AN ABSTRACT OF THE THESIS OF

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Title: IN VIVO AND IN VITRO ASSESSMENT OF VITAMIN B6 BIOAVAILABILITY IN HUMANS.

Abstract Approved: Dr. James E. Leklem

Bioavailability (BA) of vitamin B6 (B-6) from foods may be limited. The knowledge of the BA of B6 from food is important in that this would help to understand if the B6 present in the diet of individuals will meet the requirements for this vitamin.

The purpose of this study was a) to develop a method to measure the level of glycosylated vitamin B6 (GB6) in the foods; b) to investigate the relative vitamin B6 bioavailability from tuna (T), whole wheat bread (WW), and peanut butter (PB) in humans; c) to follow the excretion pattern of GB6 and relate this to the occurrence of the GB6 in foods.

To measure the level of GB6 in foods, the B6 content was determined microbiologically before and after treatment of the foods with

β-glycosidase as well as after acid hydrolysis. Animal products contained no measurable amount of GB6, but grain and legumes had 6-75% of total B6 present as GB6. Of the fruits and vegetables analyzed, orange juice (47%) and raw carrots (51%) had the highest GB6 levels.

Relative BA of B6 from T, WW, and PB was investigated in eight healthy men in a 52-day study (10-day adjustment and three 14-day experimental periods). B6 intake was set at 1.6 mg/day, with 50% coming from one experimental food and 50% from a basal diet. Urine was analyzed for 4-pyridoxic acid (4PA) and B6; feces for B6; and plasma for pyridoxal-5'-phosphate (PLP). Of these four indices used to assess B6 bioavailability, 4PA and urinary B6 were significantly ($p < 0.01$) higher in T than in either WW or PB periods. When T was fed, fecal B6 excretion was significantly ($p < 0.01$) lower than when PB was fed. The B6 in WW and PB was 75% and 63% as available as that from T, respectively.

The urine from the last day of each period for five subjects and the last fecal composite for each period was analyzed for the non-conjugated B6 and GB6. The majority of B6 in the feces was in the non-conjugated form. No GB6 was detected in the feces during either the T or PB periods. Only 4% of total B6 in the feces was in the GB6 form when WW was fed. GB6 was found in the urine in all periods.

The level of GB6 in the food was inversely related to B6 bioavailability in foods fed to humans in our study and in three other human studies. It appears that the level of GB6 in foods could be used as an index of B6 bioavailability in foods.

IN VIVO AND IN VITRO ASSESSMENT OF VITAMIN B6 BIOAVAILABILITY IN HUMANS

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DEDICATION

I would like to dedicate this dissertation to my parents, FATOMEH and MEHDI KABIR and my wife FARIDEH ASKARIAN. They made many sacrifices to make this work possible.

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IN VIVO AND IN VITRO ASSESSMENT OF VITAMIN B-6 BIOAVAILABILITY IN HUMANS

Chapter 1.

Introduction

Vitamin B-6 was first recognized by György in 1934. Since then, several metabolic functions for the vitamin have been identified (György, 1971). Vitamin B-6 functions as a coenzyme with pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate being the coenzyme forms. It acts in several biochemical reactions related to the metabolism of amino acids (Robinson, 1966). Some of the reactions include transamination, racemization, decarboxylation, dehydration and desulfhydration (Braunstein, 1960). The transaminases represent a major group of enzymes which require pyridoxal-5'-phosphate as a cofactor and are responsible for the transfer of the α-amino group of the amino acids to keto acids (Umbreit, 1954).

Pyridoxal-5'-phosphate serves as a coenzyme in reactions involving dihydroxyphenylalanine and 5-hydroxytryptophan. These reactions result in the formation of amines which have biological importance in humans. Pyridoxal-5'-phosphate also serves as a coenzyme for δ-aminolevulinic acid to δ-amino levulinic acid. This latter compound is an intermediate in the formation of porphyrin which is required for hemoglobin synthesis (Cartwright and Wintrobe, 1948).

Vitamin B-6 also plays an important role in the maintenance and functioning of the immune system, probably through its influence on nucleic acid synthesis (Axelrod and Trakatellis, 1964; Axelrod, 1971). The effect of vitamin B-6 deficiency on immune response has been

studied (Bach et al., 1973). However, the mechanism involved in the suppressive effect of a vitamin B6 deficiency on the immune system has not yet been understood.

Baranowski et al. (1957) detected the presence of significant amounts of pyridoxal-5'-phosphate in rabbit skeletal muscle glycogen phosphorylase. Cori and Illingworth (1957) showed that pyridoxal-5'-phosphate is required for the catalytic activity of this enzyme. They also showed that the phosphorylase could be reversibly changed into an enzymatically inactive apoenzyme and free pyridoxal-5'-phosphate. However, the mechanism of action of pyridoxal-5'-phosphate has not been fully determined.

Vitamin B6 may be related to hormone regulation. Litwack (1979) reported that pyridoxal-5'-phosphate competitively binds to the DNA site of activated glucocorticoid complex. Pyridoxal-5'-phosphate is capable of inhibiting or decreasing glucocortocoid induced protein synthesis in liver.

Chemistry of Vitamin B6

The term "vitamin B6" is recommended as the generic descriptor for all 3-hydroxy-2-methylpyridine derivatives that show the biological activity of pyridoxine in rats (Mayes et al., 1974). Vitamin B6 in nature exists as pyridoxine, pyridoxal, pyridoxamine and their phosphorylated forms (Rabinowitz and Snell, 1948). The ultraviolet absorption spectra of different forms of vitamin B6 varies depending on the pH of solution (Snell, 1963). This occurs because each is converted to a variety of aqueous ionic forms depending upon the pH and

other physical factors. Vitamin B-6 is quite stable in acid solutions, but rapid destruction by light occurs in neutral and alkaline solution (Brin, 1977).

Metabolism and Absorption of Vitamin B-6

Vitamin B-6 is found in many foods and occurs as the free, phosphorylated and bound forms (Orr, 1969; Siegel et al., 1943). This vitamin occurs in animal products largely as pyridoxal and pyridoxamine forms, while in vegetable products pyridoxine is the more prevalent form (Rabinowitz and Snell, 1948). The phosphorylated forms of the vitamin in food are probably hydrolyzed to the free forms in the intestinal tract (Turner, 1961; Lemeng and Li, 1975). Booth and Brain (1962) studied the absorption of tritium-labeled pyridoxine-hydrochloride (^{3}H-PN-HCl) in the rat by instilling a dose of 0.5 to 5 mg ^{3}H-PN-HC and 10 mg of unlabeled PN directly into the jejunum, ileum and colon. The percentage of radioactive dose excreted when the labeled dose was placed in the jejunum, ileum or colon was 71 percent, 50 percent and 18 percent, respectively. No absorption took place through the stomach wall. These data suggest that vitamin B-6 is primarily absorbed in the jejunum.

Brain and Booth (1964) studied the absorption of ^{3}H-PN-HCl in seven control subjects who had no sign of malabsorption. In these subjects, ^{3}H-PN-HCL was absorbed relatively fast as measured by the rate of urinary excretion of vitamin B-6. They found that patients with extensive resection of the distal small intestine absorbed appreciable amounts of the ^{3}H-PN-HCl following successive oral doses of 1.0, 5.0, 10.0, 20.0 and 100 mg of ^{3}H-PN-HCl.

The major portion of the dose given was absorbed within the first two hours after feeding. There was a linear relationship between the intake and excretion of urinary vitamin B-6. Also, normal absorption was achieved in one patient who had only 4 feet of residual proximal intestine. This is in agreement with observations in the rat that demonstrated that the absorption of pyridoxine takes place in the jejunum (Booth and Brain, 1962).

Tsuji et al. (1977) studied the uptake of ^{3}H pyridoxine by everted rat intestinal rings at concentrations of 0.1-4μM of the vitamin in the medium. Incubation of the tissue at 37°C for 60 min. in Krebs-Ringer bicarbonate medium containing glucose and labeled pyridoxine resulted in intracellular accumulation of the labeled compounds. The total intracellular concentration of the compound was always higher than the extracellular concentration during incubation, especially at low external concentrations. The tissue was partially saturated with increasing external concentration of ^{3}H pyridoxine in the medium, but this high external concentration was not effective for the first 5 minutes. Addition of 4-deoxypyridoxine to the medium considerably lowered the uptake by the cells. The concentration of intercellular compounds showed that ^{3}H-vitamin is mostly in the phosphorylated form. They concluded that pyridoxine enters the intracellular space by simple diffusion and is accumulated by metabolic conversion to the phosphate form of the vitamin. This conversion is inhibited by 4-deoxypyridoxine.

Middleton (1977) studied the kinetics of mucosal membrane transport of PN·HCl in vitro in the rat jejunum utilizing everted sacs

and a double-label isotope technique. Short-term incubation within the period of initial linear tissue uptake indicated: 1) no evidence of saturation of uptake over a wide PN·HCl concentration range (0.01 - 10 μM); 2) failure of 4-deoxypyridoxine (10 μM) to inhibit uptake of 2 μM PN·HCl significantly. They also suggested a passive diffusion for PN·HCl absorption. In another study, Middleton (1982) found a linear relationship between pyridoxal-5-phosphate concentration and disappearance from perfused segments of rat jejunum with no evidence of saturation at concentrations from 100 to 300 μM of pyridoxal-5-phosphate. At concentrations of 1mM and 3mM pyridoxal-5'-phosphate there was a significant decrease in the disappearance of pyridoxal-5'-phosphate, indicating saturation of the disappearance mechanism which has been suggested to be alkaline phosphatase.

The three free forms of vitamin B-6 are phosphorylated (as the three forms) by the enzyme pyridoxal kinase which is widely distributed in mammalian tissue (McCormick et al., 1961). Pyridoxine-5'phosphate and pyridoxamine-5-phosphate are then oxidized to pyridoxal-5'-phosphate in an irreversible reaction catalyzed by pyridoxine phosphate oxidase. In addition, pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate are interconverted by the action of various aminotransferases. No mechanism for the conversion of pyridoxine to pyridoxal is known in mammalian tissue (Shane and Snell, 1972, 1975). The tissue level of pyridoxal-5'-phosphate is controlled, in part by the action of phosphatases (Lumeng and Li, 1975). Binding of pyridoxal-5'-phosphate to protein protects it from the action of these enzymes (Lumeng et al., 1974; Anderson et al., 1974). The major excretory product of

vitamin B-6 is 4-pryidoxic acid which is formed by oxidation of pyridoxal. This oxidation reaction was believed to be mediated by aldehyde oxidase, a hepatic enzyme with wide specificity. Recent studies, however, have shown a widely distributed, NAD-specific, aldehyde dehydrogenase as being responsible for 4-pyridoxic acid formation (Stanulovic et al., 1976). The pathway is shown in Fig 1.1.

Excretion of Vitamin B-6 and its Metabolites

One approach to the assessment of requirements for B vitamins is the evaluation of vitamin excretion levels in terms of the B vitamin intake (Williams, et al., 1950). These studies, which measure both intake and output (fecal and vitamin B-6 excretion) are also used in assessing availability of nutrients (Ranhotra, et al., 1976).

^{14}C labeled pyridoxine and tritium-labeled pyridoxine have been used to study the excretion of vitamin B-6 in humans. Using an oral dose of ^{14}C labeled pyridoxine, Tillotson et al. (1966) studied the excretion of vitamin B-6 in two subjects. One was fed a self-selected diet providing 1.75 to 2.00 mg of vitamin B-6 and another one was fed a fixed intake of 1.37 mg/day. Complete urine collection was made until half of the carbon-14 activity was excreted. The half-life of ^{14}C in the subjects with the 1.37 mg intake was 20 days and for the subject with an intake of 1.75 to 2.0 mg was 15 days. Percent of total radioactive dose excreted in the feces was 2.5 - 3. In both subjects 95 percent of the administered dose was absorbed. Urinary 4-pyridoxic acid was 30-35 percent of the total dose. Urine was analyzed for the three forms, and pyridoxal represented the major form excreted in the urine. Others excreted in the urine in large amounts were pyridoxamine, pyridoxal phosphate and pyridoximine phosphate.

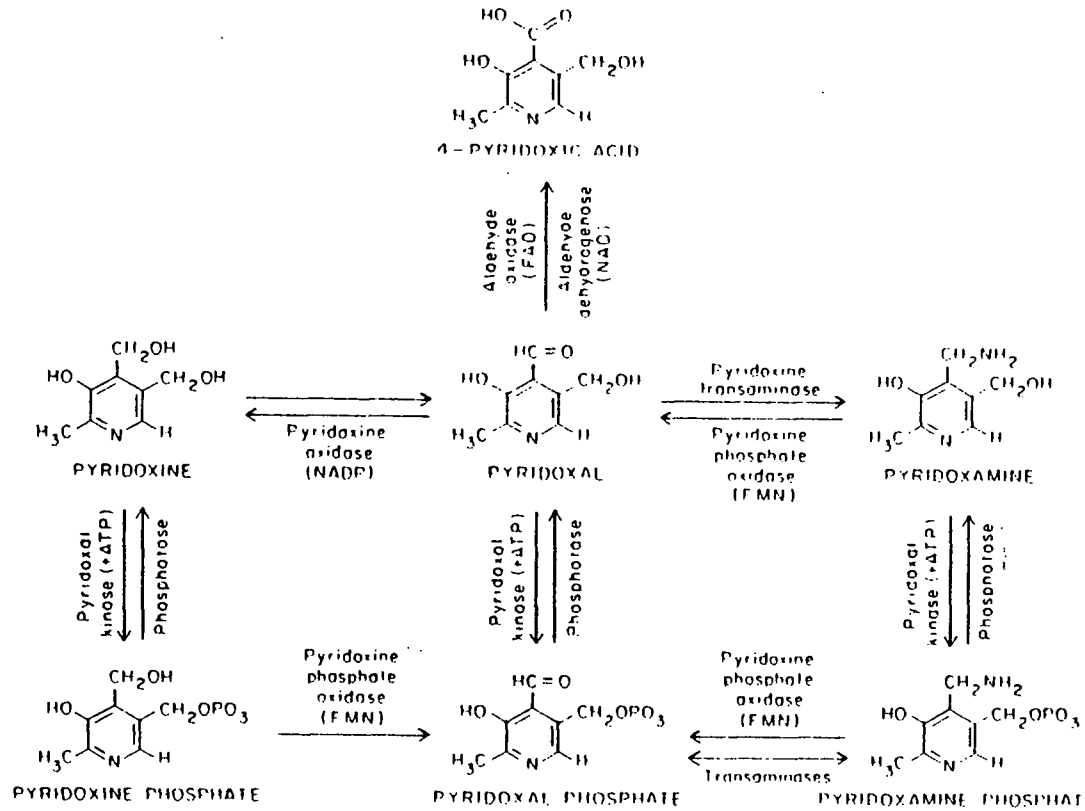

Fig. 1.1. Metabolic Interconversion of Vitamin B-6 and the Formation of 4-Pyridoxic acid. (Adapted from Wada and Snell, 1961)

Johansson et al. (1966) studied the excretion of vitamin B-6 in three subjects given tritium-labeled pyridoxine. Two subjects received 400 μg of labeled pyridoxine intravenously and the third subject an oral dose of 250 μg of labeled pyridoxine. Complete urine was collected daily for 25 days, and urinary 4-pyridoxic acid was measured. The two subjects who received the dose intravenously excreted 28-36 percent of the dose as 4-pyridoxic acid, and the one receiving the oral dose excreted 41 percent as 4-pyridoxic acid. Long-term and short-term diet studies have been conducted to study the excretion of vitamin B-6. Some of these studies are shown in Table 1.2. The study by Denko et al. (1946) showed that five subjects fed a diet containing 1.05 mg vitamin B-6/day and 40 g protein/day, excreted 19 percent and 30 percent of the vitamin as urinary and fecal vitamin B-6, respectively. The study by Linkswiler and Reynolds (1950) showed that at levels of vitamin B-6 intake of 0.76 and 2.76 mg/day the excretion of vitamin B-6 metabolites was higher than the intake of vitamin B-6. They postulated that vitamin B-6 is synthesized by intestinal microflora, but later it was shown that the method used to measure 4-pyridoxic acid gives false high values.

Baysal et al. (1966) showed that the urinary excretion of 4-pyridoxic acid of subjects fell to zero after consuming a diet providing 0.16 mg vitamin B-6 and 100 g protein/day. Kelsay et al. (1968) and Donald et al. (1971) found that ... both male and female subjects consuming 0.16, 0.34 and 0.94 mg of vitamin B-6 excreted relatively small amounts of vitamin B-6 in their urine. The study by Tarr et al. (1981) showed that at a constant protein intake, the excretion of

Table 1.1 Percent of the total vitamin B-6 intake excreted as urinary, fecal vitamin B-6 and 4-pyridoxic acid.

No. of Subjects	Duration of Study	B-6 Intake	Protein Intake	% as Urinary Vitamin B-6	% as Fecal Vitamin B-6	% as 4-Pyridoxic Acid	Reference
	days	mg/day	g/day				
5M[1]	35	1.05	40	19	30	-	Denko et al., 1946
9M	10	0.76	20-30	12	93	313	Linkawiler and Reynolda, 1950
9M	10	15.76	20-30	6	5	56	
3M	10	2.76	20-30	5	33	128	
5M	25	0.16	100	21	-	0	Baysal et al., 1966
5M	40	0.16	54	25	-	31	Kelsay et al., 1968
6M	17	1.60	150	10	-	15	
8F[2]	42	0.34	57	11	-	97	Donald et al., 1971
8F	8	0.94	57	3	-	21	
6M	35	1.1	96	4	-	-	Tarr et al., 1981
6M	35	2.3	96	4	-	-	
6M	21	2.7	96	5	-	-	
9M	7	1.5		8	46	38	Leklem et al., 1980
9M	7	1.5		8.5	29	44	
9M	7	1.5		8	28	44	
2M	1	70PM		4	-	27	Rabinowitz and Snell, 1949
2M	1	82PN		12	-	29	
2M	1	82PL		2	-	65	Ritchey and Feeley, 1966
3F	6	1.3	22.1	7 + 1	-	28 ± 5	
6F	6	1.73	40.3	5	-	20 ± 4	
8F	43	0.34	-	22-4	-	34	Donald and Bosse, 1979
8F	7	0.94	-	3.4		25	
9F	28	0.19	-	-		37	
6F	28	0.80	-	-		42	
3F	28	2.0	-	-		51	

[1]Male [2]Female

urinary vitamin B-6 increases as the intake of the vitamin increases in the range of 1.1-2.7 mg/day. Leklem et al. (1981a) showed that urinary vitamin B-6 is almost the same when whole wheat bread, white bread enriched with B-6 or white bread supplemented with B-6 was fed. However, fecal vitamin B-6 excretion increased, depending on the food which provides the vitamin B-6.

Rabinowitz and Snell (1949) studied the metabolism of the three B-6 vitamers. They found that when pyridoxal was fed, a higher percentage was converted to 4-pyridoxic acid than when either pyridoxine or pyridoxamine was fed. In contrast, more urinary vitamin B-6 was excreted when pyridoxine was fed than when either pyridoxal or pyridoxamine was fed.

Ritchey and Feeley (1966) studied the excretion of urinary vitamin B-6 and 4-pyridoxic acid at two levels of protein. In the girls studied, the increase in protein level did not change the excretion of either urinary vitamin B-6 or 4-pyridoxic acid at a vitamin B-6 intake of 1.3-1.73 mg/day and a protein intake of 22-40 g/day. The studies in Table 1.1 show ranges of 2-25 percent, 0-313 percent and 5-93 percent for urinary vitamin B-6, 4-pyridoxic acid and fecal vitamin B-6, respectively. This wide range may be due to the limited number of subjects, methodological differences and also differences between the total intake of vitamin B-6 in different studies listed in Table 1.1.

Lewis and Nun (1977) determined the percent vitamin B-6 intake excreted as urinary 4-pyridoxic acid in twenty-two children ages 32-107 months and having an intake of 1.1 mg vitamin B-6/day. The percent of the intake excreted as 4-pyridoxic acid was 48 ± 32 percent.

Vitamin B-6 Bioavailability

The vitamin B-6 content of a variety of foods has been determined (Orr, 1969), but determination of the nutrient content of a food, as such is of little value unless it can be related to bioavailability. The term bioavailability refers to the fraction of a total dietary vitamin which undergoes intestinal absorption and functions in vivo as vitamin B-6. The knowledge of vitamin B-6 bioavailability from food would help to assess the intakes required for this vitamin. The Recommended Daily Allowances for vitamin B-6 for different age groups is presented in Table 1.2 (NRC, 1980).

The first study regarding the bioavailability of B-6 from foods was done by Samra et al. (1947). A variety of animal and plant foods were studied. They compared rat growth with the amount of vitamin B-6 in the foods as measured by Saccharomyces uvarum. The apparent bioavailability was calculated from their data by dividing the vitamin B-6 content of the foods obtained from rat growth assay by the vitamin B-6 content measured microbiologically. Liver fractions, whole wheat and yellow corn exhibited a bioavailability ranging from 65 to 75 percent. This is the first indication of incomplete utilization of vitamin B-6 in natural foods. It is important to recognized that Samara et al. (1946) have shown that pyridoxal and pyridoxamine were less active than pyridoxine in promoting growth of rats. This needs to be considered when evaluating vitamin B-6 content derived from rat growth bioassays.

Toepfer et al. (1963) used values obtained from a rat bioassay and the total vitamin B-6 of the food obtained from both chromatographed (ion-exchange chromatogrphy) and non-chromatographed (i.e., total vitamin B-6) extracts of beef, lima beans, milk solids, and whole wheat

Table 1.2 Recommended Daily Dietary Allowances for Vitamin B-6.

Individuals	Age (years)	Vitamin B-6 mg
Infants	0.0 - 0.5	0.3
	0.5 - 1.0	0.6
Children	1 - 3	0.9
	4 - 6	1.3
	7 - 10	1.6
Males	11 - 14	1.8
	15 - 18	2.0
	19 - 22	2.2
	23 - 50	2.2
	51+	
Females	11 - 14	1.8
	15 - 18	2.0
	19 - 22	2.0
	23 - 50	2.0
	51+	
Pregnant		+0.6
Lactating		+0.5

(NRC, 1980)

flour to calculate vitamin B-6 bioavailability. The percent bioavailability, as calculated by dividing the rat bioassay values by the total vitamin B-6 obtained from non-chromatographed extracts, was 93 percent, 96 percent, 70 percent and 68 percent for the beef, lima beans, milk solids and whole wheat flour, respectively. When the bioavailability of vitamin B-6 was calculated based on chromatographed values, the percentages obtained were 83 percent, 106 percent, 78 percent and 84 percent for the beef, lima beans, milk solids and whole wheat flour, respectively. The higher bioavailability values obtained for lima beans and whole wheat flour when the chromatographed values were used were due to lower chromatographed values for these two foods as compared to the total vitamin B-6 content of the foods.

Yen et al. (1976) measured the bioavailability of vitamin B-6 from corn and soybean in chicks, using chick growth and serum aminotransferase activity as a measure of bioavailability. The concentration of available vitamin B-6 as μg/g of food was found to be 2.68 for non-roasted corn, 3.15 for corn heated to 80C, 2.99 for corn heated to 120°C, 2.22 for corn heated to 160°, 4.67 for hulled soybean meal, 4.95 for dehulled soybean meal and 3.32 for autoclaved soy. Corn was lower in available vitamin B-6 than soybean, and this was statistically significant. The results of this study show that as the temperature of roasting of corn increases from 80°C to 160°C, the concentration of available vitamin B-6 decreases. In the case of soybean, the autoclaving also decreased the concentration of available vitamin B-6.

Bioavailability of vitamin B-6 by rat bioassay is primarily based on rat growth. Since this is an indirect measure of bioavailability, a more direct measure of bioavailability is needed. A number of studies in humans have been done to more directly assess vitamin B-6 bioavailability. The rate of absorption of vitamin B-6 in orange juice was examined in human subjects by Nelson et al. (1977). They used a triple lumen tube to determine the uptake of vitamin B-6 from a 30 cm segment of the intestine in 15 normal subjects. Vitamin B-6 absorption was found to be significantly greater from a synthetic solution of 15 μg/100 ml vitamin B-6 than from reconstituted orange juice.

Vitamin B-6 bioavailability of whole wheat bread, white bread enriched with vitamin B-6 and white bread with an oral dose of vitamin B-6 was studied in nine men by Leklem et al. (1980). After six days of adjustment, subjects were randomly fed each of the three breads for one week. Total vitamin B-6 intake was 1.5 mg/day. The whole wheat bread, white bread enriched with B-6 and white bread supplied 1.20, 1.18 and 0.35 mg of vitamin B-6 daily. When white bread was fed, an oral dose of vitamin B-6 was given to subjects to maintain 1.5 mg/day of vitamin B-6. Fecal vitamin B-6 was significantly higher and urinary 4-pyridoxic acid was significantly lower when whole wheat bread was fed than when either white bread enriched with vitamin B-6 or white bread plus an oral dose of vitamin B-6. The plasma pyridoxal-5'-phosphate was lower when whole wheat bread was fed than when white bread enriched with vitamin B-6 was fed. There were no significant differences between urinary vitamin B-6 excretions. Their data suggested

that vitamin B-6 is 5-10 percent less available from whole wheat bread than from WB6 or white bread and an oral dose of vitamin B-6. Vitamin B-6 from beef has been reported to be more available to humans than that from soybeans (Leklem et al., 1980b).

The availability of vitamin B-6 in an average American diet was assessed in six healthy males (Tarr et al., 1981). The study was divided into three periods. During period1, a semi-purified formula diet with a daily supplement of 1.1 mg pyridoxine was fed for 35 days. For period 2, natural foods providing 2.3 mg pyridoxine/day were fed for 35 days. During period 3, a formula diet providing 2.7 mg pyridoxine/day was fed for 21 days. Compared to the pure vitamin (assumed to be 100% available), the bioavailability of vitamin B-6 ranged from 61 to 81 percent when plasma pyridoxal-5'-phosphate was used as an index. Based on urinary vitamin B-6 data, bioavailability of vitamin B-6 ranged from 73-92 percent.

These studies show that for the human the availability of vitamin B-6 from certain foods is limited. The reason for this low availability is not clear, but there are some factors that might contribute to the decreased bioavailability in foods.

One of these factors is food processing. Gregory and Kirk (1978a) studied the effect of roasting of dehydrated food and its impact on stability and bioavailability of vitamin B-6. Dehydrated food systems were used to study the stability and bioavailability of vitamin B-6 as affected by roasting at 180°C for 25 minutes. In order to see the maximum effect of roasting on vitamin B-6 bioavailability, this roasting condition was above the one normally used for commercial processing.

The relative loss of pyridoxine, pyridoxamine and pyridoxal-5'-phosphate was found to be 50-70 percent by both a microbiological and a semiautomated fluorometric procedure. The rat bioassay was used to assess the bioavailability of the remaining vitamin B-6 in food. Using rat weight gain, gain/g feed consumed and erythrocyte aspartate aminotransferase as criteria, the results indicated full bioavailability of the remaining vitamin B-6 after roasting. In another study these same investigators studied the bioavailability of the ε-pyridoxyllysine (Gregory and Kirk, 1978b). During heating and storage of foods this compound is formed from the interaction between pyridoxal-5'-phosphate and protein (Gregory and Kirk, 1977). ε-pyridoxyllysine was 60 percent as available as free pyridoxine, using rat growth, growth per gram of feed consumed and erthyocyte asparate aminotransferase as indicators of vitamin B-6 bioavailability. The bioavailability of vitamin B-6 in non-fat dry milk and a fortified rice breakfast cereal product was also studied by rat bioassay using the above mentioned criteria. The vitamin B-6 in non-fat dry milk was fully available but that of rice breakfast cereal was between 18-44 percent available (Gregory, 1980).

The effect of food processing on the vitamin B-6 content of milk was studied after Coursin (1954) reported the evidence of convulsions due to vitamin B-6 deficiency in infants who consumed a non-fat fortified heat sterilized liquid formula. Hassinen et al. (1954) found that the vitamin B-6 content of all canned liquid milk products is considerably lower than the amount present in the cow's milk. In contrast, the spray-dried milk products, when reconstituted, contained

only slightly less vitamin B-6 than was originally found. Sweetened condensed milk, a product which is not heat-sterilized by heating was found to have less vitamin B-6 than the milk used in its preparation.

Tomarelli et al. (1955) compared the vitamin B-6 content of the liquid milk products and spray-dried milk, both by microbiological assay and rat bioassay method. In the case of heat sterilized liquid milk, the value obtained by rat bioassay was lower than that of microbiological assay. In the case of spray-dried milk, there was agreement between the results of microbiological and rat assay. The above studies suggest that in addition to the decrease in total vitamin B-6 content of food as a result of processing, some compounds may be formed which have a lower bioavailability as compared to the pure form of vitamin B-6.

There are reports that diet composition, especially fiber content, may influence the bioavailability of vitamin B-6 (Gregory, 1980; Leklem et al., 1980a,b). The effect of feeding 15 g of cooked wheat bran on the bioavailability of vitamin B-6 was studied in 10 human subjects (Miller et al., 1979) after a four day adaptation period (a switch-back design was used), in which there were three periods of 18 days each. With the bran diet, there was an increased fecal vitamin B-6 excretion and a decreased 4-pyridoxic acid excretion in 8 subjects. Bran also lowered plasma B-6 in 8 men and plasma pyridoxal-5'-phosphate in 4 men. Bran did not have a consistent effect on urinary vitamin B-6 excretion. Their study suggested that wheat bran may decrease vitamin B-6 bioavailability.

Effects of cellulose, pectin and bran on the bioavailability of pyridoxine have been examined using the rats and chicks bioassay methods (Nguyen et al., 1981a). Dose response curves for growth, feed consumption, feed efficiency and either liver pyridoxal-5'-phosphate or erythrocyte aspartate aminotransferase activity and PLP stimulation in vitro were compared among animals fed experimental diets varying in amount and type of dietary fiber source. Five percent (by weight) of different sources of fiber was added to the chicks' diets and 10 percent to the rats' diets. Diets containing pectin markedly increased the fecal vitamin B-6 content. No difference in liver PLP was detected among rats fed various diets. In chicks, 5 percent dietary pectin resulted in increased feed consumption but depressed growth due to diarrhea. Bran resulted in a 17 percent decrease in bioavailability of pyridoxine in chicks as indicated by growth and feed consumption. Their results suggest that the polysaccharides tested did not have important deleterious effects on the bioavailability of pyridoxine.

In vitro binding of vitamin B-6 by selected polysaccharides has been studied (Nguyen et al., 1981b). The ability of eight purified polysaccharides, lignin, and wheat bran to bind B-6 vitamins in vitro was examined using equilibrium dialysis under physiological conditions. No significant binding was detected when 0.5 percent, 0.75 percent and 1 percent levels of pectin were dialyzed to equilibrium in the presence of 0.1 μ pyridoxine, pyridoxamine and pyridoxal. When one percent wheat bran, lignin, and other polysaccharides were incubated overnight with 0.1 M pyridoxine prior to dialysis, no binding of pyridoxine was observed.

Bound Forms of Vitamin B-6 and Their Relationship to Bioavailability

Natural sources of vitamin B-6 contain the vitamin mainly in a bound form as evidenced by the need for vitorous acid hydrolysis prior to the microbiological assay (Siegel et al., 1943; Rubin et al., 1947). The bound form of vitamin B-6 in this discussion refers to vitamin B-6 bound covalently to another molecule. A majority of vitamin B-6 in foods occur as the phosphorylated form, particularly in animal sources. Pyridoxal-5'-phosphate and pyridoxal can react with the free amine group of amino acids to form a Schiff base. While a Schiff base is not considered a bound form as defined above, this linkage may lead to a more stable bound form as discussed below. Instudies, the addition of pyridoxal and pyridoxal-5'-phosphate to amino acid of primary amines in a dilute solution resulted in an increase in teh yellow color of the mixture. However, N-substituted amino acids did not cause a similar color change. The intensification of the color change in this reaction was due to the formation of a Schiff base (Matsuo, 1957; Metzler, 1967). Pyridoxal has been reductively coupled with amino acids and amino acid esters to give pyridoxyl amino acids (Heyl et al., 1948). Twenty amino acids and esters of amino acids have been reductively coupled with pyridoxal to form pyridoxyl amino acids. These compounds have limited vitamin B-6 activity for rats and no activity for several bacteria and yeast. Eighteen pyridoxal amines of different amino acids have been synthesized and tested for their microbiological activity. All had less than 0.5 percent activity on a weight basis compared to pyridoxal hydrochloride, if the dilutions were made with air-free water (Snell et al., 1948). In another study, pyridoxal was condensed with

several amino acids to form pyridoxylideneamine which had been hydrogenated to give pyridoxyl amino acid esters (Rabinowitz et al., 1953). Their availability was tested in rats. They had 50-100 percent activity, (i.e., growth promoting activity as compared to pyridoxine) exceptfor pyridoxyl-3, 4 dihydroxyphenethylaminde and pyridoxyl-dl arternol, which had 10-20 percent activity (Rabinowitz and Snell, 1953). Pyridoxal and pyridoxal-5'-phosphate have been reported to bind to protein in a liquid model system during processing at 121°C for 20 minutes. Pyridoxal phosphate, the most reactive B-6 vitamin, was shown to bind as a Schiff base, substituted aldamine and pyridoxal amino complex (Gregory and Kirk, 1977). In a low moisture food system, during storage at 37°C and a water activity of 0.6 for 128 days, about half of the pyridoxal-5'-phosphate bound to protein existed as an ε-pyridoxyllysine complex (Gregory and Kirk, 1978 The availability of ε-pyridoxyllysine, as measured in rats, was 60 percent that of free pyridoxine (Gregory and Kirk, 1978b). A reaction involving the sulfhydryl group of milk serum protein and pyridoxal has been shown to occur in evaporated sterilized milk, resulting in the formation of bis-4-pyridoxal disulfide, a compound of low vitamin B-6 activity (Bernhart et al., 1960; Wendt and Bernhart 1960). The bound forms of vitamin B-6 mentioned so far, which include interactions between protein and vitamin B-6, may help to better understand the reactions which may take place during processing and storage of foods which contain both protein and vitamin B-6.

As early as 1942, conjugates of vitamin B-6 of unknown structure were reported in rice bran and urine (Scudi et al., 1942; Scudi, 1942). Scudi found that rice bran contained a water soluble pyridoxine conjugate

of a low molecular weight that was not precipitated by protein precipitants but was absorbed by acid clay and eluted mostly as unbound pyridoxine (Scudi, 1942). One of the bound forms of vitamin B-6 occurring in rice bran was isolated from a faintly yellowish syrup obtained by ion-exchange and chromatographic techniques (Yasumoto et al., 1977). The compound isolated was found to chromatograph in the same place as pyridoxine β-D-glucoside. When the compound was acid hydrolyzed, pyridoxine and glucose were produced. Treatment of the rice bran isolate with β-glucosidase also resulted in release of glucose and pyridoxine. Pyridoxine glucoside has also been isolated from a reaction mixture which contained 20g of pyridoxine hydrochloride, 50g of sucrose, 0.1M phosphate buffer at pH 8.0, and 28g (dry weight) of intact cells of Sarcina lutea IF3232 in 1 liter total volume, and incubated 25 hours at 28°C. The pyridoxine moiety of pyridoxine glucoside was identified by paper chromatography, and subsequent analysis showed that glucose was conjugated with pyridoxine at a molar ratio of 1:1. Ogata and coworkers proposed the linkage to be either through the 4' or 5' position of pyridoxine (Ogata et al., 1968). A conjugate form of pyridoxine and glucose (pyridoxine β-glucoside) has been synthesized by incubation of cellobiose and pyridoxine in the presence of wheat bran β-glucosidase (cellobiase)(Suzuki et al., 1979). The two resultant derivatives have been purified and identified as 4'-(β-gluconyl) pyridoxine and 5'-(β-gluconyl) pyridoxine. Treatment of both conjugates with almond β-glucosidase resulted in release of glucose and pyridoxine. Recently, the occurrence of a particulate enzyme in seedlings of podded pea (Pisum sativum l. vc. kinvsaya), which catalyzed

the transfer of D-glucose from UDP-glucose to the 5'-, but not 4'-, hydroxyl group of pyridoxine has been reported (Tadera et al., 1982). The enzyme has a high affinity for UDP-glucose. Pyridoxine was replaceable by pyridoxamine as a glucosyl acceptor.

Utilization of a chemically synthesized pyridoxine β-glucoside was examined in vitamin B-6 deficient rats (Tsuji et al., 1977). Oral administration of this compound for 12 days corrected the deficiency. But in another study, in which synthetic form was given both orally and intravenously to rats, only the oral administration of this compound was effective in correcting the deficiency. Therefore, this compound seems to be broken down in the intestinal tract (Yasumoto et al., 1979).

A bound form of vitamin B-6 in orange juice has been studied using ultracentrifugation, ultrafiltration, and membrane dialysis of this food (Nelson et al., 1977). The results of the study implies a binding of vitamin B-6 to a molecule of less than 3500 daltons molecular weight. The binding was resistant to protease treatment, although it was susceptible to heat-acid hydrolysis. The binder of vitamin B-6 in orange juice is a small dialyzable molecule which is heat stable and binds both pyridoxal and pyridoxine.

Fatty acid derivaties of vitamin B-6 have been synthesized through an acylation process. An ester linkage is formed between the carboxyl group of fatty acid and hydroxyl group of pyridoxine (Sakuragi et al., 1969a). Several long chain fatty acid esters of hte vitamin B-6 group have been prepared: pyridoxine tripalmitate, pryidoxine trilinoleate, pyridoxal dipalmitate, pyridoxamine tripalmitate. The biological activity of various esters of pyridoxine, such as pyridoxine triacetate, tripalmitate and trilinoleate, has been tested in rats. All of these

compounds had the same activity as pyridoxine hydrochloride (Sakuragi and Kummerow, 1956b). These compounds have not been found in in vivo system.

Rationale for the Research

With the importance of vitamin B-6 in amino acid metabolism, neurotransmitter formation, the immune system, and hemoglobin synthesis (Robinson, 1966; Bach et al., 1973; Cartwright and Wintrobe 1948), its presence in the diet and its bioavailability from foods to humans becomes significant. Vitamin B-6 is present in a wide variety of foods, but the level in the food does not mean it is available to humans to perform the reactions in which vitamin B-6 serves as a co-factor.

Vitamin B-6 bioavailability from natural sources is incomplete (Nelson et al., 1977; Leklem et al., 1980a; Tarr et al., 1981). There are reports that processing of foods decreases its bioavailability (Yen et al., 1976; Gregory and Kirk, 1978a; Tomarelli, 1955). There are also conjugate forms of vitamin B-6 in foods (Scudi, 1942; Yasumoto et al., 1977; Nelson et al., 1977) which might decrease the bioavailability of vitamin B-6 from foods.

Considering the facts mentioned above, the purpose of this study was a) to investigate the bioavailability of vitamin B-6 from tuna, whole wheat bread and peanut butter, foods which are commonly consumed in American diets; b) to measure the level of glycosylated vitamin B-6, a bound form of vitamin B-6 in foods; c) to observe the excretion pattern of glycosylated vitamin B-6 when foods containing this form

were fed; and d) to study the relationship of glycosylated vitamin B-6 in food to vitamin B-6 bioavailability.

CHAPTER 2

MEASUREMENT OF GLYCOSYLATED VITAMIN B6 IN FOODS

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ABSTRACT

MEASUREMENT OF GLYCOSYLATED VITAMIN B6 IN FOODS. G.H. KABIR, J.E. LEKLEM AND L.T. MILLER. Department of Foods and Nutrition, Oregon State University, Corvallis, Oregon 97331.

To examine the levels of glycosylated vitamin B6 in 22 foods, each food was stirred for 2 hr at pH 6.8 and then incubated for 2 hrs at pH 5.0 and 37°C with β-glucosidase (60 units per g food). Vitamin B6 content of foods was measured microbiologically before and after enzyme treatment as well as with acid hydrolysis. Animal products contained no measurable amount of glycosylated B6. Grains and legumes generally had a high level of this bound form (6-57% of the total vitamin B6). Of the fruits analyzed, orange juice had the highest level of glycosylated vitamin B6 (47%). Among fresh vegetables studied, raw carrots had the highest level (51%). For broccoli and cauliflower, the glycosylated value was higher for the processed food as compared to the raw food.

INTRODUCTION

Natural sources of vitamin B6 contain the vitamin mainly in a bound form as evidenced by the need for vigorous acid hydrolysis prior to microbiological assay (Siegel et al., 1943; Rubin et al., 1947). Conjugates of vitamin B6 of unknown structure have been reported in rice bran and urine (Scudi et al., 1942; Scudi, 1942). Subsequently, rice bran was found to contain a water-soluble pyridoxine conjugate of a low molecular weight which is not precipitated by protein precipitants, is absorbed by acid clay and eluted mostly as unbound pyridoxine (Scudi, 1942). It was later shown that vitamin B6 in rice bran concentrate is conjugated with glucose in a 1:1 ratio (Yasumoto et al., 1977). Also, vitamin B6 in orange juice has been found to be bound to a non-protein compound with a molecular weight less than 3500 daltons (Nelson et al., 1977). The exact structure of this conjugated form of vitamin B6 has not been determined. Conjugated forms of pyridoxine and glucose (pyridoxine β-glucoside) have been synthesized by incubation of cellobiose and pyridoxine in the presence of the wheat bran β-glucosidase (cellobiase). The two resultant derivatives have been purified and identified as 4'-(β-glucosyl) pyridoxine and 5'-(β-glucosyl) pyridoxine. Treatment of both conjugates with almond β--glucosidase results in release of glucose and pyridoxine (Suzuki et al., 1979). Recently, the occurrence of a particulate enzyme in seedlings of podded pea (Pisum sativum L. cv. Kinusaya), which catalyzed the transfer of D-glucose from UDP-glucose to the

5'-, but not 4'-, hydroxyl group of pyridoxine, has been reported (Tadera et al., 1982).

The main objectives of this study were to determine if β-glucosidase treatment of foods could be used to determine the amount of vitamin B6 which may be present as a glycosidic linkage in foods and to determine the amount of such a bound form of vitamin B6 in several commonly consumed foods.

MATERIALS AND METHODS

Food Samples

The following foods were selected to represent different food groups: cooked ground beef (approximately 20% fat), canned (oil packed) tuna, raw and baked chicken, skim milk, pressure cooked soybeans, pressure cooked navy beans, peanut butter (made from 100% Spanish peanuts), whole wheat flour, whole wheat bread (made from whole wheat flour), wheat bran, frozen corn, cooked white long grain rice, rice bran, bananas, avocados, canned peaches, freshly-squeezed orange juice, frozen orange juice concentrate, untoasted filberts, raw and canned green beans, raw and frozen broccoli, raw and frozen cauliflower, and raw carrots. With the exception of oranges, raw and processed foods were obtained from the same lot of food directly at the processing plant. Those foods that were frozen were thawed at room temperature (23-25°C) and the thawed food tested without further heating.

Enzyme Treatment of Foods

The optimum concentration of β-glucosidase and time of incubation to be used to test each food was determined using frozen orange juice concentrate. This food was used since in preliminary studies it was found to contain a substantial amount of vitamin B6 presumed to be bound to glucose. Levels of 15, 30, 60, 90 and 120 units of β-glucosidase (β-D-glucoside glucohydrolase from almonds, Sigma Chemical Company, St. Louis, MO) were incubated with 1 g of orange juice concentrate for 2 hours and the amount of

vitamin B6 released was measured microbiologically (AOAC, 1980). The enzyme used had a reported activity of 6 units per mg protein (one unit will liberate 1.0 μmole of glucose from salicin per minute at pH 5.0 and 37°C). A level of enzyme beyond 30 units did not increase the amount of vitamin B6 released. In addition to orange juice, rice bran was also used to test the optimum concentration of enzyme. The results of this study also showed that the level of enzyme beyond 30 units per gram did not result in additional release of the bound form of vitamin B6 from rice bran. The enzyme was tested for phosphatase activity and it did not show any activity with pure pyridoxal-5'-phosphate as a substrate. The enzyme does not appear to have protease activity since we did not observe the release of any bound form of vitamin B6 from animal products. To determine the optimum time for the reaction, 1 g of orange juice concentrate and 60 units of β-glucosidase were incubated for time intervals of 1/2, 1, 2, 3 and 4 hrs and the amount of vitamin B6 released was measured microbiologically. Incubation for longer than 2 hr did not increase the amount of vitamin B6 released. The optimum pH for this enzyme is at pH 5 and this pH was used for all foods (Heyworth and Walker, 1962).

Since pyridoxal 5'-phosphate is a cofactor for many enzymes, pepsin and pancreatin were tested to see if these enzymes released vitamin B6 which might be bound to protein. We treated beef and cooked soybeans with either 100 mg of pepsin (porcine stomach mucosa, Sigma Chemical Co., St. Louis, MO) at pH 2.0 and 37°C for 18 hr or 100 mg of pancreatin (porcine pancreas, Sigma Chemical Co., St. Louis, MO) at pH 6.8 for 1 hr. at 37°C. In neither

situation could we detect any release of vitamin B6 as compared to the non-enzyme treated condition. As described below and in the results, these two foods were also treated with β-glucosidase. There was no vitamin B6 released from beef by the treatment of this enzyme, but there was vitamin B6 released from soybeans. Based on these data and work of Yasumoto et al., 1977; Suzuki et al., 1979, the vitamin B6 released from foods treated with β-glucosidase will be referred to as glycosylated vitamin B6.

After the optimum concentration and incubation time were established, foods from different categories were treated by the following procedure to determine the glycosylated vitamin B6 content. One hundred to 150 g of each food were ground in a blender until a homogenous mixture was obtained. One gram of this was then added to each of duplicate flasks containing 100 ml of 0.1 M phosphate buffer, pH 6.8. To control mold growth, 25 mg of thymol was added to each flask. The contents of the flask were stirred for 2 hr at room temperature (22-25°C). This two hr initial stirring in phosphate buffer was done to further break-up the food material and obtain a homogeneous mixture for the subsequent enzyme treatment. A pH of 6.8 was selected to reflect the pH of the intestine. The pH was then adjusted to 5.0 with 1N HCl and to one of the duplicate flasks, 60 units β-D-glucosidase were added. From this stage on, all flasks were incubated at 37°C for 2 hr in a shaking water bath (GCA/Precision Scientific Company) at 40 cycles/min. Enzyme action was stopped by addition of 10 ml of 1N HCl to all flasks followed by steaming for 5 min. This acidification and

steaming process by itself did not result in any release of bound vitamin B6 using rice bran (a food high in vitamin B6) as a test food. The pH was adjusted to 4.5 using 6 N KOH, the volume diluted to 250 ml, and the mixture filtered through Whatman No. 1 filter paper. The filtrate was further diluted as necessary and the vitamin B6 activity was measured by microbiological assay using Saccharomyces uvarum. In addition, the total vitamin B6 content of each food was measured following acid hydrolysis of 2 gram sample (AOAC, 1980). The enzyme source used contained no measurable vitamin B6. Recovery of pyridoxine added to orange juice carried through each of the procedures for total vitamin B6, non-enzyme treated and enzyme treated assays averaged 97.6 ± 12.0% (n=6 samples). Glucose levels in the foods before and after enzyme treatment were not determined.

Calculation of % glycosylated vitamin B6 in each sample was as follows: $\%\text{glycosylated vitamin B6} = \frac{G - F}{T} \times 100$. Where: G = mg B6/g of sample treated with β-glucosidase; F = mg B6/g of sample without enzyme; and T = mg B6/g of sample as determined by microbiological assay of the acid hydrolyzed sample.

To assess which of the B6 vitamers was conjugated with glucose, the levels of the three B6 vitamers, pyridoxine, pyridoxal and pyridoxamine, were determined in orange juice and peanut butter before and after the 2 hr treatment with β-glucosidase. The three vitamers were separated on an ion exchange resin (Dowex AG50W-X8 [K+] 100-200 mesh)(AOAC, 1980).

RESULTS AND DISCUSSION

The total, non-conjugated and glycosylated levels of vitamin B6 in the 22 foods are shown in Table 2.1. There was a loss of 53%, 46% and 29% in total vitamin B6 when comparing the raw to the processed foods from the same lots for green beans, cauliflower and broccoli, respectively. However, this is in agreement with Schroeder (1971), who did not do actual analyses but reviewed work of others and calculated losses of 57-77% for canned vegetables and 37-56% for frozen vegetables. These values calculated by Schroeder were not necessarily from the same lot. Our values should be considered in view of the fact that we did not measure water uptake or loss which could influence the values when expressed on a per 100 g basis.

In Table 2.1 the term "non-conjugated vitamin B6" refers to the amount of vitamin B6 from a sample which had been mixed with 0.1 M phosphate buffer pH 6.8 for two hours, was neither acid-hydrolyzed nor enzyme-treated, but was further incubated at pH 5.0 for 2 hr Glycosylated vitamin B6 refers to the difference in vitamin B6 between the enzyme treated sample and the non-conjugated vitamin from the paired sample. The results (Table 2.1) show that animal products, including skim milk, contained no measurable glycosylated vitamin B6. Filberts, bananas and avocados contained very small amounts compared to soybeans, navy beans, orange juice concentrate, wheat bran and rice bran. Almonds, the food from which the β-glucosidase enzyme is extracted, contained no glycosylated vitamin B6. When raw vegetables were compared to the processed forms (frozen broccoli and cauliflower), the percent of glycosylated vitamin B6

was found to increase with processing. Raw foods may contain some β-glucosidase activity and this activity might release the glycosylated vitamin B6 underthe condition of this assay. In contrast, the enzyme could be denatured during processing. This may be the reason for the increase in the level of glycosylated vitamin B6 observed for the processed foods. To address this possibility, one gram of raw cauliflower was mixed with one gram of frozen cauliflower and carried through the assay procedure. No enzyme was added to this mixture of foods. Using the value obtained from this assay and those values from non-enzyme and enzyme treated samples for the individual foods, it was calculated that 52% of the glycosylated vitamin B6 in the frozen cauliflower was released in the presence of added raw cauliflower. This suggests that there is some enzyme activity in the raw food and that the enzyme is denatured during processing. The increase seen in the percent of glycosylated vitamin B6 in canned green beans, compared to the raw green beans was small and might be due to the loss of total vitamin B6 content during processing. It is also important to point out that there was only one value for the raw green beans as well as for certain other foods. In the case of the animal products the values for the total vitamin B6 generally agreed with published values (Orr, 1969); and there was consistently no glycosylated vitamin B6 detected. The total vitamin B6 content of the foods presented in Table 2.1 agree with the published values (Orr, 1969). However, there are some differences which may be due to varietal differences, stage of harvest, processing conditions, handling process either

after harvest or processing, sample preparation and inter-laboratory variations. There were major differences between the total vitamin B6 content of frozen corn, frozen cauliflower, canned peaches and bananas of the reported values and our values. Our values for these foods were lower than the reported ones. The increase in glycosylated vitamin B6 in frozen orange juice concentrate as compared to the freshly-squeezed orange juice was minimal. It should be pointed out that the freshly-squeezed orange juice and the concentrate were not from the same lot.

The three forms of vitamin B6, pyridoxal, pyridoxine and pyridoxamine, were measured in peanut butter and orange juice, both with and without enzyme treatment. β-glucosidase treatment resulted in an increased level of pyridoxine in both orange juice and peanut butter as compared to the level in the non-enzyme treated foods (Table 2.2). Thus it appears that pyridoxine was the predominate form bound to glucose in these two foods. Saccharomyces uvarum is known to show little or no growth in the presence of phosphorylated forms of vitamin B6 as compared to the unphosphorylated forms. The relative growth for pyridoxal 5'-phosphate is 2% and for pyridoxamine phosphate is less than 0.4% (Morris et al., 1959). Since there was an increase in vitamin B6 content of the enzyme treated sample which can be measured microbiologically, it appears that the conjugated form is non-phosphorylated. Further support for this is indicated by a lack of phosphatase activity of the enzyme when pyridoxal 5'-phosphate was used as a substrate and incubated for two hr in the presence of 60 units of glucosidase enzyme. This is in agreement with findings on rice bran concentrate

from which β-5-(β-D-glycopyranosyl) pyridoxine was isolated (Yasumoto et al., 1977). These authors did not quantitate the amount in the rice bran. Vitamin B6 in orange juice has been reported to be bound to a compound with a molecular weight less than 3500 daltons (Nelson et al., 1977). This latter compound might be glucose, since in our study we found that 47% of vitamin B6 in orange juice concentrate was in the glycosylated form.

As shown in Table 1, the sum of the free and glycosylated form was less than the total for several foods tested. Exceptions to this were most vegetables, bananas and peaches, foods for which the sum was close to or equal to the total B6 (a mean ± SD of 94 ± 7% for these nine values). This raises the question of what other forms of vitamin B6 may be present in some of these foods. One likely explanation would be as the phosphorylated forms. There also may be as yet unidentified forms, or vitamin B6 might be bound to amino acids or proteins. Some of the reactions of vitamin B6 with amino acids and proteins are summarized as follows: (a) vitamin B6 is reported to form Schiff base with amino acids (Matsuo, 1957); (b) pyridoxal and pyridoxal-5'-phosphate have been reported to bind to protein in a food model system as a Schiff base, substituted aldamine and pyridoxal amino complex (Gregory and Kirk, 1977) and during storage of a low moisture food at 37°C for 128 days, half of the pyridoxal-5'-phosphate was found to be bound to protein as ε-pyridoxyllsyine complexes (Gregory and Kirk, 1978); (c) a reaction of sulfhydryl groups of milk serum proteins and pyridoxal occurs in evaporated sterilized milk, resulting in the formation of bis-4-

pyridoxal a compound with low B6 activity (Bernhart et al., 1960; Wendt and Bernhart, 1960).

Interestingly, with filberts the amount of free vitamin B6 was higher than the total amount of vitamin B6. One reason for this might be that other components in the filberts interact with the free forms of vitamin B6 during acid hydrolysis, a process which is required to assay total B6. This process is not used for free vitamin B6 determination. There may also be a growth stimulating factor for the yeast in filberts that is destroyed by heat during hydrolysis. We found that recovery values of added pyridoxine to the acid-hydrolyzed treated, non-enzyme treated and enzyme treated assays for filberts were 86%, 122% and 125% respectively. This is in line with heat inactivation of a growth promoting factor. Recovery data for orange juice did not show this trend.

The significance of our findings is confirmation of a conjugated form of vitamin B6 in a variety of plant foods and its absence in animal foods. The nutritional importance of this compound is not yet clear. One area of nutrition where this form of vitamin B6 may be of significance is that of bioavailability. The bioavailability of vitamin B6 from orange juice has been determined in humans using a triple lumen technique. Based on samples taken at points along a 30 cm segment of the intestinal lumen, the uptake of vitamin B6 from orange juice and from a synthetic solution containing a mixture of three forms of vitamin B6 was 35% and 65%, respectively of the amount contained in the original mixture infused into the lumen (Nelson et al., 1976). In our study, 47% of

the vitamin B6 in orange juice was found to be bound to glucosylated. It has been reported that vitamin B6 from whole wheat bread is less available than from white bread or white bread supplemented with vitamin B6 (Leklem et al., 1980). In this study we found 36% of vitamin B6 in wheat bran is glycosylated. Preliminary reports of other studies in our laboratory suggest a relationship between the amount of glycosylated vitamin B6 and its bioavailability from the food. From studies in humans, vitamin B6 in tuna was more available than that in peanut butter (Kabir et al., 1982). The amount of glycosylated vitamin B6 in tuna and peanut butter was 0 and 18%, respectively. Vitamin B6 in bananas and filberts was more available than in soybeans (Gonzalez, 1982). The amount of glycosylated vitamin B6 in bananas, filberts and soybean was 3%, 4% and 57%, respectively.

In summary, plant foods contain a bound form of vitamin B6 which appears to be a glycosylated form of vitamin B6. The level of this bound form of vitamin B6 seems to increase during processing, based on the limited number of foods studied.

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Table 2.1. Vitamin B-6 and Glycosylated Vitamin B-6 Content of Different Foods.

Food	Total Vitamin B-6[1]	Nonconjugated Vitamin B-6[1]		Glycosylated Vitamin B6[1]		Sum[5]
	µg/100 g		%	µg/100 g	%	
VEGETABLES						
Broccoli, raw	168±0.8[3]	140±4	84	n.d.[4]	-	84
Broccoli, frozen	119±13	48±2	23	78±10	65	88
Cauliflower, raw	156±0.8	148±0.2	95	9±0.5	5	100
Cauliflower, frozen	84±7	20±4	23	69±8	82	105
Green beans, raw	60	51	85	6	10	95
Green beans, canned	28±2	16±0.1	56	8±0.1	28	84
Carrots, raw	170	75	44	87	51	95
FRUITS						
Bananas	313±6	308±31	98	10±14	3	101
Avocados, fresh	443±4	221±1	50	15±6	3	53
Orange juice, fresh	43±0.1	18±0.5	42	16±0.6	37	79
Orange juice, concentrate	165±2	54±1	33	78±0.8	47	80
Peaches, canned	9±0.3	7±0.5	71	2±0.1	21	92
NUTS						
Filberts, raw	587±15	707±16	120	26±29	4	124
Almonds, raw	86	69	81	n.d.	0	81
GRAINS						
Corn, frozen	88±14	38±9	44	6±1	6	50
Rice(white),cooked	138±2	50±2	37	19±1	14	51
Rice bran	3515±84	600±6	17	153±30	4	21
Whole wheat bread	169±2	69±1	40	29±3	17	57
Wheat bran	903±1	117±3	13	326±17	36	49
Whole wheat flour	265	129	48	29	11	59
LEGUMES						
Navy beans, cooked	381±35	143±6	37	159±9	42	79
Peanut butter	302±21	49±5	16	54±11	18	34
Soybeans, cooked	627±11	130±4	21	357±4	57	78

Table 2.1 (continued)

ANIMAL PRODUCTS						
Beef, ground, cooked	263	83	31	n.d.	-	31
Tuna, canned	316	158	50	n.d.	-	50
Chicken						
Breast, raw	700	454	65	n.d.	-	65
Breast, cooked	684	316	46	n.d.		46
Leg, raw	388	176	45	n.d.		45
Leg, cooked	306	150	49	n.d.		49
Milk, skim	5±1	4±0.3	79	n.d.		79

[1]Total vitamin B-6 refers to the amount of vitamin B-6 measured microbiologically after acid hydrolysis. Nonconjugated vitamin B-6 is the amount measured in a sample that was mixed with 0.1M phosphate buffer for 2 hr. Glycosylated vitamin B-6 refers to the difference between the enzyme treated value and the free vitamin B-6 value. All values are as pyridoxine equivalents per 100 g of food.

[2]The percent of nonconjugated and glycosylated forms was calculated by dividing the amount of free or glycosylated forms by the total vitamin B-6 content of each corresponding food.

[3]The values listed are means ± standard deviation for duplicate samples. Values without a standard deviation are based on a single analysis.

[4]n.d. = not detected by the enzyme treatment.

[5]Sum of the % nonconjugated and glycosylated vitamin B-6 as a percentage of the total vitamin B-6.

Table 2.2. Level of the Three B-6 Vitamers in Frozen Orange Juice Concentrate and Peanut Butter Before and After β-glucosidase Treatment.

Food	Pyridoxine		Pyridoxal		Pyridoxamine	
	before	after	before	after	before	after
	µg/100 g					
Orange juice concentrate	10.10±0.07[1]	71.4±0.2	9.6±0.1	15.7±0.1	16.6±0.3	16.1±0.7
Peanut butter	14.4±0.2	50.6±1.4	14.7±0.7	16.2±2.4	15.6±0.4	15.0±1.7

[1]Values for each form were derived from standard curves for the respective forms. Each value is the mean of duplicate samples. The levels of each of the three B-6 vitamers were determined on 12 g of sample before and after β-glucosidase treatment for 2 hr using the procedure described in the text. Separation of the three B-6 vitamers was done on an ion exchange resin (Dowex AG50Wx8 [K^+] 100-200 mesh) (AOAC, 1980). Values for vitamin B-6 (expressed as pyridoxine equivalents) for samples which were not acid hydrolyzed and not subjected to ion-exchange chromatography were 49.0±0.5, 118±3.0, 53±2.0 and 95±2.0 µg/100 for the orange juice without enzyme (nonconjugated), orange juice plus enzyme, peanut butter without enzyme (non-conjugated) and peanut butter plus enzyme, respectively.

CHAPTER 3

COMPARATIVE VITAMIN B6 BIOAVAILABILITY FROM TUNA, WHOLE WHEAT BREAD AND PEANUT BUTTER IN HUMANS. [1,2]

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ABSTRACT

COMPARATIVE VITAMIN B6 BIOAVAILABILITY FROM TUNA, WHOLE WHEAT BREAD AND PEANUT BUTTER IN HUMANS. G.H. KABIR, J.E. LEKLEM AND L.T. MILLER. Department of Foods and Nutrition, Oregon State University, Corvallis, Oregon 97331.

Relative bioavailability of vitamin B6 from tuna, whole wheat bread and peanut butter was investigated in eight healthy men. The study was divided into a 10-day adjustment and three, 14-day experimental periods in a 3x3 latin square design. Total vitamin B6 intake was set at 1.6 mg/day, with 50% of the intake coming from one of the three experimental foods and 50% from a basal diet. Complete 24-hour urine and fecal collections were made throughout the study. Urine was analyzed for 4-pyridoxic acid (4PA) and vitamin B6, fecal samples for vitamin B6 and plasma (sampled every five days) for pyridoxal 5'-phosphate (PLP). Mean values ± SD for the adjustment, tuna, whole wheat bread and peanut butter periods were: 5.65±1.76, 4.89±1.0, 3.62±0.66 and 2.80±0.50 μmoles/day for 4-pyridoxic acid; 0.98±0.34, 1.05±0.2, 0.76±0.09 and 0.68±0.19 μmoles/day for urinary vitamin B6; 2.72±0.94, 3.08±0.73, 3.80±0.78 and 4.42±1.03 μmoles/day for fecal vitamin B6 and 65±23.3, 64.8±29.8, 49.3±14.4 and 48.4±20.2 nm for plasma pyridoxal 5'-phosphate, respectively. Of the four indices used to assess vitamin B6 bioavailability, 4PA and urinary vitamin B6 were significantly ($p \leq 0.01$) higher in the tuna period than in either the whole wheat bread or peanut butter periods. The fecal

vitamin B6 excretion during the tuna period was significantly lower than during the peanut butter period. Based on the four indices used to assess vitamin B6 bioavailability, the vitamin B6 in whole wheat bread and peanut butter is 75% and 63%, respectively, as available as that from tuna.

INTRODUCTION

The amount of vitamin B6 present in various foods has been measured (Orr, 1969). However, this does not necessarily represent the amount of the vitamin that is available to humans. In fact, studies in humans indicate that the bioavailability of vitamin B6 from natural sources is limited (Tarr et al., 1981; Leklem et al., 1980). Vitamin B6 in nature exists mainly as bound and phosphorylated forms. These forms are not utilized by the test microorganism until released by acid hydrolysis (Rubin et al., 1947; Siegel et al., 1943). This vigorous acid treatment of food is not entirely representative of the digestive process in the human or animal gastrointestinal tract.

Both humans and animals have been used to assess vitamin B6 bioavailability, but animal studies are not always applicable to humans. Among the factors to be considered in animal studies are: (a) when using growth as a criteria of bioavailability, other components in the diet may affect growth; (b) the physiological state of the experimental animals (Melnick et al., 1945). Also in the case of B complex vitamins, synthesis of the vitamin and utilization by intestinal microflora could be a major factor that is different in rats as compared to humans (Samra et al., 1946).

The purpose of this study was to investigate the bioavailability to humans of vitamin B6 from tuna, whole wheat bread and peanut butter, three foods which are commonly consumed in the American diet. Urinary vitamin B6 and 4-pyridoxic acid, fecal

vitamin B6 and plasma pyridoxal 5'-phosphate were the four indices of vitamin B6 bioavailability that were utilized and compared.

Indexing Key Words: vitamin B6, bioavailability, urinary 4-pyridoxic acid, fecal vitamin B6.

MATERIALS AND METHODS

Subject Selection

Nine apparently healthy men, 21-30 years and not taking any vitamin supplementation, were selected as subjects from a group of respondents to a campus advertisement. The study was explained and informed consent obtained. This project was approved by the Oregon State University Committee on Human Subjects. The subjects' ages, body weights and heights are presented in Table 3.1. A fasting blood sample was drawn from each subject 1 week before the start of the study for routine clinical testing. Serum enzymes, electrolytes, cholesterol and triglyceride levels were normal for all subjects. Intestinal absorption was assessed using a 5 g xylose absorption test (Buttery et al., 1975). All subjects had normal xylose absorption.

Experimental Design

The study involved three variables: tuna, peanut butter, and whole wheat bread. The experiment was based on a 3 x 3 latin square design, which is shown in table 3.2.

Diet

The 52-day feeding study was divided into a 10-day adjustment and three 14-day experimental periods. The whole wheat bread for the entire study was prepared in a local bakery and was kept frozen (-20°C) until the day it was fed. Canned tuna was purchased in one lot. The entire lot was opened, drained, mixed together and frozen (-20°C) in one-day portions until fed. The peanut butter

(made from 100% Spanish peanuts) was also purchased in one lot.

The vitamin B6 content of the tuna, peanut butter and whole wheat bread was 0.27, 0.31 and 0.17 mg/100 g, respectively, as measured microbiologically, using Saccharomyces uvarum (AOAC, 1980). The total vitamin B6 content of the diet was set at 1.6 mg/day, 50% of which was from a constant basal diet and the remaining 50% from one of the experimental foods. During the 10-day adjustment period, 50% of the vitamin B6 in the diet was supplied by 270 g of ground beef (weight prior to cooking) and 70 g of cheddar cheese. The beef and cheese were replaced by either 293 g of tuna, 252 g of peanut butter or 415 g of whole wheat bread in each experimental period.

The amount of each food and the partial nutrient composition of the basal diet and experimental foods are given in Table 3.3. The nitrogen levels of the daily diets as assayed (Scales and Harrison, 1929) were 20.5, 19.5, 16.9 and 20.0 g/day for adjustment, tuna, whole wheat bread and peanut butter periods, respectively.

In addition to the adjustment and experimental foods listed in Table 3.3, margarine, honey, hard candy and a carbonated beverage were provided for the subjects to maintain a constant body weight. Tea and coffee were also made available. Salad dressing was provided during the tuna period. No alcoholic beverages were permitted throughout the study. A record of the daily consumption of these optional items was maintained by each subject. A three-meal regimen was served in the metabolic unit of the Foods and Nutrition Department. Subjects weighed themselves each day before breakfast

and maintained their usual daily activities. Strenuous physical activity (i.e., running more than 1 mile per day or participation in competitive sports) was not permitted.

Vitamin B6 content of constant diet and experimental foods was determined weekly (AOAC, 1980). Mean values for the microbiologically active vitamin B6 content of the basal diet and the three experimental foods are given in Table 3.4. The vitamin B6 content of the adjustment diet was 1.48 mg/day (n = 2 samples).

Sample Collection

(a) Urine: Complete 24-hour urine collections were made throughout the study and an aliquot of each was frozen at -20°C until analyzed. (b) Feces: All fecal samples were collected throughout the study. The subjects were given a fecal marker of FDC blue No. 1 dye in a gelatin capsule (50 mg of dye plus 200 mg of methylcellulose) to mark the beginning of each composite collection. One 4-day and two 5-day composites were made for each experimental period. The fecal excretion from each subject was weighed daily and stored at -20°C. For each subject the fecal specimens corresponding to a composite were pooled together in a large plastic bag, mixed thoroughly and a portion was freeze-dried for subsequent analysis. (c) Blood: On the first and last day of the adjustment period, a morning fasting blood sample was drawn from the antecubital vein. Similarly, a fasting blood sample was drawn on the 4th, 9th, and 14th day of each experimental period. After centrifugation, plasma was stored at -40°C until analyzed.

Analytical Methods

Total vitamin B6 was determined on 10 ml of the acid hydrolyzed urine samples from days 1, 4, 7 and 10 of the adjustment period and from days 2, 5, 8, 11 and 14 of the three experimental methods (Miller and Edwards, 1982). The inter-assay variation for nine determinations of a control sample assayed along with other samples was ± 5%. Total vitamin B6 content of the feces was determined on 1 g of the freeze dried sample which had been hydrolyzed (AOAC, 1980). The inter-assay variation for eight determinations of a sample assayed along with the other samples was ± 7%. The 4-pyridoxic acid content of the urine, which was assayed for the same days for which urinary B6 was measured, was determined by the ion exchange-fluorometric method of Reddy et al. (1958). The inter-assay variation for twelve determinations of a control sample assayed along with other samples was ± 7% and the recovery for thirty-six samples (4-5 urines per subject) was 94 ± 8%. The creatinine content of the daily urine excretion was measured by an automated procedure (Pino et al., 1965). Plasma pyridoxal 5'-phosphate was measured on the samples of the 1st and 10th day during the adjustment period and on the 4th, 9th and last day of each experimental period by a tyrosine apodecarboxylase radio-assay method (Chabner and Livingston, 1970). Recoveries were determined by adding pyridoxal 5'-phosphate to the plasma prior to the extraction step. Recoveries done on these samples averaged 92 ± 8%.

Statistical Analysis

The data were analyzed by analysis of variance for latin square and L.S.D. test (Snedecor and Cochran, 1978). For personal reasons, subject No. 6 left the study during the first experimental period. Missing values for this subject were calculated (Snedecor and Cochran, 1978) and used in the latin square analysis.

RESULTS

There was no significant change in the subjects' weights during the study. The mean starting weight for the subjects was 72 ± 9 as compared to 73 ± 8, 73 ± 9 kg and 73 ± 9 kg during the tuna, whole wheat bread and peanut butter periods, respectively.

The initial vitamin B6 status of the subjects was adequate based on the criteria of Shultz and Leklem (1981). At the end of the adjustment period in two of the eight subjects (subjects 3 and 7), there was a drop of 33% in urinary vitamin B6 excretion and a drop of 30% in urinary 4-pyridoxic acid. None of the other subjects showed any appreciable change in vitamin B6 excretion. This drop in excretion of 4-PA and urinary vitamin B6 suggests that the level of B6 in the study diet was less than that ingested by these two subjects prior to the study.

Urinary vitamin B6 and 4-PA excretion stabilized within seven to eleven days for all subjects following all diet changes. The mean excretion levels of urinary B6 and 4-PA for days 11 and 14 of each experimental period, along with the mean levels of fecal vitamin B6 excretion and plasma PLP concentration for the same period

are given in Table 3.5. The mean values for these days for each subject were used for statistical analyses and bioavailability comparisons. All subjects had a significantly higher ($p < 0.01$) excretion of 4-PA and urinary vitamin B6 during the tuna period than during either the whole wheat bread or peanut butter period. The subjects excreted significantly less ($p < 0.01$) fecal vitamin B6 during the tuna period as compared to the peanut butter period. With the exception of subjects 3 and 7, all subjects had a lower excretion of fecal vitamin B6 during the tuna period as compared to the whole wheat bread period. During the tuna period, as compared to the whole wheat period, subjects 3 and 7 had a 19% and 11% higher fecal B6 excretion, respectively. The percent of the intake excreted as 4-PA, urinary vitamin B6 and fecal vitamin B6 is shown in Figure 3.1. Collectively, these three measures accounted for 98 $\pm$ 12%, 89 $\pm$ 9% and 86 $\pm$ 13% of the ingested vitamin B6 excreted during the tuna, whole wheat bread and peanut butter periods, respectively. For the adjustment period this value was 104 $\pm$ 29% for the 8 subjects. The higher average percentage and standard deviation for the adjustment period were due to the 4-PA values of two subjects (subjects 4 and 9). For these two, this higher 4-PA excretion on day 10 of the study indicated they had not fully adjusted to the level of vitamin B6 being fed. However, by day 5 of the first experimental period their 4-PA excretion was similar to others fed a comparable diet. The PLP level was higher in most subjects during the tuna period than in either the whole wheat bread or peanut butter periods. The exception was subject 7 who had a slightly lower

value in the tuna period than either the whole wheat bread or peanut butter period.

The relative vitamin B6 bioavailability was evaluated by four different measurements: 4-PA, urinary vitamin B6, fecal vitamin B6 excretion levels and the plasma PLP level. Using 4-PA excretion as a criterion, the mean bioavailability of vitamin B6 from whole wheat bread and peanut butter, as compared to that of tuna, was 74 ± 11% and 57 ± 6%, respectively. Based on urinary vitamin B6 excretion, bioavailability of vitamin B6 from whole wheat bread, as compared to that of tuna, was 72 ± 11% and from peanut butter, 65 ± 8%. Vitamin B6 bioavailability based on fecal excretion showed that the vitamin B6 in whole wheat bread was 77 ± 23% and that in peanut butter was 57 ± 18% as available as the vitamin B6 from tuna. Using plasma PLP as a basis, vitamin B6 bioavailability, as compared to tuna, was 76 ± 16% and 74 ± 18% from whole wheat bread and peanut butter, respectively. When compared to tuna, the average bioavailability of vitamin B6, based on these four indices mentioned above, was 75% from whole wheat bread and 63% from peanut butter.

The analyses of data dicusssed so far was done using statistics for the latin square design. Because one subject did not complete the study, we also evaluated the data statistically using a completely randomized design (Snedecor and Cochran, 1978), with $p \leq 0.05$ as the level of significance. The results were as follows: urinary 4-pyridoxic acid and urinary vitamin B6 excretion were statistically higher during the tuna period that either the whole wheat bread or the peanut butter period, but there was no significant difference in fecal vitamin

B6 excretion between the tuna period and the whole wheat period bread period. The changes in plasma pyridoxal 5'-phosphate were not statistically significant.

DISCUSSION

The indices used to evaluate vitamin B6 bioavailability in humans in this study were urinary vitamin B6 and 4-pyridoxic acid, fecal vitamin B6 and plasma PLP. Among these, urinary vitamin B6, fecal vitamin B6 and 4-PA excretion showed the most significant changes. The vitamin B6 bioavailability from whole wheat bread and peanut butter was compared to that of tuna. There was a decrease of 26% in 4-PA, 28% in urinary vitamin B6 and an increase of 23% in fecal vitamin B6 excretion during whole wheat bread as compared to the tuna period. Similarly, for peanut butter there was a decrease of 43% in 4-PA, 35% in urinary vitamin B6 and an increase of 43% in fecal vitamin B6 excretion as compared to that of the tuna period. Using the above criteria, vitamin B6 in tuna was significantly more available than either that in whole wheat bread or peanut butter. Further evidence for a higher vitamin B6 bioavailability was seen in the higher plasma PLP level in the tuna period than either the whole wheat bread or peanut butter period. There was a good agreement between the percent decrease in urinary vitamin B6 and 4-PA excretion and the increase in fecal vitamin B6 excretion during both the whole wheat bread and peanut butter as compared to that of the tuna period. This would again suggest that vitamin B6 in tuna was more available than either

whole wheat bread or peanut butter. Using 4-pyridoxic acid, urinary vitamin B6, fecal vitamin B6 and plasma pyridoxal 5'-phosphate to assess vitamin B6 bioavailability, the mean values for whole wheat bread were 74, 72, 77, 76% and for peanut butter, 57, 65, 57 and 74% than that of tuna, respectively. The mean bioavailability values are in close agreement regardless of the index used, with the exception of plasma pyridoxal 5'-phosphate for peanut butter which gave a higher value as compared to the other three measurements.

The amount of urinary vitamin B6 excreted during the whole wheat bread period was similar to the previous results of Leklem et al. (1980). In both studies 8% of the vitamin B6 intake was excreted as urinary vitamin B6. For the three foods studied, there was a range of excretion of 7-11% of the intake as urinary vitamin B6. In a separate study, 10% of the total vitamin B6 intake was found to be excreted as urinary vitamin B6, during an 18-day period in six subjects on a controlled diet which provided 1.66 mg of vitamin B6 and 150 g of protein per day (Kelsay et al., 1968). Tarr et al. (1981) have reported that approximately 5% of the total vitamin B6 intake was excreted in the urine of their subjects who had vitamin B6 intakes of 1.1, 2.3 or 2.7 mg/day. This is slightly lower than we saw in our study. Part of this difference might be due to Tarr et al. (1981) using a higher ratio of urine to acid for hydrolysis than we did. We have observed a 15% lower urinary vitamin B6 value as the ratio of urine to acid was increased from 1:10 to 1:5 (unpublished observation).

The percent of the total intake excreted as 4-PA was 53, 39

and 30% during the tuna, whole wheat bread and peanut butter periods, respectively. For humans, different percentages of excretion have been reported for 4-PA. Isotopic study in humans (3 subjects) has shown that a range of 28-41% of vitamin B6 intake is excreted as 4-PA (Johansson et al., 1966). Also, it has been reported that normal subjects excrete 0.5 to 1.3 mg (3 to 7 μmoles) of 4-PA/day (Sauberlich et al., 1972). A range of 30-40% of the vitamin B6 ingested in a normal diet has been reported to be excreted in the urine as 4-PA (Tillotson et al., 1966). In another study, a range of 53-60% of vitamin B6 as 4-PA has been reported (Kelsay et al., 1968). The range of 4-PA excretion in our study agrees with these studies.

The fecal vitamin B6 excretion in the whole wheat bread and peanut butter periods was 23% and 43% higher as compared to the excretion in the tuna period, respectively. This increase in fecal vitamin B6 excretion agrees well with a decrease of 26% and 43% in 4-PA excretion during whole wheat and peanut butter as compared to the tuna period, respectively. Therefore, it seems that the increase in fecal vitamin B6 excretion during the whole wheat bread and peanut butter periods was due to incomplete absorption of vitamin B6 from the foods.

There are reports that the vitamin B6 is synthesized by intestinal microflora of both rats and humans (Linkswiler and Reynolds, 1950; Sumi et al., 1977). Additionally, it has been reported that the growth rate of rats increased if dextrin was substituted for sucrose in the pryidoxine deficient diet. The growth-promoting

effect of dextrin was decreased by adding sulfathaladine drugs to the diet, an indication that pyridoxine synthesized by the intestinal flora in the presence of dextrin became available to the rats (Samra et al., 1946). While there is more fiber and carbohydrate in whole wheat bread and peanut butter than tuna which might promote vitamin B6 synthesis (Samra et al., 1946), we did not see any evidence of intestinal microflora synthesis of vitamin B6 and its subsequent utilization during the feeding of peanut butter or whole wheat bread. This is based on the lower excretion of both 4-PA and urinary vitamin B6 excretion in the whole wheat and peanut butter periods as compared to the tuna period. Other evidence comes from the data presented in Figure 3.2 which show that 98 ± 12, 89 ± 9 and 86 ± 13% of the ingested vitamin B6 was excreted during tuna, whole wheat bread and peanut butter periods, respectively. This shows that, on the average, intake was greater than excretion. While we cannot rule out intestinal synthesis of vitamin B6, if it did occur, it was not of sufficient magnitude to overcome the difference in vitamin B6 bioavailability between the tuna and either peanut butter or whole wheat bread.

One could not account for all of the ingested vitamin B6, especially when the whole wheat bread and peanut butter were fed. There are at least two possible explanations: (a) Some of the vitamin B6 from whole wheat bread and peanut butter might be broken down by intestinal microflora to metabolites of vitamin B6 which were not measured in the microbiological or fluorometric assays; (b) Since the quality of protein in whole wheat bread and peanut butter is different from that in tuna, the amino acid pool

and subsequent rate of transamination could result in retention of more pyridoxal 5'-phosphate in liver and other tissues during the whole wheat bread and peanut butter periods as compared to the tuna period. This situation would be greater for the tuna versus the peanut butter and for the tuna versus the whole wheat bread because the quantity of protein fed was essentially the same during the tuna and peanut butter periods. It has been reported that a high protein diet causes an increase in transaminase level (Wang and Appelhanz, 1956). In rats fed different levels of protein, the activity of alanine transaminase increased in direct proportion to the level of casein (Nicholas and Rosen, 1963). In another study, the activities per gram of liver of glutamate oxaloacetate transaminase and glutamic pyruvate transaminase increased as the protein content of the diet increased (Waldorf et al., 1963).

Vitamin B6 bioavailability from whole wheat bread, white and white bread enriched with vitamin B6, has been studied in men (Leklem et al., 1980). The bioavailability of vitamin B6 from whole wheat bread was 5-10% less than from white bread supplemented with vitamin B6 or vitamin B6 from oral doses of vitamin B6. The percent bioavailability of vitamin B6 from whole wheat bread in our study was 75% of that from tuna. This difference in percent bioavailability between the two studies is probably related to both the base of comparison as well as the fact that in the previous study 80% of the total vitamin B6 intake came from the bread. Since the total vitamin B6 intake was nearly the same in these two

studies, the level of excretion of urinary vitamin B6, 4-PA and fecal vitamin B6 was compared and found to be similar. In the previous study the excretion level was 0.74, 3.40 and 4.06 μmoles/day and in our study was 0.76, 3.62 and 3.80 μmoles/day for urinary vitamin B6, 4-PA and fecal vitamin B6, respectively.

Differences between foods in the bioavailability of vitamin B6 in our study might be due to several factors. There is evidence that heat treatment of foods results in decreased bioavailability (Yen et al., 1976; Tomarelli et al., 1955). Formation of ε-pyridoxyllysine during heating of protein and pyridoxal or pyridoxal 5'-phosphate has been reported (Gregory and Kirk, 1977). This compound has been found to be 60% available to the rat (Gregory and Kirk, 1978). If processing were a factor, then the fact that there is a greater percentage of pyridoxal in tuna than in the other two foods (Orr, 1969) would have tended to reduce the difference in vitamin B6 bioavailability between the tuna and the other two foods. There is also evidence that foods with a high fiber content have a weak inhibitory effect on vitamin B6 bioavailability in humans (Leklem et al., 1980; Miller et al., 1979). Based on data from Southgate et al. (1976), the dietary fiber content of the whole wheat bread fed was 35 g and that of the peanut butter was 24 g. While the whole wheat diet contained more dietary fiber than the peanut butter diet, the vitamin B6 in whole wheat bread was relatively more available than that in peanut butter. However, this difference was not statistically significant. Therefore, it seems that the relatively higher dietary fiber content of the

whole wheat diet did not affect the vitamin B6 bioavailability. This is in agreement with the work of Nguyen et al. (1981) which showed that polysaccharides tested in vitro did not bind to vitamin B6. We have found glycosylated vitamin B6 in plant foods but not in animal foods. The proportion of this compound in foods appears to be inversely related to the vitamin B6 bioavailability of the limited number of foods tested (Kabir et al., 1982).

In summary, vitamin B6 in tuna was found to be more available than that in either whole wheat bread or peanut butter. The vitamin B6 in whole wheat bread was more available than that in peanut butter, but this difference was not statistically significant. Among the indices used to assess vitamin B6 bioavailability, urinary 4-pyridoxic acid, urinary vitamin B6 and fecal B6 excretion agreed more closely with each other than did plasma PLP levels.

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Table 3.1. Description of Subjects.

Subject	Age	Height	Beginning Weight
	years	cm	kg
1	24	173	69
2	29	175	84
3	28	190	90
4	27	173	64
5	21	170	67
7	30	178	67
8	21	180	70
9	21	173	79
Mean ± SD	25 ± 4	176 ± 6	72 ± 9

Table 3.2. Experimental Design[a].

Period	Subject								
	1	8	2	6	9	3	4	5	7
I	PB	WW	T	PB	WW	T	WW	T	PB
II	WW	T	PB	WW	T	PB	PB	WW	T
III	T	PB	WW	T	PB	WW	T	PB	WW

[a]PB = peanut butter, T = tuna, WW = whole wheat bread

Table 3.3. Composition[a] of the Basal, Adjustment and Experimental Diets.

Item	Amount	kcal	Protein
	g		g
Basal Diet			
Orange juice, frozen, diluted 1:3	250	122	1.7
Rice cereal, ready-to-eat	35	140	2.0
Bread, white, enriched	75	202	6.5
Milk, dry, non-fat	45	163	16.0
Carrots, raw	80	34	0.8
Raisins	56	162	1.4
Pears, canned	100	66	3.4
Tomato juice, canned	100	19	0.8
Rice, dry	45	163	3.0
Green beans, canned	65	12	0.7
Corn, canned	30	24	0.6
Peaches, canned	100	77	0.4
Celery, raw	80	12	0.6
Ice cream, vanilla	80	154	3.6
Vanilla cream cookies	54	271	2.6
Subtotal		1621	44.6
Adjustment Diet			
Beef, ground (before cooking)	270	591	80.0
Cheese, cheddar	70	278	17.5
Subtotal		869	97.5

Continued

Continued, 3.3

Experimental Diet			
Tuna	293	844	71.0
Whole wheat bread	415	1007	43.5
Peanut butter	252	1494	67.0
Total, Adjustment Diet		2490	141.5
Total, Tuna Diet		2465	115.0
Total, Whole Wheat Bread Diet		2628	87.5
Total, Peanut Butter Diet		3115	111.0

[a]Calculated from reference #11. The total nitrogen content consumed during the adjustment, tuna, whole wheat bread and peanut butter periods was 20.5, 19.5, 16.9, and 20.0 g/day, respectively.

Table 3.4. Total Vitamin B6 Level on the Daily Diet.

Diet	Tuna	Peanut Butter	Whole Wheat Bread
	mg/day	mg/day	mg/day
Experimental Diet	0.70 ± 0.06[a]	0.74 ± 0.03	0.73 ± 0.05
Basal Diet[b]	0.82 ± 0.04	0.82 ± 0.04	0.82 ± 0.04
Total Diet	1.52 ± 0.10	1.56 ± 0.07	1.56 ± 0.09

[a]Mean ± standard deviation of five samples for each period.

[b]The value for the basal diet represents five samples taken at five different weeks during the study.

Table 3.5 Fecal vitamin B6, urinary vitamin B6, urinary 4-pyridoxic acid excretion and plasma pyridoxal 5'-phosphate levels of eight subjects.[1]

Periods	Fecal vitamin B6	Urinary vitamin B6	Urinary-4-pyridoxic acid	Plasma pyridoxal 5'-phosphate
	umoles/24 hr	umoles/24 hr	umoles/24 hr	nM
Adjustment Period				
Day 1	-	1.00 ± 0.35	5.64 ± 1.98	81.5 ± 36.0
Day 10	2.72 ± 0.94[2]	0.97 ± 0.32	5.53 ± 1.96	65 ± 23.3
Experimental Period				
Tuna	3.08 ± 0.73[a]	1.05 ± 0.2[b,c]	4.89 ± 1.10[d,e]	64.8 ± 29.8
Whole Wheat Bread	3.80 ± 0.78	0.76 ± 0.09[b]	3.62 ± 0.66[d]	49.3 ± 14.4
Peanut Butter	4.42 ± 1.03[a]	0.68 ± 0.19[c]	2.80 ± 0.50[e]	48.4 ± 20.2

[a,b,c,d,e]Values sharing the same letter are significantly different from each other ($p<0.01$).

[1]The values listed for the experimental periods are the means and standard deviations of eight subjects for two samples during the last four days for urinary vitamin B6 and 4-pyridoxic acid, for one composite samples of the last five days for fecal vitamin B6 and for two values during the last five days for plasma pyridoxal 5'-phosphate.

[2]The fecal vitamin B6 value listed is for a composite sample from the last five days of the adjustment period.

Figure 3.1. Legends.

Percentage of the vitamin B6 intake excreted as urinary vitamin B6, fecal vitamin B6, 4-pyridoxic acid and total of these three for the tuna (T), whole wheat bread (WW) and peanut butter (PB) periods.

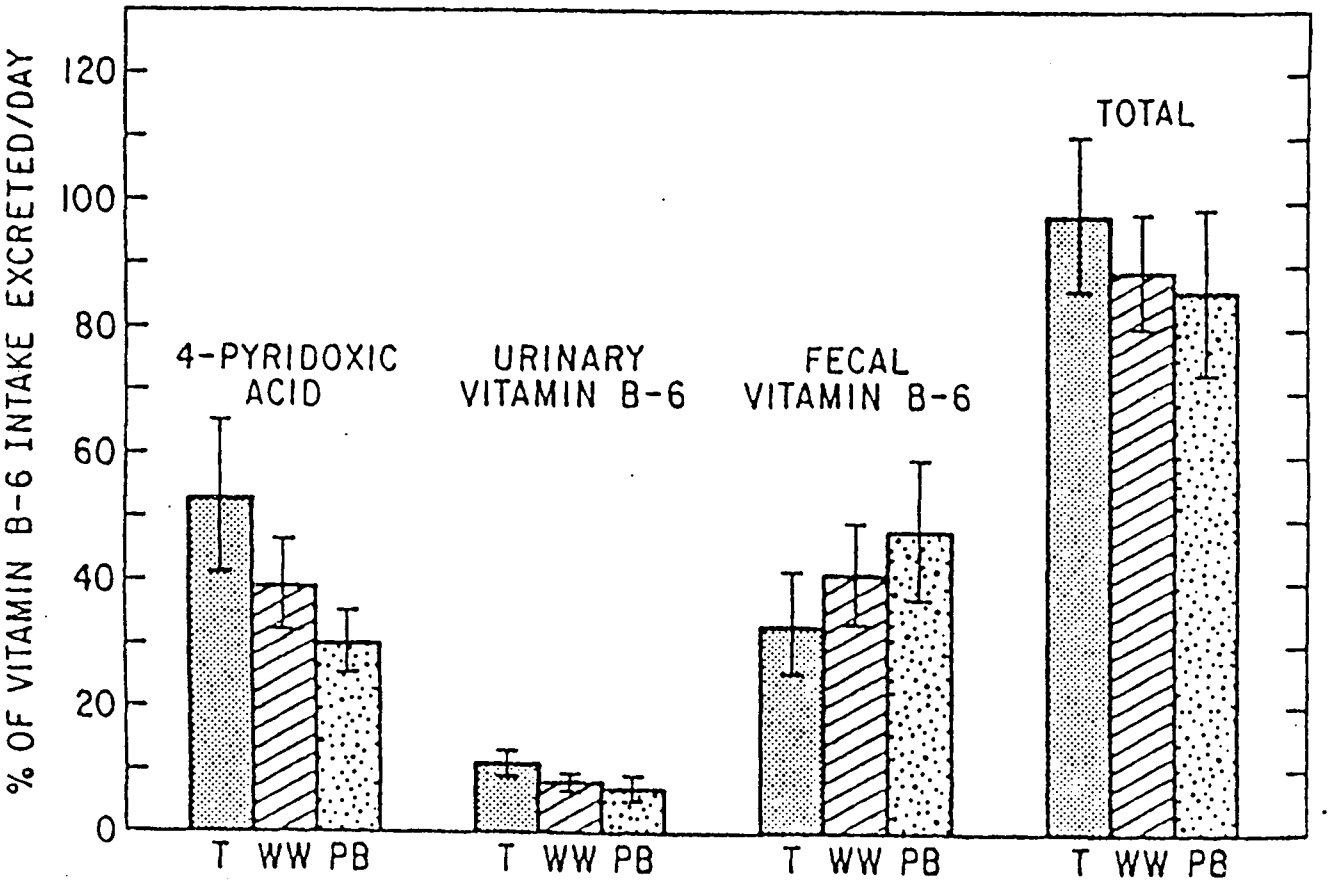
% OF VITAMIN B-6 INTAKE EXCRETED/DAY
120
100
80
60
40
20
0
4-PYRIDOXIC ACID
URINARY VITAMIN B-6
FECAL VITAMIN B-6
TOTAL
T WW PB
T WW PB
T WW PB
T WW PB

Figure 3.1

CHAPTER 4

RELATIONSHIP OF THE GLYCOSYLATED VITAMIN B6 CONTENT OF FOODS TO VITAMIN B6 BIOAVAILABILITY IN HUMANS

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ABSTRACT

RELATIONSHIP OF THE GLYCOSYLATED VITAMIN B6 CONTENT OF FOODS TO VITAMIN B6 BIOAVAILABILITY IN HUMANS. G.H. KABIR, J.E. LEKLEM AND L.T. MILLER. Department of Foods and Nutrition, Oregon State University, Corvallis, Oregon 97331.

The purposes of this study were (a) to determine the urinary and fecal excretion patterns of glycosylated vitamin B6 when tuna, whole wheat bread and peanut butter were fed to humans; (b) to examine the relationship between glycosylated vitamin B6 and vitamin B6 bioavailability and determine if the glycosylated vitamin B6 content of a food could be used as an in vitro index of vitamin B6 bioavailability. To address part (a), eight males participated in a 52-day diet study that was divided into a 10-day adjustment and three 14-day experimental periods. Twenty-four hr urines and feces were collected throughout the study and urinary 4-pyridoxic acid and plasma pyridoxal 5'-phosphate were measured in the above study. Foods from this study along with representative foods from three other studies were analyzed for total, free and glycosylated vitamin B6.

Only 4% of total vitamin B6 in the feces was in the glycosylated vitamin B6 form when whole wheat bread was fed and undetectable amounts on the other diets. The percentage of urinary vitamin B6 excreted as the glycosylated form was 12 ± 7%, 18 ± 10% and 26 ± 9% when tuna, whole wheat bread and peanut butter were fed, respectively.

Based on all studies, there was an inverse relationship between the glycosylated vitamin B6 content of a food and its vitamin B6 bioavailability in humans.

INTRODUCTION

Vitamin B6 exists naturally as free and bound forms. It is not entirely clear as to what compounds the vitamin is bound to and if these bound forms are utilized by humans.

There are reports that vitamin B6 is bound to both protein and non-protein compounds. After storage of a low moisture food at 37°C and an a_w of 0.6 for 128 days, about half of the pyridoxal 5'-phosphate bound to protein existed as an ε-pyridoxal lysine complex (Gregory and Kirk, 1978). The bioavailability of this complex to rats was 60% that of pyridoxine (Gregory and Kirk, 1978). Vitamin B6 in orange juice is reported to be bound to a non-protein compound with a low molecular weight (Nelson et al., 1977). Vitamin B6 in rice bran concentrate has been found to be conjugated with glucose in a 1:1 ratio (Yasumoto et al., 1977). We have found that vitamin B6 is bound to glucose in a majority of plant foods tested (Kabir et al., 1983).

The purpose of the present study was to investigate the excretion pattern of glycosylated vitamin B6 when various foods were fed and to determine if the glycosylated vitamin B6 content of a food was related to the bioavailability of vitamin B6 from that food.

MATERIALS AND METHODS

The vitamin B6 bioavailability portion of the study reported for tuna, whole wheat bread and peanut butter has been described elsewhere (Kabir et al., 1983). A brief description of the experimental procedure used in that study is presented below.

Subject Selection

Eight healthy men, ages 21-30 years and not taking any vitamin supplements, were selected as subjects. The study was explained and informed consent obtained. This project was approved by the Oregon State University Committee on Human Subjects. The mean age and weight of the subjects were 25 ± 4 years and 72 ± 9kg, respectively.

Experimental Design and Diet

The study involved three variables: tuna, peanut butter and whole wheat bread. The experiment was based on a 3 x 3 latin square design. The 52-day feeding was divided into a 10-day adjustment and three 14-day experimental periods. The foods were purchased in one lot where possible and, where necessary, kept frozen (-20°C) until the day they were fed.

Based on values from food composition tables (Orr, 1969), the total vitamin B6 content of the diet was set at 1.6 mg/day, 50% of which was from one of the experimental foods and the remaining 50% of the vitamin B6 in the diet was supplied by 270 g of ground beef (weight prior to cooking) and 70 g of cheddar cheese. The beef and cheese were replaced by either 293 g of tuna, 252 g of

peanut butter or 415 g of whole wheat bread in each experimental period. The actual vitamin B6 content of the basal constant diet and the tuna, whole wheat bread and peanut butter which were fed in addition was 0.82 ± 0.04, 0.70 ± 0.06, 0.74 ± 0.03 and 0.73 ± 0.05 mg/day, respectively, as measured weekly by microbiological assay (AOAC, 1980).

Sample Collection

(a) Urine: complete 24-hr urine collections were made throughout the study, an aliquot of each collection was frozen at -20°C until analyzed. (b) Feces: all feces were collected throughout the study. One 4-day and two-5 day composites were made in each period by giving subjects a fecal marker at the beginning of each composite. After mixing the feces for each individual composite it was freeze-dried for subsequent analysis. (c) Blood: a fasting blood sample was drawn from the antecubital vein on the 4th, 9th and 14th day of each experimental period.

Food Samples for Glycosylated Vitamin B6

In addition to the food from the above study, glycosylated vitamin B6 was determined in foods from other bioavailability studies conducted in our laboratory (Gonzalez, 1982) as well as foods of the same types used in two other vitamin B6 bioavailability studies (Tarr et al., 1981; Nelson et al., 1976). These foods were purchased locally.

Analytical Methods

Total vitamin B6 was determined on 10 ml of the acid-hydrolyzed urine samples from days 2, 5, 8, 11 and 14 of the three experimental periods (AOAC, 1980). Total vitamin B6 of the feces was determined on 1 g of freeze-dried sample which had been acid-hydrolyzed (AOAC, 1980). The 4-pyridoxic acid content of the urine was assayed for the same days for which urinary vitamin B6 was measured. An ion exchange-fluorometric method (Reddy et al., 1958) was used to determine 4-pyridoxic acid. Plasma pyridoxal 5'-phosphate was measured on samples from the 4th, 9th and 14th day of each experimental period (Chabner and Livingston, 1970). The glycosylated vitamin B6 content of the urine from the last day of each experimental period, the last fecal composite for each period, and foods from this study and representative foods from other studies was determined by mixing 5 ml of urine, 0.5 g feces or 1 g of food with 100 ml of 0.1M phosphate buffer at pH 5.00, treating with 10 mg of β-glucosidase and incubating for 2 hr at 37°C in a shaking water bath. The enzyme was omitted from the above procedure for free vitamin B6 determination. The vitamin B6 content of the enzyme and non-enzyme treated samples were then assayed by a microbiological procedure (AOAC, 1980).

RESULTS AND DISCUSSION

The percent free and glycosylated vitamin B6 content of the experimental foods and constant basal diet of our study along with representative foods from the three other studies are given in Table 4.1. The plant products contained a measurable amount of glycosylated vitamin B6 in contrast to animal products which contained no detectable amount.

The level of urinary vitamin B6 and 4-pyridoxic acid for days 11 and 14 of each experimental period, along with plasma pyridoxal 5'-phosphate concentration for days 9 and 14 of each experimental period and fecal vitamin B6 for the last 5 days of each period are given in Table 4.2. The percent free and glycosylated vitamin B6 in urine of five subjects for the last day of each period and for the last fecal composite of each period are given in Table 4.2.

The relative vitamin B6 bioavailability was evaluated by four different indices: 4-pyridoxic acid, urinary vitamin B6, fecal vitamin B6 and plasma pyridoxal 5'-phosphate. The relative vitamin B6 bioavailability from whole wheat bread and peanut butter as compared with that of tuna, is given in Table 4.3. When regression analysis was performed between the percent relative vitamin B6 bioavailability and percent glycosylated vitamin B6 of the three experimental foods, the correlation coefficients were 0.99, 0.94, 0.94 and 0.92; when urinary vitamin B6, 4-pyridoxic acid, plasma pyridoxal 5'-phosphate and fecal vitamin B-6, respectively, were used as indices to

assess relative vitamin B6 bioavailability. As discussed later, the resultant regression equations were subsequently used to estimate vitamin B6 bioavailability for other foods. We recognize that only three points were used in the regression analysis and therefore bioavailability data obtained must be viewed with this in mind.

The results of this study showed that the majority of vitamin B6 in the feces exists in the free form. This was seen for fecal samples from all three periods. Only during the whole wheat bread period was there a detectable amount of glycosylated vitamin B6 in the feces. During all the periods, some glycosylated vitamin B6 was excreted in the urine, but a lower percentage was excreted during the tuna period than either the whole wheat bread or peanut butter periods. Of the total glycosylated vitamin B6 in the diet, 9%, 13% and 7% of this was excreted in the urine and feces during the tuna, whole wheat bread and peanut butter periods, respectively. The question of what happened to the remainder of the glycosylated vitamin B6 remains. There are several possible explanations: (a) glycosylated vitamin B6 may have been hydrolyzed in the intestine and then absorbed; (b) it may have been absorbed as such and then hydrolyzed; (c) or it might have been hydrolyzed by intestinal microflora and not absorbed. Synthetic glycosylated vitamin B6 given orally to vitamin B6 deficient rats corrected the symptoms of this deficiency, but if given intravenously it was not as effective (Yasumoto et al., 1979). This suggests that this bound form is

broken down in the intestinal tract. This would support (a) and rule out (b) as given above. Tsuji et al. (1977) have also found that oral glycosylated vitamin B6 corrected a vitamin B6 deficiency in rats. In neither of these reports was it mentioned if coprophage was prevented. If this had occurred, then hydrolysis of the bound form by intestinal microflora cannot be ruled out.

In our study there was a lower excretion of both 4-pyridoxic acid and urinary vitamin B6 coupled with a higher fecal vitamin B6 excretion during the whole wheat bread and peanut butter periods as compared to the tuna period. Further, the level of glycosylated vitamin B6 was 0%, 17% and 18.5% for tuna, whole wheat bread and peanut butter, respectively. This suggests a relationship between the amount of vitamin B6 absorbed from a food and its percent glycosylated vitamin B6 content.

If this bound form had been absorbed intact and then hydrolyzed or broken down in the small intestine and then absorbed, this would have minimized the difference in the excretion of 4-pyridoxic acid, urinary and fecal vitamin B6 seen between the different periods. However, since there was some glycosylated vitamin B6 excreted in the urine, it seems some of the bound form was absorbed. It is unlikely that the glycosylated vitamin B6 was synthesized in vivo, because animal products tested contained no detectable amount of this form. There also is the possibility that vitamin B6 exists in another as yet unidentified bound form, but that this form is not digested and thus contributes to a high fecal excretion of vitamin B6.

The nutritional importance of this compound is not yet clear. One area of nutrition where this form of vitamin B6 may be of significance is that of bioavailability. In our study we saw an inverse relationship between the amount of glycosylated vitamin B6 and its relative bioavailability. Based on this observation, we tested foods from other studies where vitamin B6 bioavailability was measured. Nelson et al. (1976) used a triple lumen profusion tube technique in humans to measure the bioavailability of vitamin B6 from orange juice. The uptake of vitamin B6 from orange juice was 54% that of a synthetic form. In our study we found that 47% of the vitamin B6 in orange juice was bound to glucose. The existence of this compound in orange juice might be related to lower absorption. The representative plant portion of foods of the diet used by Tarr et al. (1981) was analyzed for total, free and glycosylated vitamin B6. Since we did not detect any measurable amount of glycosylated vitamin B6 in animal products, we did not analyze the animal portion of their diet. Ten percent of the total vitamin B6 intake in their study was found to exist as the glycosylated form. Based on the total glycosylated vitamin B6 content in their diet and using the regression equation from our study for urinary vitamin B6 and plasma pyridoxal 5'-phosphate, a bioavailability of 82% and 80% was estimated as compared to 79% and 71%, respectively, as determined by Tarr et al. (1981). In another human study in our laboratory (Gonzalez, 1981), the vitamin B6 bioavailability of bananas, beef, filberts and soybeans was studied. The vitamin B6 in bananas and filberts was

found to be more available than that from soybeans. The amount of glycosylated vitamin B6 in bananas and filberts and soybeans was 3%, 4% and 57%, respectively. Using the 4-pyridoxic acid values reported by Gonzalez (1982) as an index and using the regression equation developed from the present bioavailability study, the percent vitamin B6 bioavailability was 98% for bananas, 96% for filberts and 41% for soybeans as compared to mean values of 131 ± 68, 88 ± 14, and 58 ± 24%, respectively as obtained by Gonzalez (1981). While differences exist between the extrapolated bioavailability values and those reported, the values are in general agreement. The differences seen may be due to different experimental designs being used in these two studies. Taken together, these observations suggest an inverse relationship between the percent glycosylated vitamin B6 content of a food and the bioavailability of vitamin B6 in humans.

In summary, the majority of the vitamin B6 in feces was present in the free form. Only during the whole wheat bread period was vitamin B6 found in the feces in the glycosylated form (4% of the total). On the average, 12-29% of total urinary vitamin B6 was in the glycosylated form. There was an inverse relationship between the level of glycosylated vitamin B6 present in a food and the bioavailability of vitamin B6 of that food in humans. Therefore, the data from this study suggest that the level of glycosylated vitamin B6 may be useful as an index of vitamin B6 bioavailability.

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Table 4.1. Total, Free and Glycosylated Vitamin B6 Content of Different Foods.

Sample	Total Vitamin B6	% Free Vitamin B6	% Glycosylated Vitamin B6
Foods from present study	ug/100 g		
Peanut butter	302±21[2]	16	18
Tuna (canned)	316	50	0
Whole wheat bread	169±2	40	17
Plant food portion of[1] the present study	58±0.3	38	40
Foods for Tarr et al. study (10)			
White bread & shredded wheat[1]	34±0.3	33	24
Fruits & vegetables[1]	64±0.8	39	50
Foods from Gonzalez study (9)			
Bananas	313±6	98	3
Filberts	587±15	120	4
Soybeans (cooked)	627±11	21	57
Orange juice as in study of Nelson et al. (11)	165±2	33	47

[1]Composites of these plant foods were analyzed.
[2]All samples, except tuna, were analyzed in duplicate. The value listed is the mean ± standard deviation. Values are as pyridoxine equivalents.

Table 4.2. Mean excretion of fecal and urinary vitamin B6, glycosylated vitamin B6, urinary 4-pyridoxic acid and the mean plasma level of pyridoxal 5'-phosphate during the three experimental periods.

Experimental Period	Fecal			Urinary			Urinary		
	Vitamin B6	Free Vitamin B6	Glycosylated Vitamin B6	Urinary Vitamin B6[5]	Urinary 4-pyridoxic acid	Plasma pyridoxal 5'-phosphate	vitamin B6[6]	Free vitamin B6	Glycosylated vitamin B6
	µmoles/day	%	%	µmoles/day	µmoles/day	nM	µmoles/day	%	%
Tuna	3.08 ± 0.73[1,2a]	98 ± 11[3]	n.d.	1.05 ± 0.20[b,c]	4.89 ± 1.1[d,e]	81.5 ± 36.0	1.09 ± 0.24[f,g]	82 ± 6	12 ± 7
Whole Wheat Bread	3.8 ± 0.78	82 ± 8	4 ± 5[4]	0.79 ± 0.09[b]	3.62 ± 0.66[d]	64.8 ± 29.8	0.77 ± 0.11[f]	65 ± 7	18 ± 10
Peanut Butter	4.42 ± 1.03[a]	83 ± 12	n.d.	0.68 ± 0.19[c]	2.8 ± 0.5[e]	48.4 ± 20.2	0.71 ± 0.19[g]	63 ± 9	26 ± 9

[1]Mean ± SD.
[2]Mean fecal excretion for the last five days of each experimental period for all eight subjects.
[3,4]Percent free and glycosylated vitamin B6 for the last five days of each experimental period for all eight subjects; n.d. = not detected.
[5]Mean urinary vitamin B6 excretion for days 9 and 14 of each experimental period for all eight subjects.
[6]Mean urinary vitamin B6 excretion for the last day of each experimental period for five subjects.

All values for vitamin B6 (including glycosylated vitamin B6) are as pyridoxine equivalents.

[a,b,c,d,e,f,g,]Values sharing the same superscripts are significantly different from each other at $p \leq 0.01$.

Table 4.3. Percent Relative Bioavailability of Tuna, Whole Wheat Bread and Peanut Butter as Assessed by Using Fecal B6, Urinary B6, 4-Pyridoxic acid excretion and Plasma Pyridoxal 5'-phosphate Concentration.

Experimental period	Criteria used to assess vitamin B6 bioavailability				
	4-pyridoxic acid	Urinary vitamin B6	Fecal vitamin B6	Plasma pyridoxal 5'-phosphate	Average bioavailability
Tuna	100	100	100	100	100
Whole Wheat Bread	74	72	77	76	75
Peanut Butter	57	65	57	74	63

RECOMMENDATIONS FOR FUTURE WORK

Based on results from this study, the following experiments are suggested:

1. The effect of the quality of protein on plasma pyridoxal-5'-phosphate level, urinary vitamin B6 and urinary 4-pyridoxic acid excretion.

2. Comparison of the excretion pattern of glycosylated vitamin B6 when human subjects are fed a diet that does not contain any glycosylated vitamin B6 in contrast to one which contains an appreciable amount of glycosylated vitamin B6.

3. The effect of processing on glycosylated vitamin B6 to see if the increased level of glycosylated vitamin B6 in processed foods is due to denaturation of naturally occurring β-glucosidase or if it is formed during processing.

4. Production and testing of glycosylated vitamin B6 synthetically in vivo.

5. In addition, a methodological change is suggested. Based on the observation that the non-conjugated vitamin B6 content of filberts was higher than the total vitamin B6 content, recovery of added pyridoxine should be done for all samples to be analyzed to see if there is a promoting or inhibiting factor in the particular sample studied.

SUMMARY AND CONCLUSION

Bioavailability of vitamin B6 from certain foods may be limited. The knowledge of vitamin B6 bioavailability from food is important in the sense that this would help to understand the extent to which the vitamin B6 present in the diet will meet the requirements for this vitamin in the body.

The purpose of the study were a) to develop a method to measure the level of glycosylated vitamin B6 in foods, b) to measure the level of glycosylated vitamin B6 in foods of both animal and plant origin, c) to investigate the relative vitamin B6 bioavailability from tuna, whole wheat bread, and peanut butter in humans, d) to follow the excretion patterns of glycosylated vitamin B6 when foods containing this compound are fed and to study the relationship between the levels of glycosylated vitamin B6 and vitamin B6 bioavailability in humans.

A method was developed to measure the glycosylated vitamin B6 content of foods. The optimum concentration for the β-glucosidase was found to be 60 units per gram of food and the incubation time of 2 hr at 37°C. To measure the level of glycosylated vitamin B6 in foods, the vitamin B6 content was determined microbiologically before and after treatment with β-glucosidase as well as after acid hydrolysis of the food. Animal products contain no measurable amount of glycosylated vitamin B6, but grains and legumes had levels of from 6-57%

of the total vitamin B6. Of the fruits and vegetables analyzed, orange juice (47%) and raw carrots (51%) had the highest level of glycosylated vitamin B6.

The relative bioavailability of vitamin B6 from tuna, whole wheat bread and peanut butter was investigated in eight healthy men in a 52-day study (10 day adjustment and three 14-day experimental periods). Vitamin B6 intake was set at 1.6 mg/day, with 50% coming from one experimental food and 50% from a basal diet. Urinary 4-pyridoxic acid, urinary vitamin B6, fecal vitamin B6 and plasma pyridoxal-5'-phosphate were used as indices to determine bioavailability. Of these four indices used to assess vitamin B6 bioavailability, the level of 4-pyridoxic acid and urinary vitamin B6 excreted were significantly ($p \leq 0.01$) higher when tuna was fed than when either whole wheat bread or peanut butter were fed. When tuna was fed, fecal vitamin B6 excretion was significantly ($p < 0.01$) lower than when peanut butter was fed. The vitamin B6 in whole wheat bread and peanut butter was 75%, and 63% as available as that from tuna, respectively.

The urine from the last day of each period for five subjects and the last fecal composite from each period were analyzed for the amount of non-conjugated and glycosylated vitamin B6. The majority of vitamin B6 in the feces was in the non-conjugated form. No glycosylated vitamin B6 was detected in the feces during either the tuna or peanut butter periods, and only 4% of total vitamin B6 in the feces was in the glycosylated form

when whole wheat bread was fed. The level of glycosylated vitamin B6 excreted in the urine was 12 ± 7%; 18 ± 10%; and 26 ± 9% of the total urinary vitamin B6 for tuna, whole wheat bread and peanut butter periods, respectively.

The level of glycosylated vitamin B6 in the foods was inversely related to the percent vitamin B6 bioavailability for the foods in our study and for foods of the type used in three other human studies. It appears that the level of glycosylated vitamin B6 in foods could be used as an index of vitamin B6 bioavailability in humans.

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APPENDICES

Appendix A

INFORMED CONSENT

The purpose of this experiment is to study the absorption of vitamin B6 from various foods. The study will run a total of 52 days. The first 10 days of the study you will be fed a standard diet. These foods will be a major source of vitamin B6 in the diet. This new food will replace one of the foods fed in the previous diet. The foods to be fed are tuna, peanut butter, and whole wheat bread. During the initial 10 days, cheese and hamburger will be the major source of vitamin B6.

The absorption of vitamin B6 will be studied by examining blood, urinary and fecal levels of vitamin B6 and its metabolites. Changes in excretion of vitamin B6 and its metabolic products will be used as a measure of the influence of food. As an indication of adequate intestinal absorption, a xylose test will be done. Five grams of xylose is given orally and urine collected for five hours. This test will be done prior to beginning the study.

This metabolism will be examined in nine men. Blood, urine, and fecal samples will be collected. Twenty-four urines and all fecal samples will be collected throughout the study. A fecal marker will be given at intervals each week. This is given in a capsule and is of no risk to you. Blood samples (about 30 ml) will be collected about twice a week. All meals will be provided and no other food is permitted except as indicated. No

alcohol is allowed while on this study. Also we ask that each person maintain their normal activity level throughout the study and not engage in any unusual physical activity. Meals will be prepared for you at facilities located in the Department of Foods and Nutrition. The diet will be adequate in all nutrients for which an RDA (Recommended Dietary Allowance) has been established. The diet consists of normal foods you would find in most diets. Body weight will be kept constant by adjusting caloric intake (adjustment of carbohydrate intake). This may mean a slightly higher intake of sugar than you may now be eating. The frequency and amount of blood drawn should not be a physical stress for a normally active healthy man. Blood will be monitored for iron levels. This study will last 52 days (March 31 to May 22, 1981). Breakfast will be provided on the morning following the last day of the study (May 22).

Information obtained from this study can be used in nutrition education and in recommendations on methodology of absorption of vitamin B6. This information will further be used in later studies which detemrine how much vitamin B6 present in foods is available for use by the body. Such information can provide a more accurate estimate of the RDA for this nutrient.

INFORMED CONSENT

I have discussed the rationale, procedure and safety of this investigation with the interviewer. All of my questions have been answered. I understand that I am free to leave this experiment at any time.

While participating in this study I give my consent to take oral doses of xylose and fecal markers. In addition, I agree to have blood drawn and to collect timed urine and fecal samples. I will consume only the food and beverages that are allowed on this diet, and I agree to record my food and beverage consumption for the period of the study. In return for these services, the Department of Foods and Nutrition will pay me $3.00 a day for complete urine and fecal collections. I will be paid at the end of the study. Deductions from this payment may be made if urine collections have been incomplete, as determined by urinary creatinine.

The Department of Foods and Nutrition reserves the right to remove a subject from the study if he is uncooperative in following the protocol of this investigation.

I am not taking any drug or vitamin supplement.

Signed_____

Witness_____

Date_____

Appendix B

Name_____

Date_____

DAILY ACTIVITY SHEET

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

Activity	Length of time (fraction of hours)	Time of Day
Sleep		
Sitting		
Walking		
Running		
Bicycling		
Other sports (indicate type)		

2. Record all "free" foods

Sugar (teaspoons)_____

7-up (ounces)_____

Hard candies_____

Life Savers (rolls)_____

Coffee (cups)_____

Tea (cups)_____

3. How do you feel today? Excellent_____
Good_____
Fair_____
Poor _____

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

5. Unusual events- exams! and other.

6. Other comments.

Appendix C, Table C1. Blood Chemistry of the Subjects at the Beginning of the Study

Test	units	Subjects 1	2	3	4	5	7	8	9	Normal Range
LDH[1]	u/L	128	138	106	162	126	133	126	150	85 - 162.0
SGOT	u/L	17	19	18	33	25	25	24	28	14.0 - 40.0
Phos	mg/dl	3.2	4.4	3.6	3.8	3.4	3.5	4.3	4.8	2.4 - 4.1
Calcium	mg/dl	9.8	10.0	9.4	9.9	10.1	9.9	9.4	9.8	8.7 - 10.3
Alkaline-Phosphatase	u/L	19	23	34	23	27	28	33	30	12 - 27
Bilirubin	mg/dl	0.7	0.40	0.50	0.50	0.90	1.7	0.50	0.50	0.4 - 1.4
U.A.	mg/dl	3.9	4.8	4.3	4.5	3.7	3.9	4.7	4.4	2.5 - 6.50
T.P.	g/dl	3.2	8.1	7.1	7.8	7.6	7.0	7.50	7.8	6.3 - 7.9
Alb	g/dl	4.60	5.20	4.50	4.90	5.10	4.70	4.60	4.70	3.9 - 5.1
CHOL	mg/dl	172	252	199	172	164	212	182	194	146 - 329
CREA	mg/dl	1.20	1.10	1.20	1.10	1.10	1.40	1.10	1.0	0.7 - 1.40
BUN	mg/dl	15	16	12	15	14	14	15	17	9.0 - 24.0
GLUC	mg/dl	94	93	86	91	89	83	93	96	69 - 121
NA	meq/l	142	144	141	143	145	143	142	143	134 - 145
K	meq/l	3.5	4.2	3.9	5.0	4.10	4.70	3.60	3.90	3.5 - 5.0
Cl	meq/l	103	102	104	104	105	106	105	106	100 - 107

1. LDH = Lactate Dehydrogenase, SGoT = Serum Glutamate-oxaloacetate transaminase, Phos = phosphorous, U.A. = uric acid, T.P. = Total Protein, Alb = Albumin, CHoL = Cholesterol, CREA = Creatinine, BUN = Blood urea nitrogen, GLU = Glucose, NA = Sodium, K = Potassium, Cl = chloride.

Table C2. Hematocrit and Hemoglobin Values

Day of the Study		Subjects 1	2	3	4	5	7	8	9
1	Hct%	49.0	50.5	44.5	50.0	48.0	51.0	47.0	45.0
	Hgb g/dl	17.5	18.1	14.9	17.9	16.9	17.6	15.7	16.1
11	Hct%	48.5	51.5	47.0	47.5	47.5	49.0	45.0	44.0
	Hgb g/dl	17.3	18.0	15.40	17.0	16.60	17.0	14.7	15.0
25	Hct%	46.5	50.5	46.5	48.4	49.0	48.0	45.0	46.5
	Hgb g/dl	16.9	17.7	15.3	17.2	17.1	16.7	15.2	16.10
39	Hct%	46.5	51.0	46.0	47.5	48.0	49.0	45.0	42.5
	Hgb g/dl	16.7	18.1	16.0	16.8	16.7	17.2	15.0	15.1
52	Hct%	47.5	51.0	44.5	49.0	49.50	48.5	47.0	41.50
	Hgb g/dl	16.5	17.9	15.0	17.3	17.1	16.5	15.7	14.6

1 - Hct = Hematocrit
2 - Hgb = Hemoglobin

Table C3. Vitamin B6 content of the daily diet for various foods.

Food	Day 21	Day 28	Day 35	Day 42	Day 49
			mg/day		
Tuna	0.62	0.77	0.72	0.65	0.74
Peanut Butter	0.69	0.75	0.78	0.73	0.75
Whole Wheat Bread	0.66	0.75	0.72	0.74	0.80
Constant Diet	0.78	0.82	0.85	0.77	0.88

Table C4. Total Fecal Weight for Each Diet Period

Period	Subjects 1 PB,WW,T[1]	2 T,PB,WW	3 T,PB,WW	4 WW,PB,T	5 T,WW,PB	7 PB,T,WW	8 WW,T,PB	9 WW,T,PB
	g/period							
Adjustment	1323	714	680	1830	957	988	1410	1300
Tuna	1681	1074	986	2113	1207	1644	2463	1568
Whole Wheat Bread	3827	2795	1976	2192	4230	3137	4162	4405
Peanut Butter	3011	1670	1763	3052	2824	3349	3242	3603

1. T = Tuna, PB = Peanut butter, WW = Whole Wheat Bread.

Table C6. Creatinine Excretions

Day of the Study	Subjects 1 PB,WW,T	2 T,PB,WW	3 T,PB,WW	4 WW,PB,T	5 T,WW,PB	7 PB,T,WW	8 WW,T,PB	9 WW,T,PB
	g/24 hours							
Adjustment								
1	1.87	1.41	2.03	—	1.71	1.76	2.02	—
2	1.85	1.72	1.76	1.53	1.57	1.73	1.64	2.14
3	1.62	1.78	2.17	1.71	1.62	1.82	2.98	1.34
4	1.84	1.82	2.17	0.95	1.84	1.72	2.10	1.56
5	1.70	1.52	2.33	0.89	1.69	1.88	1.83	1.77
6	1.68	1.73	2.12	1.45	1.78	1.62	2.42	1.79
7	1.83	1.84	2.45	1.12	1.74	1.64	2.09	1.83
8	1.9	1.85	2.47	1.64	1.70	1.79	2.29	1.97
9	2.29	2.15	2.37	1.53	1.99	1.85	2.16	1.90
10	1.79	1.77	2.40	1.70	1.75	1.86	2.10	1.88
Period I								
11	1.72	2.05	2.49	1.56	1.92	1.59	1.98	2.00
12	1.60	1.88	2.46	1.12	1.95	1.67	1.43	1.12
13	1.58	1.93	2.18	1.32	1.79	1.59	2.15	1.84
14	1.64	1.94	2.59	1.50	1.90	1.57	2.06	1.76
15	1.66	2.05	2.27	1.53	1.87	1.67	1.77	1.56
16	1.57	1,96	2.49	1.59	1.85	1.64	1.81	1.68
17	1.66	2.03	2.52	1.59	1.71	1.63	1.95	1.51
18	1.66	2.05	2.58	1.68	2.01	1.60	1.91	1.45
19	1.65	2.07	2.42	1.48	2.01	1.68	1.81	1.72
20	1.68	2.0	2.43	1.50	1.98	1.71	1.98	1.62
21	1.68	2.11	2.35	1.59	1.97	1.65	1.74	1.66
22	1.62	2.00	2.45	1.42	1.77	1.66	1.37	1.42
23	1.63	2.03	2.46	1.46	2.02	1.70	1.44	1.62
24	1.64	2.05	2.55	1.51	1.93	1.63	2.59	1.62
Period II								
25	1.64	1.52	2.26	1.29	1.65	2.05	1.58	1.60
26	1.55	1.70	2.23	1.87	1.90	1.96	2.85	1.96
27	1.57	1.63	—	1.47	1.48	1.86	0.82	1.83
28	1.67	1.92	2.28	1.32	1.63	1.89	3.38	1.81
29	1.68	1.69	2.16	1.70	1.60	1.95	1.99	1.93
30	1.68	1.75	1.81	1.79	1.57	2.06	2.17	1.94
31	1.69	1.68	2.34	1.62	1.53	1.95	2.34	1.98
32	1.86	1.76	2.00	1.77	1.67	2.09	2.12	2.13
33	1.68	1.80	2.21	1.70	1.68	2.08	2.22	1.80
34	1.60	1.75	2.03	1.54	1.45	1.88	2.01	1.99
35	1.68	1.70	2.14	1.69	1.64	1.98	2.20	1.93

Table C6 continued

	1	2	3	4	5	7	8	9
36	1.54	1.77	2.07	1.76	1.62	2.05	1.93	1.84
37	1.58	1.73	2.19	1.55	1.60	1.86	2.15	1.77
38	1.60	1.66	2.17	1.60	1.60	1.92	2.46	2.03
Period III								
39	1.95	1.93	2.23	2.00	1.64	1.78	2.28	1.53
40	1.94	1.79	2.33	2.07	1.58	1.66	—	—
41	1.96	1.68	2.03	1.78	1.47	1.73	1.66	1.38
42	1.90	1.72	2.21	2.02	1.63	1.59	1.86	1.58
43	2.04	1.75	2.10	2.02	1.56	1.69	1.93	1.55
44	1.92	1.67	2.17	1.84	1.64	1.77	2.07	1.53
45	1.80	1.81	1.98	2.12	1.68	1.60	1.78	1.60
46	2.08	1.85	1.92	2.18	1.73	1.90	1.97	1.79
47	2.00	1.86	2.46	2.06	1.65	1.75	1.74	1.68
48	2.03	1.73	1.97	2.08	1.51	1.65	1.94	1.58
49	1.86	1.82	—	1.97	1.68	1.60	1.86	1.32
50	2.02	1.75	2.16	2.06	1.62	1.80	1.98	1.53
51	1.91	1.75	2.05	1.05	1.69	1.92	1.94	1.62
52	1.96	1.64	2.03	1.94	1.55	1.72	1.91	1.58

1. T = tuna, PB = Peanut butter, WW = Whole wheat bread.

Table C7. Urinary Vitamin B6 Excretion

Day of the Study	Subjects 1 PB.WW,T	2 T,PB,WW	3 T,PB,WW	4 WW,PB,T	5 T,WW,PB	7 PB,T.WW	8 WW,T,PB	9 WW,T,PB
			µmoles/24 hours					
Adjustment								
1			1.50		1.59	0.93	0.80	
2	1.03	0.71		0.85				1.29
3								0.63
4	1.02	0.79	1.42	0.59	1.73	0.85	0.69	
5							0.94	0.86
6								
7	0.96	0.79	1.38	0.74	1.75	0.64	0.89	0.85
8								
9						0.66		
10	0.90	0.66	1.08	0.92	1.69		0.85	1.02
Period I								
11								
12	0.68	0.81	1.17	0.94	1.25	0.77	0.69	0.62
13				0.85			1.04	0.83
14			1.35					
15	0.72	0.97		0.89	1.007	0.53	0.90	0.84
16								
17								
18	0.62	0.94	1.25	1.04	1.22	0.53	0.95	0.69
19				0.95				0.81
20							0.98	
21	0.63	0.93	1.14	0.99	0.96	0.48	0.77	0.77
22								
23							0.85	
24	0.61	0.89	1.40	0.94	1.14	0.55	1.26	0.78
Period II								
25	0.73			1.36		0.78	0.83	
26	0.65	0.72	0.95		0.84	0.75	1.26	0.85
27					0.76		0.52	
28		0.52					1.40	
29	0.68		0.93	1.02	0.71	0.77	0.97	0.86
30							1.18	0.79
31	0.76	0.62	0.95					0.92
32			0.84		0.68	0.75	1.09	
33				1.16				
34								
35	0.84	0.62	0.92	1.17	0.80	0.84	1.14	0.97
36								
37								

Table C7 continued

	1	2	3	4	5	7	8	9
38	0.79	0.60	0.86	0.97	0.70	0.83	1.08	0.92
Period III								
39								0.63
40	0.96	0.71	0.90	1.39	0.57	0.65		
41				1.35		0.61	0.76	
42			0.82				0.96	0.65
43	1.00	0.69	0.79	1.50	0.46	0.60	0.83	0.59
44								
45						0.65	0.61	
46	1.04	0.74	0.68	1.41	0.65	0.70		0.68
47			0.63				0.57	
48	0.90		0.69				0.69	0.55
49	0.91	0.73		1.41	0.52	0.62	0.80	0.53
50								
51		0.8	0.76	1.41	0.51			
52	0.92	0.82	0.84	1.48	0.54	0.66	0.63	
							0.57	

1. T = tuna, PB = Peanut butter, WW = Whole wheat bread

Table C8. Urinary 4-Pyridoxic Acid Excretion

Day of the Study	Subjects 1 PB,WW,T	2 T,PB,WW	3 T,PB,WW	4 WW,PB,T	5 T,WW,PB	7 PB,T,WW	8 WW,T,PB	9 WW,T,PB
	µmoles/24 hours							
Adjustment								
1	3.81				6.34	6.81	3.99	
2		4.84	8.42	3.09				12.20
3				2.90				7.83
4	3.75	5.15	8.49		6.81	5.31	5.65	
5							4.42	5.37
6								
7	3.75	4.70	8.49	3.29	6.34	6.16	4.89	7.79
8								
9								
10	3.88	4.26	6.10	4.93	5.95	4.75	4.77	10.10
Period I								
11								
12	3.54	4.48	6.28	3.15	6.42	4.03		5.35
13				4.68			4.68	8.22
14			6.17					
15	3.02			3.48	5.53	3.90	4.50	5.32
16	2.59							
17								
18	2.59	4.03	4.91	3.29	5.68	3.22	4.18	5.14
19				2.90			3.93	5.56
20						3.03		
21	2.24	4.03	4.43	3.67	4.94	2.94	3.63	4.06
22								5.27
23							2.73	
24	2.16	3.81	4.25	3.09	5.45	2.82	4.50	5.14
Period II								
25	2.46		4.19		4.04	3.23	2.77	
26	2.16	2.24	1.90	3.87	4.49	4.03	5.67	5.14
27				3.15	4.12		4.56	
28							5.75	
29	2.72	2.46	2.8	2.50	4.67	4.23	3.55	5.78
30							3.74	5.18
31			3.06				4.68	5.14
32	2.63	2.46	2.77	—	4.67	4.43	3.71	5.18
33								
34								

Table C8 continued

	1	2	3	4	5	7	8	9
35	2.59	2.66	2.55	3.43	3.72	4.35	5.08	6.14
36								
37								5.40
38	2.24	2.09	2.51	3.29	4.28	4.23	5.67	6.78
Period III								
39								4.44
40	2.76	2.54	2.95	5.22	3.50	4.02	—	—
41								
42			3.43		2.99			3.63
43	3.24	2.91	3.62	6.38	2.77	3.94	3.72	3.94
44								
45								
46	3.49	3.36	2.70	5.61	3.04	4.10	1.56	4.41
47			2.40				2.54	
48	3.81	—	3.62	—	—	—	2.54	3.40
49	3.45	3.14	—	5.99	3.31	4.04	—	3.42
50								
51		3.81	3.43	6.81	2.42			
52	3.15	3.43	3.43	6.57	2.40	3.94	2.74	4.06

1. T = Tuna, PB = Peanut Butter, WW = Whole Wheat Bread.

Table C9. Plasma Pyridoxal-5'-Phosphate

Day of the Study	Subjects 1 PB,WW,T	2 T,PB,WW	3 T,PB,WW	4 WW,PB,T	5 T,WW,PB	7 PB,T,WW	8 WW,T,PB	9 WW,T,PB
	nM							
Adjustment								
1[1]	75.5	95.9	15.1	86.2	43.8	31.3	86.5	82.0
11[1]	73.7	84.6	95.9	81.5	46.3	26.4	48.8	62.7
Period I								
15	65.0	81.6	86.9	65.8	49.3	20.8	55.4	77.1
19	61.8	81.8	91.2	61.6	54.1	20.8	50.1	4.30
25	39.7	73.7	96.	68.5	51.1	26.0	47.7	33.6
Period II								
29	47.5	50.3	68.1	61.9	45.1	23.2	58.9	34.6
34	55.2	51.9	65.6	86.5	47.1	21.8	53.9	38.0
39	54.9	51.0	51.6	93.8	36.9	20.9	62.1	31.4
Period III								
43	53.2	50.8	59.1	91.8	37.0	19.8	50.4	32.3
48	68.2	62.1	63.1	12.35	45.9	20.7	46.0	30.2
53	69.7	61.6	60.2	10.00	33.6	24.6	37.5	33.6

1. The value listed for hte first day of periods I, II and III actually represent the previous period as these blood samples were collected on the morning of the respective days.