volume and magnesium content of white cells and platelets. As Abraham et al. point out, the former contributes less than 0.2 mg per dl and the latter about 0.1 mg per dl in the opposite direction.

RESULTS

The results are outlined in Table 4.1. Initially all subjects had magnesium levels within the normal range of 2.0 to 2.3 mg per dl for plasma (Jackson and Meren, 1968) and 5.3 to 7.3 mg per dl for RBC (Girard et al., 1966). At no stage during the experiment did the average values for either group fall significantly outside of these expected ranges. There were no significant differences between any of the sampling times with vitamin B-6 intake of two mg versus ten mg. There was no significant difference in the plasma or RBC magnesium levels of the pre-menopausal women when compared with the post-menopausal subjects over the study.

DISCUSSION

There is no indication from the results of this study that vitamin B-6 is involved in altering plasma or RBC magnesium in normal women on adequate intakes of both vitamin B-6 and magnesium. Therefore, these results do not support the postulate of Abraham et al. (1981) that vitamin B-6 plays a role in the active transport of magnesium across cell membranes. However, several differences between these two studies should be pointed out. First, all of the subjects in our study were healthy women with both plasma and RBC magnesium within the normal range at the outset of the study. Those in the other study were below the normal range at the outset, with three out of the nine subjects having even lower values for RBC than for plasma magnesium. Second, in our study, the diet was controlled throughout for 48 days, with every portion of every food for each subject each day being weighed and consumed under supervision in the Metabolic Unit of the Department of Foods and Nutrition. No dietary intake information was published in the other study. Finally, our study was based on physiological doses of vitamin B-6 (the RDA vs five times the RDA) while the other study used a pharmacological intake of vitamin B-6 (100 time the RDA).

Also included in our study was a group of post-menopausal women about whom very little nutritional information has been published from controlled studies. No difference was found by us in their plasma or RBC magnesium levels on either normal or supplemented

vitamin B-6 diets when compared with the pre-menopausal group. This supports the statement by Lindeman (1981) that concentrations of various electrolytes, including magnesium, do not vary significantly with age.

In Table 4.1 are also compared the values of plasma and RBC magnesium for the pre-menopausal women in our study with those of Abraham et al. (1981). Their "mean $\pm$ SE" values have been recalculated as "mean $\pm$ SD," which more clearly reflects individual variation. It is clear from these data that throughout our study magnesium concentrations were constant. Also, it seems that their subjects represented a different population than ours, since the mean RBC magnesium concentrations are more than two standard deviations apart for all comparisons.

CONCLUSION

In healthy adult females, magnesium levels in plasma and RBC are not affected by changing the vitamin B-6 intake from the RDA (two mg per day) to five times that level over a 48-day trial.

Table 4.1 Plasma and erythrocyte (RBC) magnesium in pre- and postmenopausal women on controlled diets with vitamin B-6 at 2 mg or 10 mg/day*

	Premenopausal Women (n = 10)		Postmenopausal Women (n = 7)	
	Plasma Mg mg/dl	RBC Mg mg/dl	Plasma Mg mg/dl	RBC Mg mg/dl
B-6 = 2.3 mg per day				
Day 1	2.1 ± 0.2	6.7 ± 0.8	2.1 ± 0.2	7.1 ± 0.5
Days 10,13	2.1 ± 0.1	6.5 ± 1.0	2.1 ± 0.2	6.8 ± 0.9
Days 17,20	2.1 ± 0.1	6.8 ± 0.8	2.1 ± 0.2	6.6 ± 0.8
B-6 = 10.4 mg per day				
Day 31	2.0 ± 0.2	6.2 ± 0.6	2.0 ± 0.1	6.4 ± 0.6
Days 38,41	2.1 ± 0.1	6.4 ± 0.5	2.1 ± 0.1	6.4 ± 0.7
Days 45,48	2.0 ± 0.1	6.4 ± 0.5	2.1 ± 0.2	6.5 ± 0.6
B-6 = 200 mg per day**				
Control	1.7 ± 0.2	2.3 ± 0.8		
Week 1	1.8 ± 0.2	4.0 ± 1.2		
Week 2	2.0 ± 0.2	4.2 ± 1.0		
Week 4	1.9 ± 0.2	4.9 ± 0.5		

* Values are mean ± SD.

** Data from Abraham et al. (1981) were recalculated to give mean ± standard deviation. There were nine subjects in their study.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Differences in vitamin B-6 metabolism and status in eight middle-aged and five young women were studied under controlled, constant dietary conditions. It was hypothesized that older women would have lower vitamin B-6 status, which might reflect an age-related metabolic difference. This metabolic difference was hypothesized to be at least in part due to differences in activity of pyridoxine kinase enzyme. The primary objective was to investigate the responses of vitamin B-6 status indicators when young and older women were fed identical constant diets. Secondarily, the activity of pyridoxine kinase enzyme and the level of the divalent cationic cofactor magnesium were studied in both groups of women.

This study consisted of two groups of three experimental weeks separated by one adjustment week. The diet provided 2.3 $\pm$ 0.1 mg of vitamin B-6 per day and at 2000 Kcal consisted of 16% protein, 35% fat and 49% carbohydrate for all except weeks 3 and 7. During weeks 3 and 7, minor alterations were made to increase the carbohydrate level, correspondingly decreasing the fat level, with constant protein and vitamin B-6 levels to facilitate a simultaneous research project by other investigators and which did not alter the parameters measured for this research.

Twenty-four hour urine collections were made throughout the study and monitored for completeness by creatinine analysis. Urinary

vitamin B-6 and 4-pyridoxic acid were measured each Tuesday, Wednesday and Friday. Diet samples were analyzed each Tuesday for total vitamin B-6. Fasting blood samples were drawn from the antecubital vein prior to breakfast each Wednesday and Saturday for analysis of plasma pyridoxal 5'-phosphate and plasma vitamin vitamin B-6. Hemoglobin and hematocrit values were determined each week. A two-gram L-tryptophan load test was given with breakfast the last two Wednesdays of each period for each vitamin B-6 level.

The data were statistically evaluated by Student's t-tests, analysis of variance and correlations based on linear regression of best fit equations to assess relationships between parameters and to determine the differences in parameters due to age and dietary vitamin B-6.

The data obtained demonstrate, for the first time, that healthy, middle-aged women have significantly different vitamin B-6 metabolism than comparable younger women under conditions where dietary intake was adequate and constant for extended periods of time. The older women had significantly lower plasma pyridoxal 5'-phosphate and total vitamin B-6 as well as total urinary vitamin B-6 on normal vitamin B-6 intakes. The excretion of urinary 4-pyridoxic acid was slightly higher in the older women. With supplementation, both age groups, the circulating status indicators increased 3-4 fold and excreted indicators increased 5-6 fold. During the supplemented weeks, only the difference in urinary total vitamin B-6 remained significant between the age groups. The tryptophan load test revealed no

significant difference between age groups in excretion of kynurenic acid or xanthurenic acid. Differences in erythrocyte pyridoxine kinase were documented between the age groups, with the older women being 50% higher throughout. Supplementation did not alter the activity of this enzyme.

Plasma and erythrocyte magnesium did not differ significantly between age groups. All subjects were within normal ranges during all experimental periods for both plasma and erythrocyte magnesium. Changing the vitamin B-6 intake from 2.3 to 10.3 had no effect on the blood magnesium levels. These results suggest that at usual intakes, vitamin B-6 does not influence the levels of plasma and red blood cell magnesium in normal women.

The implication of the evidence obtained in this investigation could be important for aging persons. The older women in this study had lower circulating levels of B-6 vitamers than the younger women on normal intakes, accompanied by an increase in urinary vitamin B-6 excreted. This suggests that they are activating less of the absorbed vitamers and/or catabolizing more of them. The disappearance of significant age differences in three of four status indicators with vitamin B-6 supplementation suggests there may be age differences in the requirement for this vitamin. However, this is not supported by the results of the functional test administered. Results of the tryptophan load test suggest that 2.3 mg/day of vitamin B-6 is probably an adequate intake for the women studied.

These results demonstrate an age-related difference in vitamin B-6 status in women under controlled conditions of dietary intake with adequate vitamin B-6. They support the results of studies done under less controlled conditions. They also reveal an age-related metabolic difference in vitamin B-6 metabolism. In addition they provide useful information for establishing a requirement for vitamin B-6 in older women.

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APPENDIX

Appendix Illustrations

Consent Form

I, _____, give my consent to participate in this study. The study has been explained to me and all my questions have been answered. I agreed to consume all food and vitamin supplements provided and to provide a record of food consumed for three days prior to beginning of the experiment. I agree to participate in the five exercise periods scheduled in this experiment on the bicycle ergometer. I understand that there is a risk of a heart attack during a test such as this and that the type of exercise procedure to be conducted has been explained. I further agree to participate only after clearance of a physician is given. I agree to allow 23-30 mls of blood to be drawn periodically throughout the experimental period to monitor B-6 status and four times during each exercise test. I also agree to collect 24 hour urine samples throughout the experimental period.

I understand that I will be paid $100.00 should I complete the study. I understand that I am free to withdraw from the study at any time and that I can terminate the exercise part of the study at any time. I also understand that the investigators reserve the right to withdraw me from this study at any time.

All information concering me will be kept confidential.

Name _____ Date _____

Witness _____ Date _____

General Instructions for Fall '82 Diet & Exercise Study

COLLECTION OF URINE:

1. Collect all urine in containers provided (24 hr. urine collection). You will receive clean urine containers each morning.

2. Label all containers carefully and clearly with your initials and date.

3. Each day:

   Urine collections will be made on a 24-hr. basis and run, for examole, from 6:45 am one day until the same time the next day. Therefore, the collection made on rising in the morning belongs with the urine collected on the previous day and should be dated accordingly. It is important that the collection made on rising is done at the same time each day.

4. Urine will be collected starting with breakfast on the day you start on the diet study. Return urine samples daily at any time convenient for you to Rm 106, Mlm Hall.

5. Store urine in a cool place and protected from light.

6. Please be careful not to spill or lose any urine. If this does happen, however, let us know immediately. The urine collections are a very critical part of this study.

7. Drink approximately the same amount of fluids each day if possible.

EXERCISE TEST (4 times during the study)

1. Come at your scheduled time to Mlm Hall, Rm 107 in comfortable clothes for exercising.

2. Exercising tests will be done fasting (before breakfast).

3. Use only decaffinated beverages the day before and the day of your exercise test (ie. after the test is completed).

4. Breakfast will be provided after the exercise testing period has been completed.

OTHER

1. Eat all food given to you each day. Let us know if you are receiving too much or too little food and we will adjust your diet accordingly.

2. Record all activities every day. A journal will be provided for you at breakfast to fill in for the previous day's activities (# hrs. slept, working, exercising, etc.).

3. No alcoholic beverages, including beer and wine, are to be consumed during the study.

4. No vitamin or mineral supplements are to be consumed during the study.

5. No smoking or use of nicotine during the study.

FREE FOODS

May be eaten in any amount desired, but amounts eaten must be recorded except for condiments:

Coffee, tea	Condiments:
Diet beverages	Salt
	Pepper
	Spices, etc.

Note: Coffee and tea should be used black except for adding honey and milk already included in your diet allotment.

Name _____

Date _____

DAILY ACTIVITY SHEET

1. Record all activity for the previous day and length spent at each.

Activity	Length of Time (fraction of hours)	Time of Day	Intensity
Work			
Sleep			
Sitting			
Walking			
Running			
Bicycling			
Swimming			
Other sports or activities (indicate type)			

2. Record all "free" foods in exact amounts used. Indicate type also used, decaf, etc.

Coffee (cups) _____

Tea (cups) _____

Diet Pop _____

3. How do you feel today? Excellent _____
Good _____
Fair _____
Poor _____

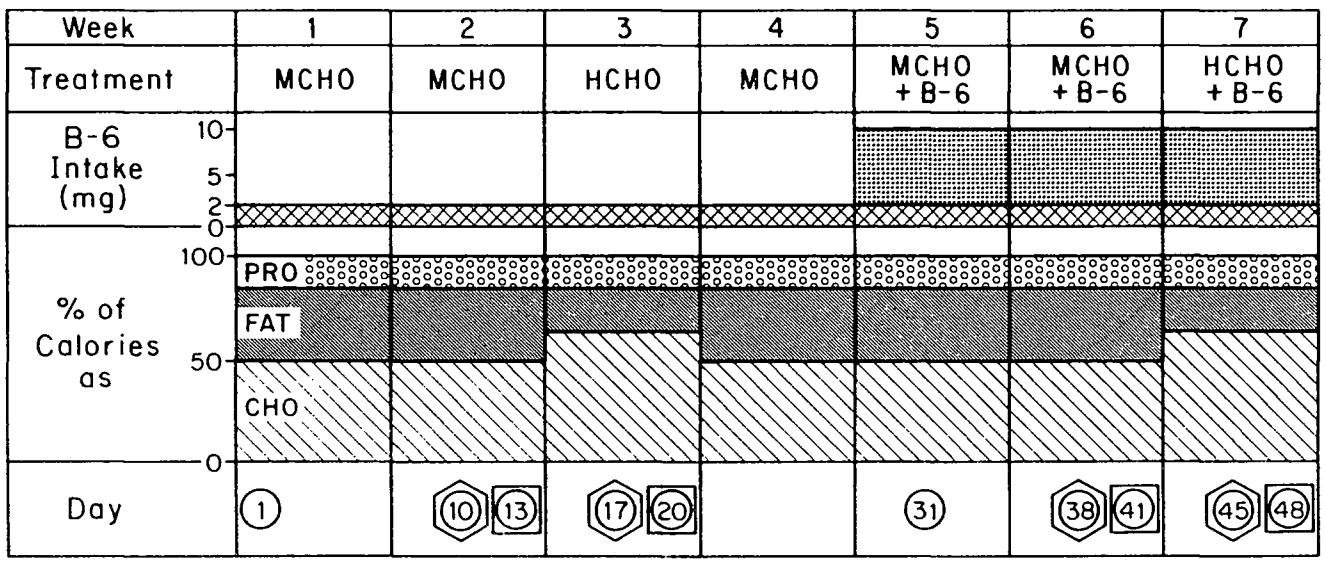
4. Any medications? (i.e., aspirin, etc.)

5. Other unusual events, exams, injuries, etc.

6. Did you turn your urine bottles in and pick up clean ones? _____

7. Other comments.

Experimental Design



Legend: MCHO = moderate carbohydrate diet
HCHO = high carbohydrate diet
+ B-6 = 8.0 mg/day pyridoxine supplement
PRO = protein
CHO = carbohydrate

Blood sample
Tryptophan load test
20 minutes exercise on stationary bicycle

Table 1. Vitamin B-6 in diets and supplements

	Normal Carbohydrate				High Carbohydrate	
	Week 1	Week 3	Week 5	Week 6	Week 4	Week 7
Vegetables & Fruit	1.53[1]	1.63	1.59	1.48	1.66	1.70
Turkey	0.36	0.32	0.32	0.32	0.32	0.30
Tuna	0.21	0.21	0.21	0.22	0.21	0.21
Milk	0.186	0.157	0.157	0.181	0.194	0.172
Ice Cream/Ice Milk	0.036	0.036	0.024	0.028	0.037	0.035
Total in Diet	2.32	2.35	2.30	2.23	2.42	2.42
Supplement[2]	--	--	7.97	7.97	--	7.97
Total B-6 Consumed	2.32	2.35	10.27	10.20	2.42	10.39

[1]Values are averages of three measurements for mg vitamin B-6 contributed by each food group per day.

[2]Assayed four times over the experiment: 7.97 ± 0.38 mg.

Table 2. Urinary creatinine excretion of young and middle-aged women (g/24 hours)

Middle-aged Subjects	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
2	0.86 ± 0.10[1]	0.91 ± 0.05	0.90 ± 0.05	0.91 ± 0.03	0.89 ± 0.02	0.88 ± 0.05	0.89 ± 0.04
4	1.28 ± 0.26	1.10 ± 0.10	1.18 ± 0.10	1.12 ± 0.07	1.06 ± 0.03	1.16 ± 0.13	1.11 ± 0.07
6	1.04 ± 0.06	1.05 ± 0.08	1.25 ± 0.08	1.09 ± 0.07	1.03 ± 0.07	1.10 ± 0.06	1.09 ± 0.04
7	0.94 ± 0.05	0.97 ± 0.04	1.06 ± 0.06	0.95 ± 0.03	0.94 ± 0.01	0.92 ± 0.05	0.98 ± 0.06
9	0.91 ± 0.08	0.98 ± 0.06	1.12 ± 0.06	0.92 ± 0.12	0.98 ± 0.04	0.95 ± 0.14	0.99 ± 0.06
12	0.95 ± 0.06	0.93 ± 0.08	1.10 ± 0.04	1.03 ± 0.08	1.01 ± 0.06	1.01 ± 0.06	1.05 ± 0.07
15	1.02 ± 0.09	1.08 ± 0.02	1.16 ± 0.07	1.08 ± 0.08	1.03 ± 0.05	1.05 ± 0.06	1.11 ± 0.09
16	1.15 ± 0.02	1.15 ± 0.10	1.14 ± 0.04	1.10 ± 0.09	1.10 ± 0.05	1.13 ± 0.05	1.14 ± 0.10
Young Subjects							
1	1.23 ± 0.14	1.19 ± 0.13	1.30 ± 0.07	1.33 ± 0.08	1.29 ± 0.06	1.28 ± 0.11	1.32 ± 0.13
11	1.26 ± 0.17	1.22 ± 0.14	1.23 ± 0.13	1.23 ± 0.14	1.32 ± 0.17	1.22 ± 0.14	1.30 ± 0.07
14	1.10 ± 0.07	1.14 ± 0.07	1.24 ± 0.09	1.19 ± 0.08	1.15 ± 0.09	1.19 ± 0.12	1.23 ± 0.10
17	1.06 ± 0.05	1.04 ± 0.12	1.11 ± 0.04	1.10 ± 0.05	1.08 ± 0.05	1.13 ± 0.04	1.18 ± 0.03
18	1.04 ± 0.04	0.94 ± 0.15	1.02 ± 0.07	1.01 ± 0.10	1.04 ± 0.07	1.02 ± 0.11	1.14 ± 0.10

[1] Means ± S.D. of seven values for each week

Table 3. Urinary xanthurenic and kynurenic acid excretion before (basal) and after (load) a 2 g L-tryptophan load

		Urinary Xanthurenic Acid Excretion (μmol/24 hr)								Urinary Kynurenic Acid Excretion (μmol/24 hr)							
		Week 2		Week 3		Week 6		Week 7		Week 2		Week 3		Week 6		Week 7	
		Basal	Load	Basal	Load	Basal	Load	Basal	Load	Basal	Load	Basal	Load	Basal	Load	Basal	Load
Middle-Aged Women	2	10.5	39.5	16.2	44.0	10.9	35.3	14.9	35.6	8.5	55.0	9.3	45.7	9.3	78.5	10.3	58.5
	4	25.4	33.4	33.3	42.5	26.2	33.4	21.3	33.8	16.6	47.7	15.8	62.1	18.4	55.9	13.5	64.1
	6	17.8	35.6	19.2	39.3	18.6	33.5	16.7	35.6	12.5	48.5	11.8	56.4	13.4	59.1	13.0	67.6
	7	19.1	33.5	19.2	37.8	23.6	34.9	18.0	38.2	13.0	82.4	13.2	94.3	15.3	88.2	14.2	107.8
	9	14.7	28.0	18.6	27.7	14.9	26.8	14.1	24.8	8.8	39.6	8.3	37.0	8.0	43.5	8.5	45.1
	12	13.5	29.7	19.8	28.0	14.6	31.0	13.9	26.5	10.9	45.3	14.0	47.7	12.0	54.1	14.1	51.2
	15	13.3	28.7	17.4	29.9	15.7	28.0	19.1	29.0	14.0	94.7	12.5	76.6	15.1	98.1	15.5	73.6
	16	23.4	41.9	22.1	36.1	22.7	34.8	20.4	34.5	16.2	87.3	14.2	62.5	14.7	68.3	13.1	72.5
Young Women	1	12.2	34.4	13.5	34.4	13.4	36.6	11.3	35.0	9.8	72.4	10.8	72.7	11.3	76.2	14.4	73.7
	11	14.5	35.4	16.0	28.7	14.7	29.3	12.9	26.4	13.7	77.3	15.2	70.8	10.8	67.6	10.2	70.8
	14	16.2	33.2	17.8	36.1	16.3	35.1	23.7	42.4	12.3	85.7	13.0	93.7	11.4	84.4	13.4	69.2
	17	15.5	36.4	15.6	--[1]	14.1	30.6	15.1	35.1	11.6	84.4	14.2	--[1]	11.3	66.0	14.7	96.6
	18	15.6	26.7	15.6	32.2	18.4	33.2	18.5	39.3	14.5	63.8	13.1	75.7	15.5	89.8	20.6	128.8

[1] Urine sample missing for second tryptophan load test for subject No. 17.

Table 4. Analysis of variance results for tryptophan metabolites in young and middle-aged women: Effect of subject age, dietary vitamin B-6, and dietary carbohydrate.

	Xanthurenic Acid						Kynurenic Acid					
	Change [1]		Basal [1]		Load [1]		Change		Basal		Load	
	F	p	F	p	F	p	F	p	F	p	F	p
Age	.88	.37	1.82	.20	.05	.83	3.07	.11	.02	.89	2.43	.15
B-6	.51	.49	.16	.70	.86	.38	1.79	.21	.49	.50	2.15	.17
	.16	.70	2.30	.16	.17	.69	.22	.65	1.77	.21	.16	.69
Age x B-6	.13	.73	1.66	.22	2.39	.15	.03	.86	.08	.79	.00	.96
Age x CHO	.32	.56	.05	.83	.23	.64	.56	.47	4.61	.06	.59	.46
B-6 x CHO	.59	.46	4.21	.06	1.26	.29	.01	.92	.32	.58	.20	.66
Age x B-6 x CHO	1.53	.24	4.29	.06	.11	.75	.10	.76	1.05	.32	.01	.91

1 Basal refers to the day before the tryptophan dose.
Load refers to the day of the dose.
Change is the difference between basal and load day excretion.

Table 5. Selected biochemistry parameters

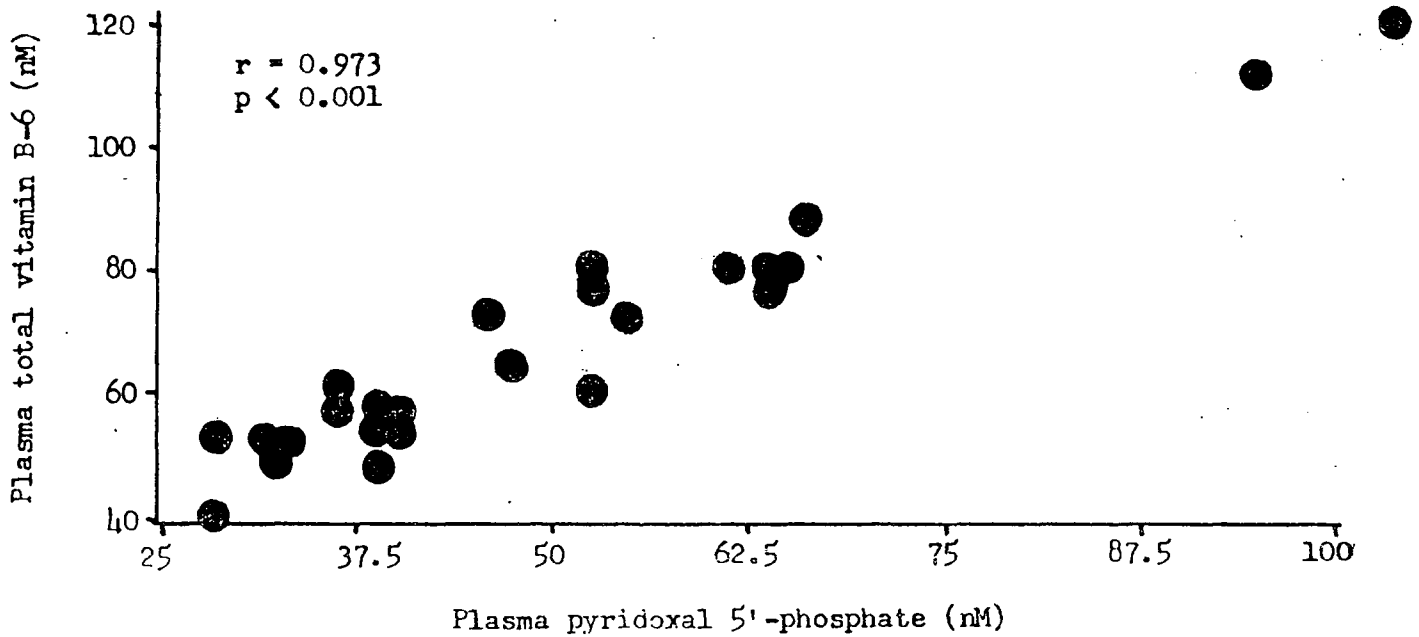
		Alkaline Phosphatase U/l		Albumin gm/dl		Cholesterol mg/dl		Lactic Dehydrogenase U/l	
		week 1	week 7	week 1	week 7	week 1	week 7	week 1	week 7
Middle-aged Women	2	40	48	4.3	4.3	180	154	155	159
	4	42	44	4.1	4.3	214	207	198	(254)
	6	40	40	3.9	3.8	232	250	228	196
	7	65	59	4.1	4.3	183	171	200	195
	9	32	36	4.7	4.2	247	196	200	193
	12	69	67	3.7	4.0	177	172	142	121
	15	50	45	4.1	4.0	211	166	191	147
	16	71	69	4.4	4.2	257	243	186	179
	$\bar{X}$	51	51	4.2	4.1	213	195	188	170
	SD	15	12	0.3	0.2	31	36	27	29
Young Women	1	49	38	4.7	4.3	133	120	137	184
	11	27	30	4.5	4.4	184	149	174	167
	14	24	46	4.7	4.4	195	163	171	106
	17	24	23	4.4	4.2	121	155	129	109
	18	40	40	4.8	4.5	186	139	147	121
	$\bar{X}$	33	39	4.6	4.4	164	145	152	137
	SD	11	6	0.2	0.1	34	17	20	36

Table 6. Blood levels of copper, zinc and magnesium in young and middle-aged women fed constant diets[1]

		Plasma Cu (µg/dl)		Plasma Zn (µg/dl)				Plasma Mg (mg/l)						Whole Blood Mg (mg/l)						Erythrocyte Mg (mg/l)					
		Wk 2	Wk 6	Day 10	Day 13	Day 38	Day 41	Wk 1	Wk 2	Wk 3	Wk 5	Wk 6	Wk 7	Wk 1	Wk 2	Wk 3	Wk 5	Wk 6	Wk 7	Wk 1	Wk 2	Wk 3	Wk 5	Wk 6	Wk 7
Middle-aged Women	2	122.2	111.2	77.4	72.4	83.8	76.5	21	19	21	19	20	20	42	37	36	38	35	38	69	60	56	62	55	63
	4	124.5	136.7	96.5	91.8	95.6	--[2]	21	22	22	21	21	20	39	45	38	39	40	41	65	78	61	64	69	70
	6	121.1	121.1	95.1	105.6	98.0	94.2	24	23	24	22	23	23	41	40	43	40	45	42	66	66	72	66	76	70
	7	141.5	146.3	101.9	131.1	107.8	89.5	23	22	22	22	22	22	45	36	45	35	39	36	79	57	80	56	64	58
	9	146.3	133.0	94.2	87.7	101.9	93.0	21	20	20	21	21	20	40	42	40	41	38	40	72	76	73	73	66	73
	12	72.7	76.4	94.2	94.2	99.5	99.5	21	19	20	19	20	19	40	37	34	34	34	35	70	64	55	57	56	58
	15	134.2	131.2	83.0	89.5	90.0	82.0	18	20	20	20	19	19	45	43	43	41	40	39	80	71	72	65	68	64
	16	106.5	105.3	107.2	127.9	100.1	99.5	21	21	21	22	21	21	39	45	38	39	40	41	65	78	61	64	69	70
	$\bar{X}$	121.2	117.8	93.7	100.0	97.1	90.6	21	21	21	21	21	21	41	40	39	38	38	39	71	67	65	63	63	65
	SD	23.3	22.9	9.6	20.4	7.4	8.7	2	1	1	1	1	2	3	3	4	3	3	3	7	8	8	6	6	6
Young Women	1	104.6	99.1	101.4	88.7	94.5	84.6	21	22	21	21	22	21	43	42	36	41	40	36	75	73	59	70	66	58
	11	109.3	93.6	112.5	104.9	84.7	79.4	21	21	20	20	21	20	30	38	34	36	35	34	44	66	57	61	60	58
	14	93.2	93.2	132.1	100.7	109.0	103.7	22	21	21	20	21	21	37	41	40	39	41	41	61	71	68	69	71	72
	17	99.3	95.6	85.3	102.5	80.0	87.7	23	21	22	24	22	22	43	43	39	39	38	38	74	77	64	62	62	63
	18	107.0	95.6	114.9	139.8	124.4	122.0	22	22	21	21	20	20	41	35	37	34	37	35	68	54	60	54	64	58
	$\bar{X}$	102.7	95.4	109.2	107.3	98.5	95.5	22	21	21	21	21	21	40	42	37	39	38	37	68	72	62	66	65	62
	SD	6.5	2.3	17.3	19.2	18.2	7.4	1	1	1	2	1	1	4	1	2	2	2	3	10	1	4	5	4	6

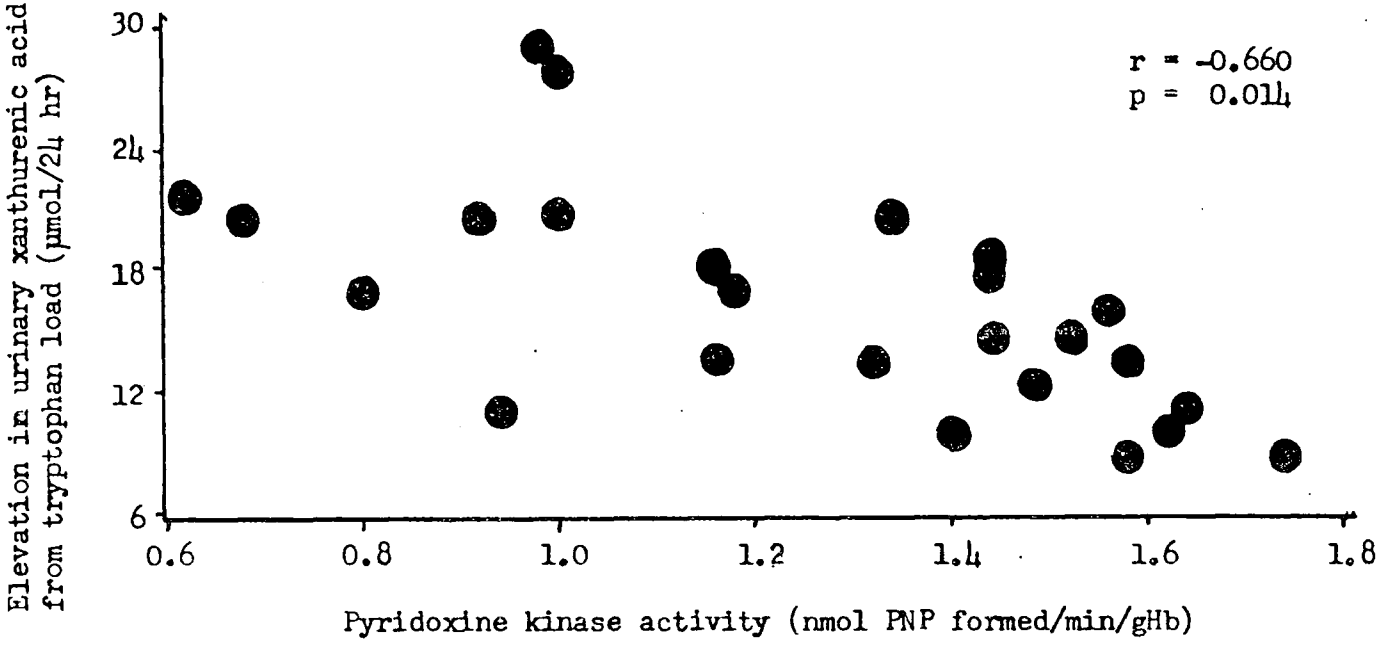
(1) Dietary Cu = 1.23 mg/day for weeks 3 & 7; other weeks = 1.18. Zn = 6.7 mg/day for weeks 3 & 7; other weeks = 7.7. Dietary Mg = 231 mg/day for weeks 3 & 7; other weeks = 268. (2) Zn data missing for subject 4, day 41.

Figure 1. Correlation between plasma pyridoxal 5'-phosphate and plasma total vitamin B-6 at 2.3 mg/day intake



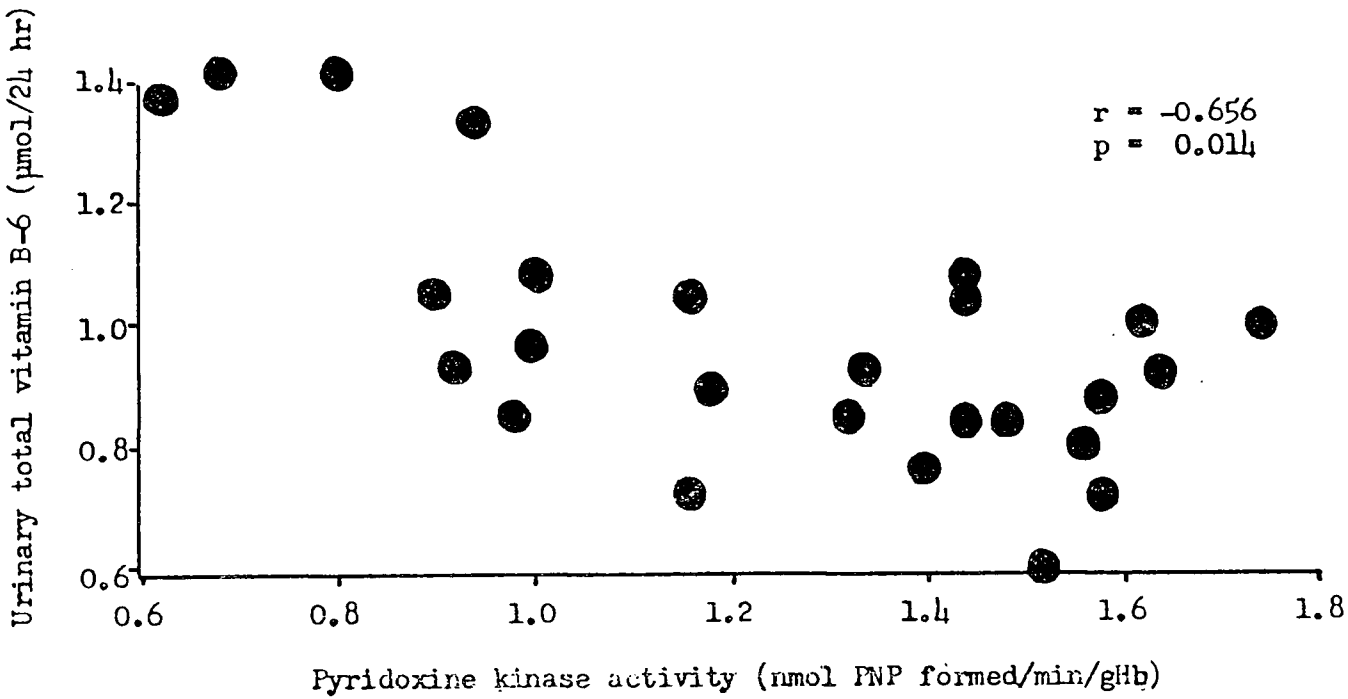
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 2. Correlation between pyridoxine kinase activity and elevation in urinary xanthurenic acid excreted from the tryptophan load test at 2.3 mg/day intake



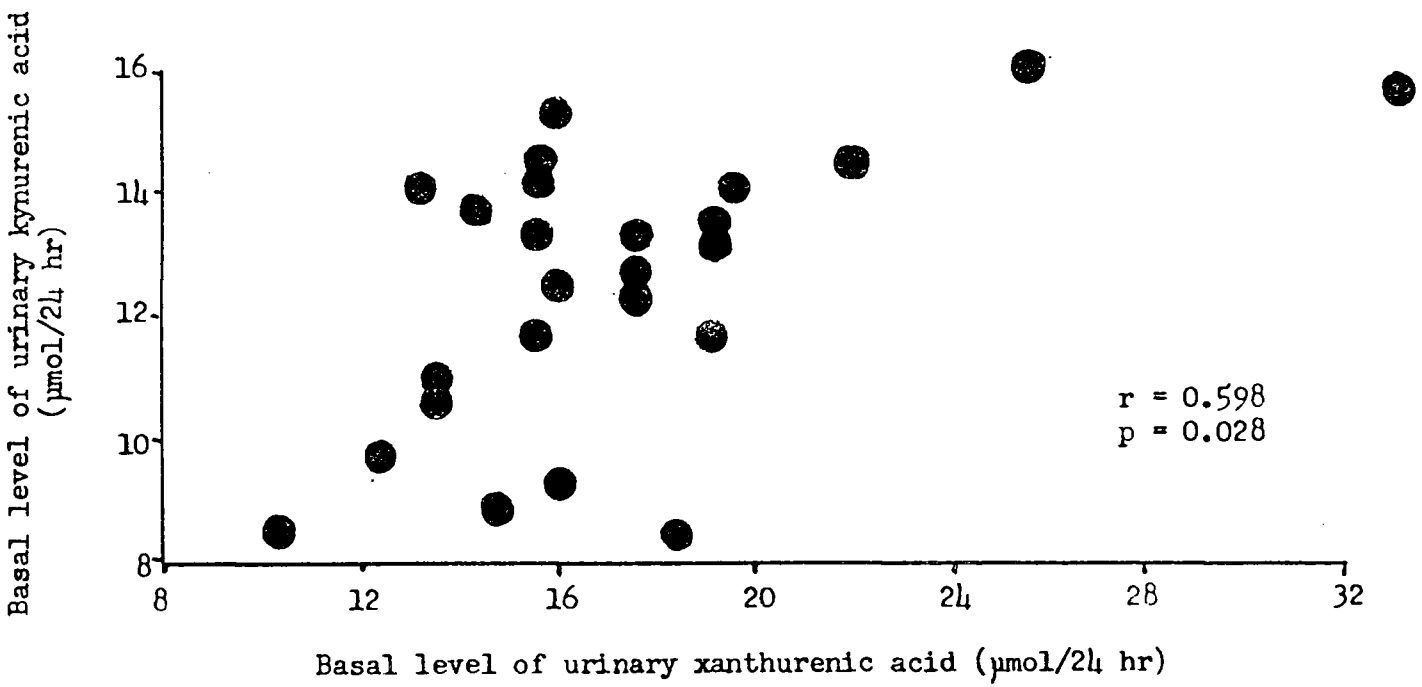
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 3. Correlation between pyridoxine kinase activity and urinary total vitamin B-6 at 2.3 mg/day intake



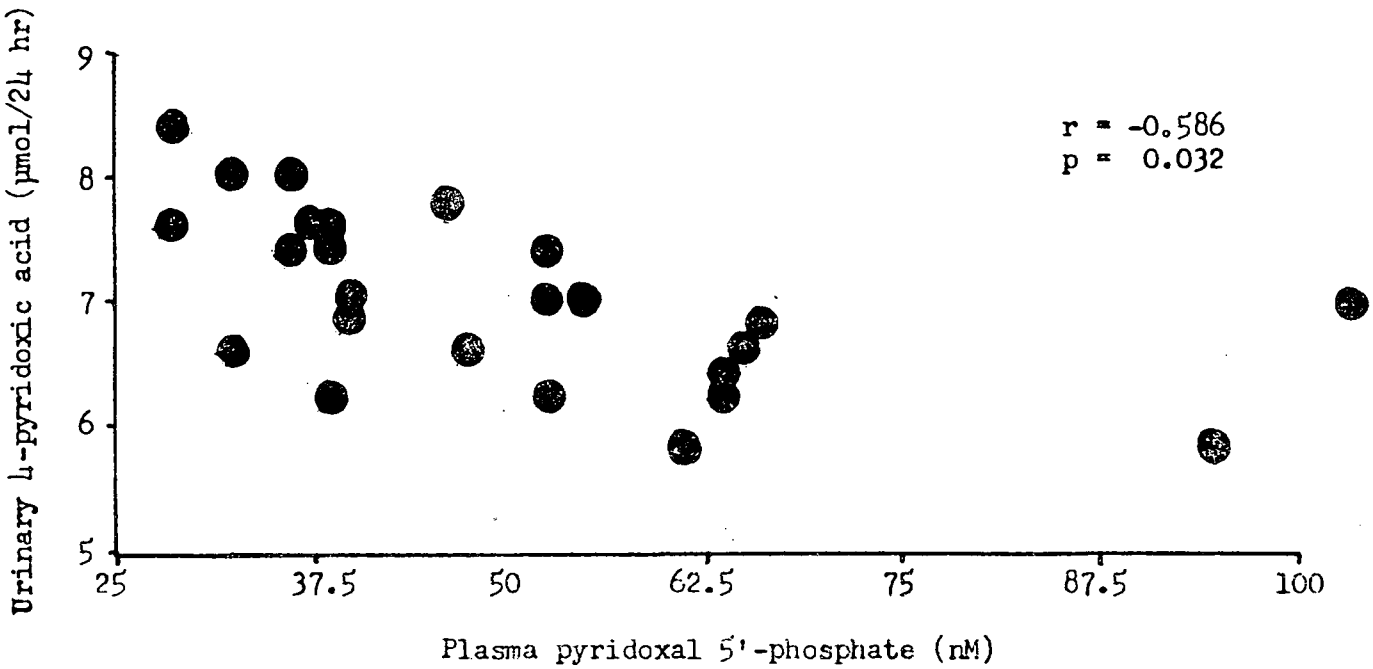
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 4. Correlation between basal level of urinary xanthurenic acid and kynurenic acid at 2.3 mg/day intake



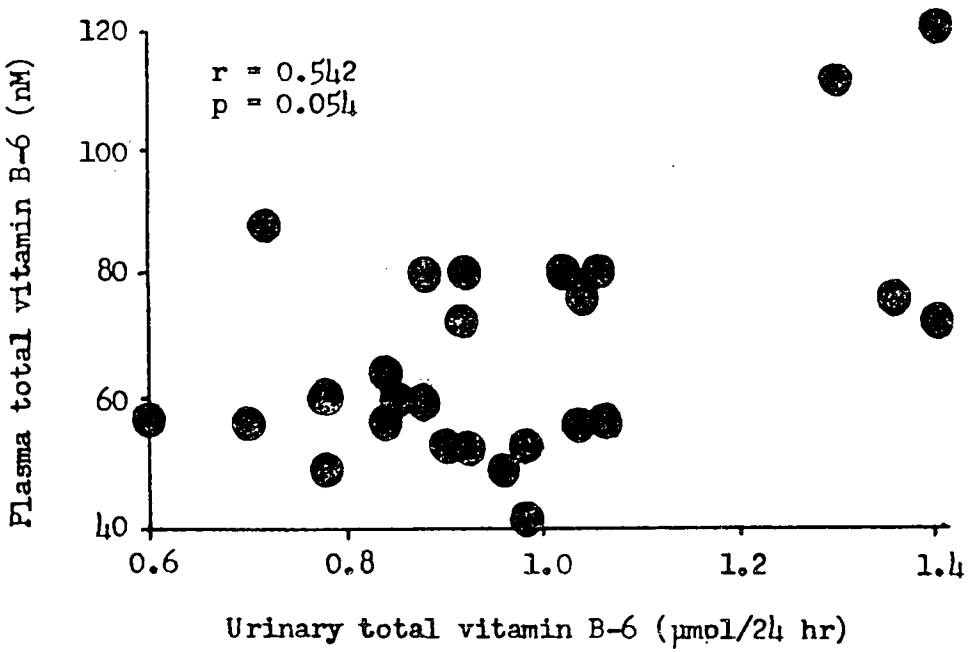
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 5. Correlation between plasma pyridoxal 5'-phosphate and urinary 4-pyridoxic acid at 2.3 mg/day intake



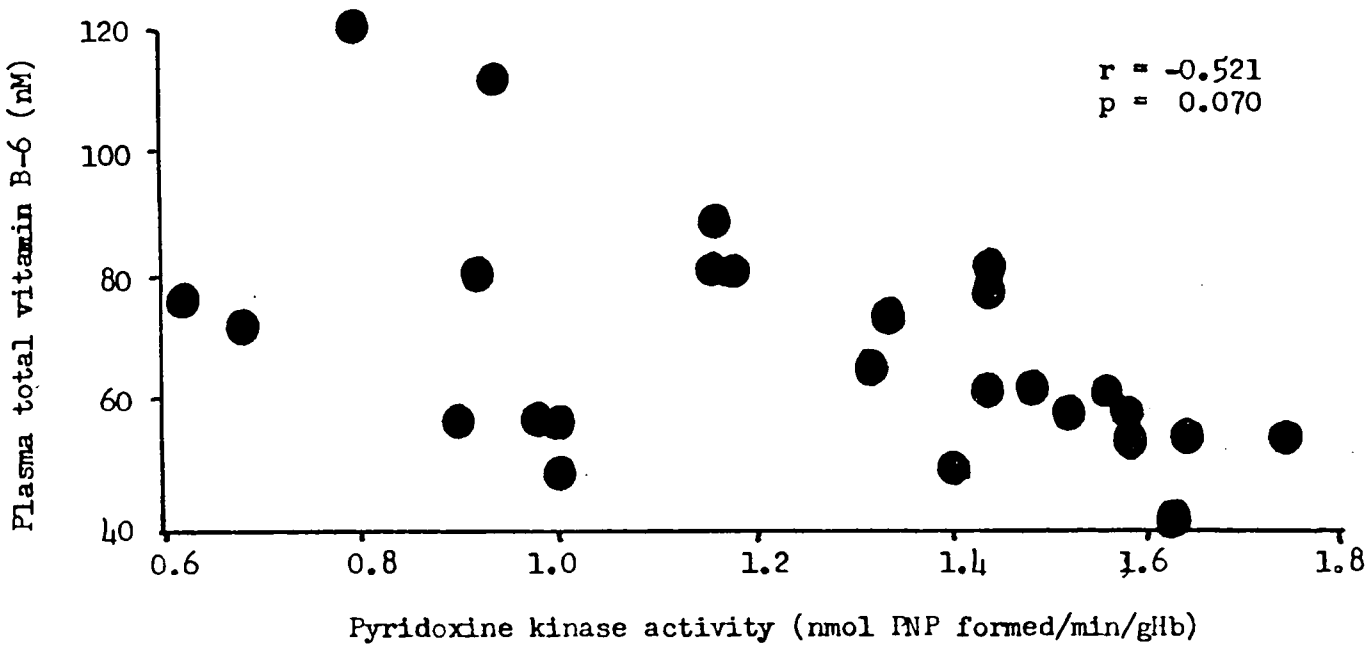
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 6. Correlation between urinary total vitamin B-6 and plasma total vitamin B-6 at 2.3 mg/day intake



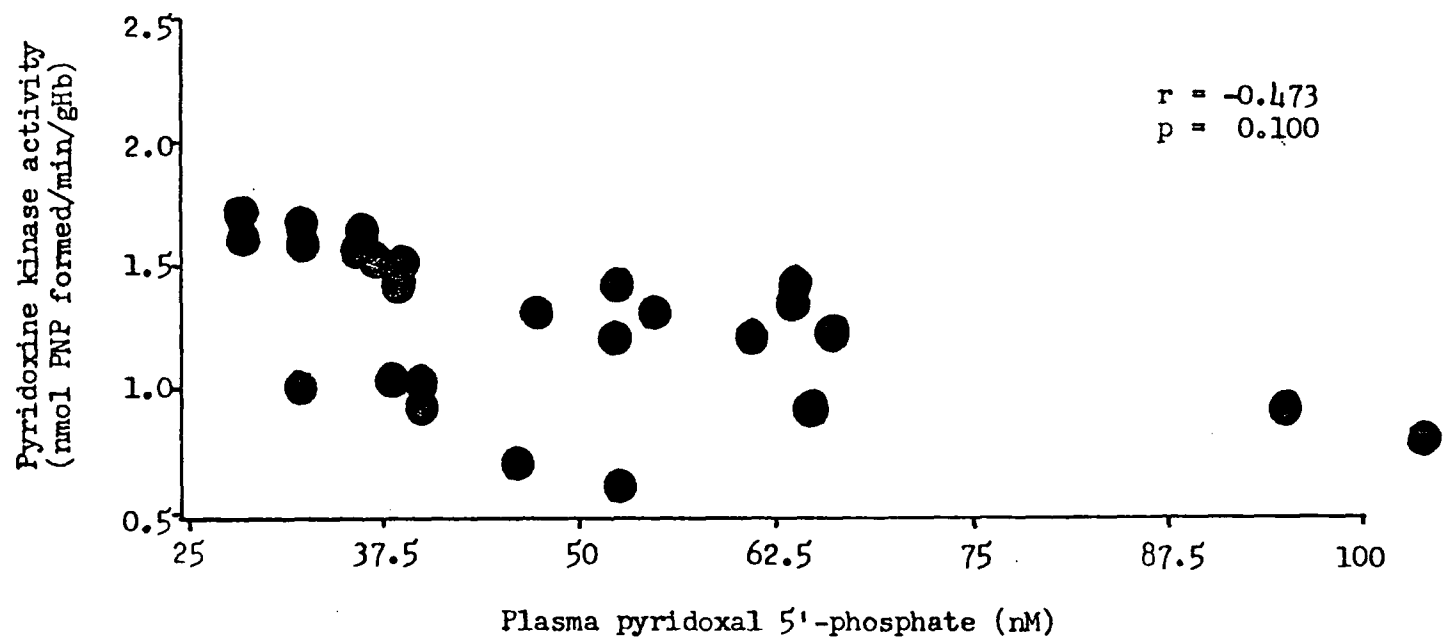
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 7. Correlation between pyridoxine kinase activity and plasma total vitamin B-6 at 2.3 mg/day intake



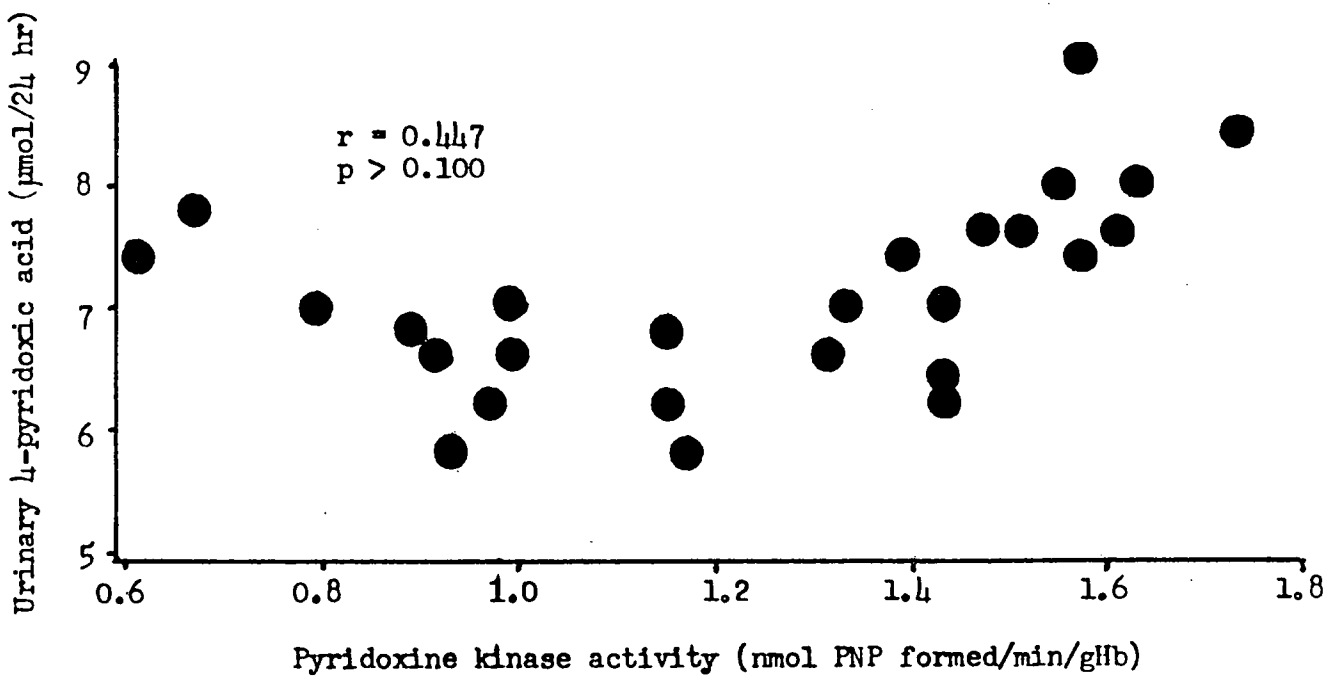
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 8. Correlation between plasma pyridoxal 5'-phosphate and pyridoxine kinase activity at 2.3 mg/day intake



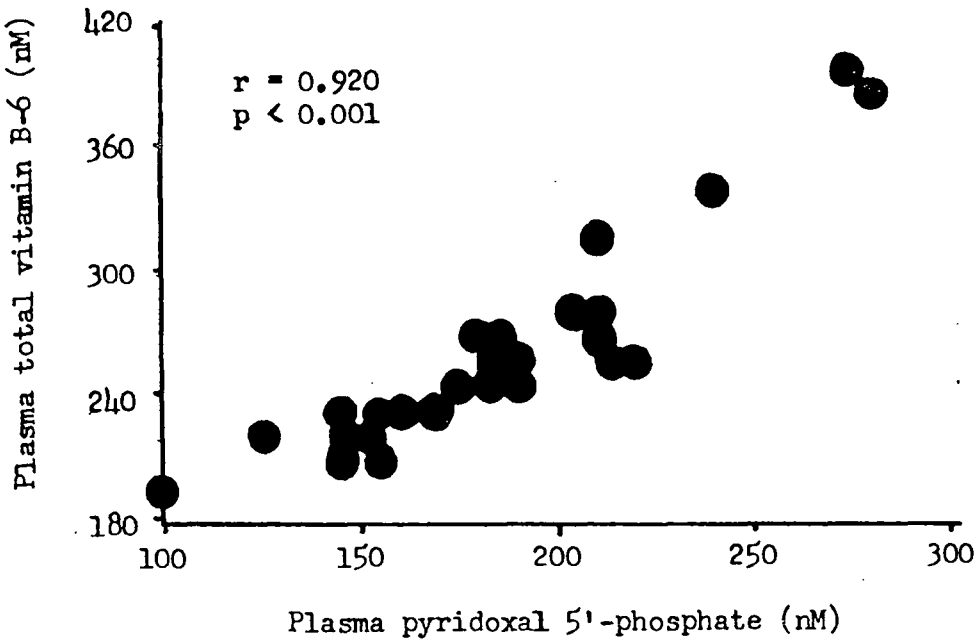
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 9. Correlation between pyridoxine kinase activity and urinary 4-pyridoxic acid at 2.3 mg/day intake



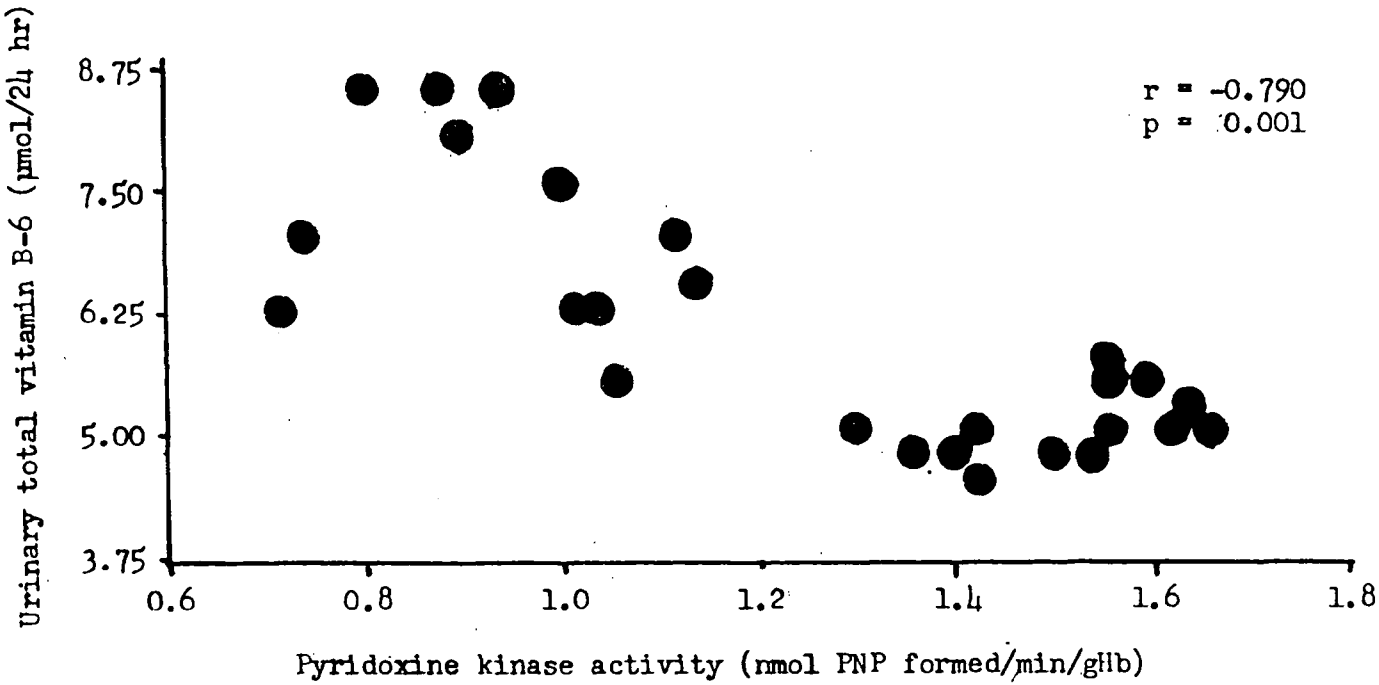
Each circle represents one analysis for week 2 and another for week 3 for each subject.

Figure 10. Correlation between plasma pyridoxal 5'-phosphate and plasma total vitamin B-6 at 10.3 mg/day intake



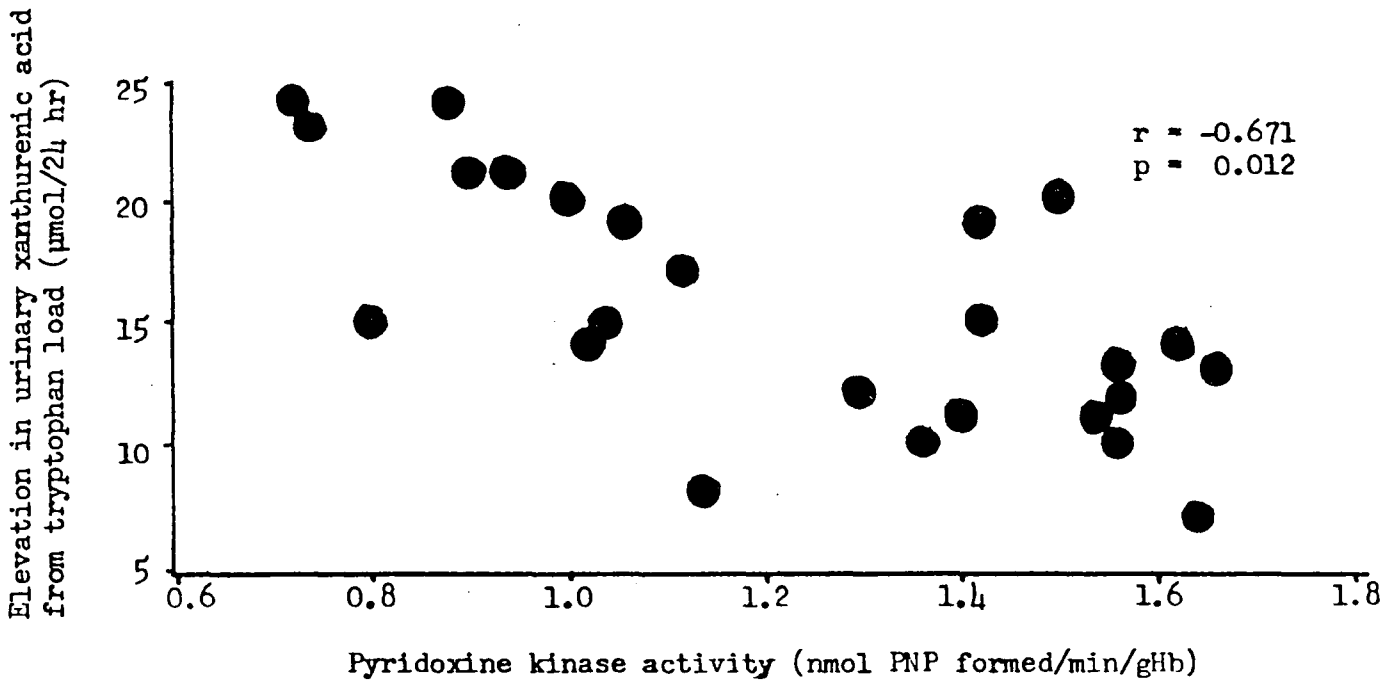
Each circle represents one analysis for week 6 and another for week 7 for each subject.

Figure 11. Correlation between pyridoxine kinase activity and urinary total vitamin B-6 at 10.3 mg/day intake



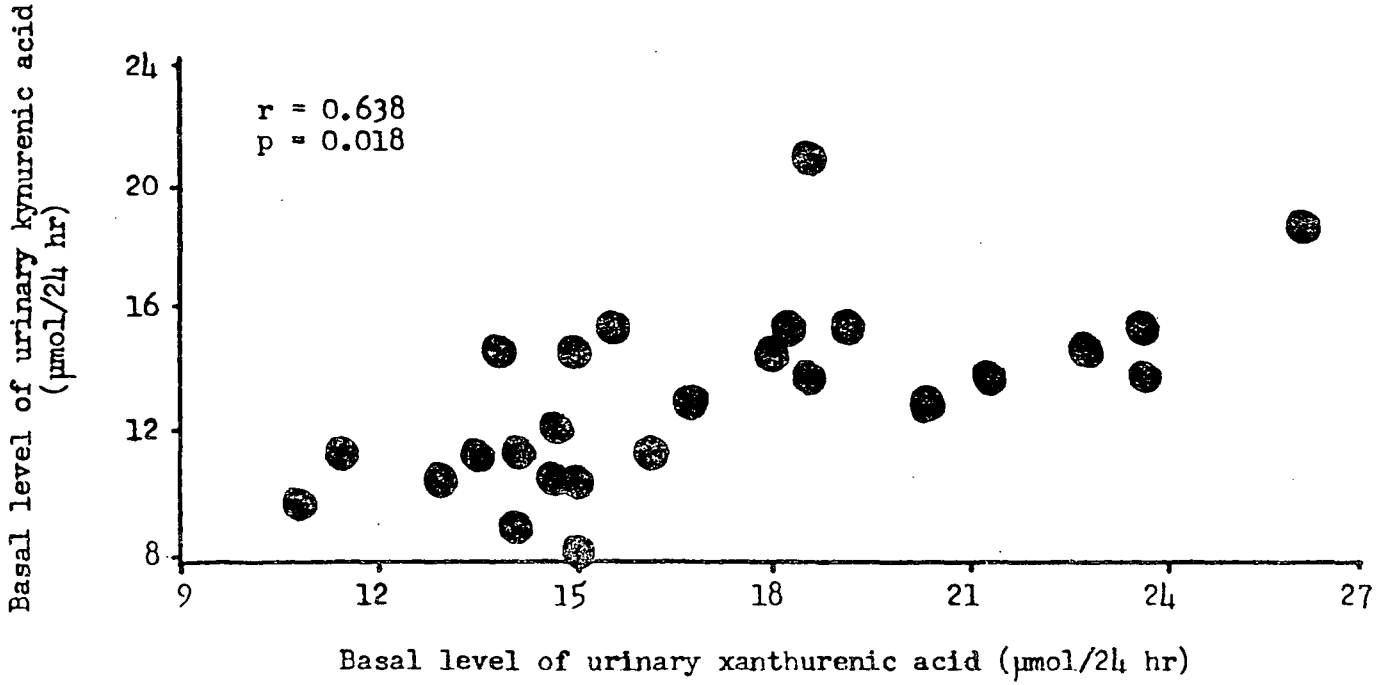
Each circle represents one analysis for week 6 and for week 7 for each subject.

Figure 12. Correlation between pyridoxine kinase activity and the elevation in urinary xanthurenic acid excreted from the tryptophan load test at 10.3 mg/day intake



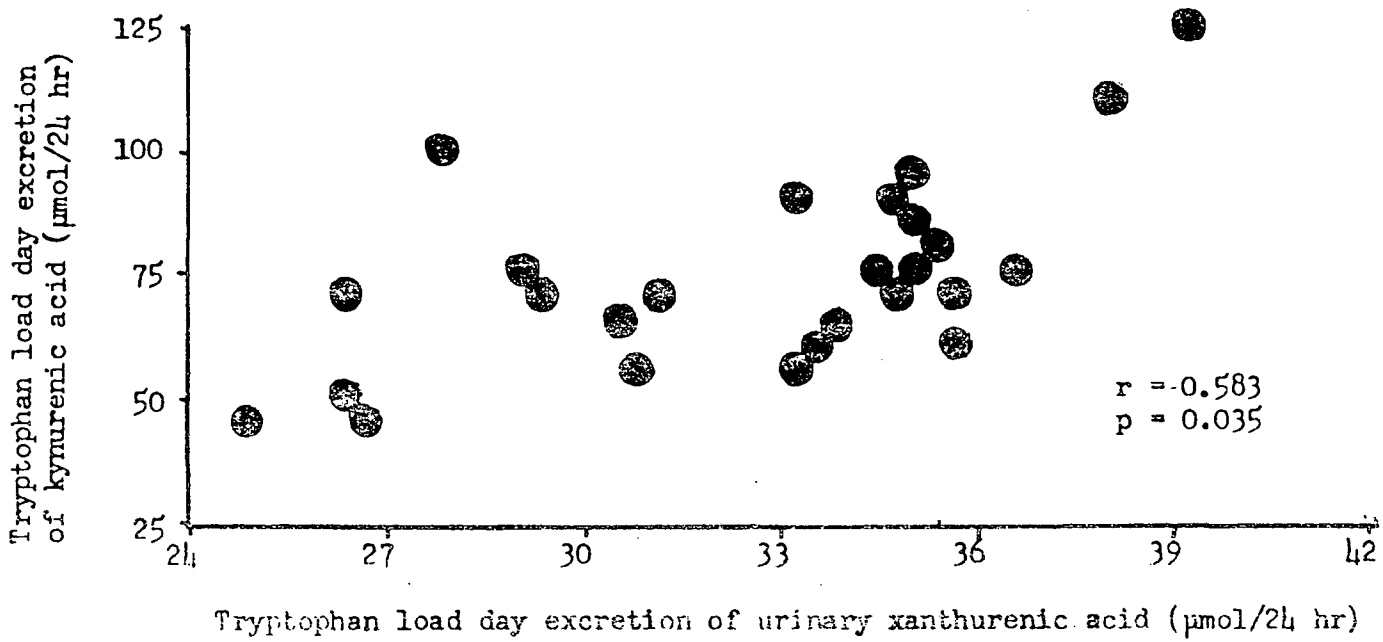
Each circle represents one analysis for week 6 and another for week 7 for each subject.

Figure 13. Correlation between basal level of urinary xanthurenic acid and kynurenic acid at 10.3 mg/day intake



Each circle represents one analysis for week 6 and another for week 7 for each subject.

Figure 14. Correlation between tryptophan load day excretion of urinary xanthurenic acid and kynurenic acid at 10.3 mg/day intake



Each circle represents one analysis for week 6 and another for week 7 for each subject.