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Title: VITAMIN B_6 ENRICHMENT OF WHEAT FLOUR: STABILITY

AND BIOAVAILABILITY

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This investigation consisted of two parts: A. stability, B. bioavailability of vitamin B_6 in wheat. Three variables; whole wheat flour (WHW), white flour (W) and W enriched with vitamin B_6 (WB_6) were tested.

Stability of vitamin B_6 during bread making and storage of bread and flour was determined. Bread was prepared from the three types of flour under commercial and home conditions. Two methods, straight dough and sponge dough for bread making were compared under home conditions. Vitamin B_6 content of dough before fermentation, after proofing and of bread was determined by a microbiological method. The WB_6 dough prepared using the sponge dough method showed a significant increase in the vitamin B_6 content during fermentation ($P < 0.05$). A significant baking loss of 10-15% was observed in the WHW and W breads prepared under

commercial conditions ($P < 0.05$), and of 5-12% in WHW, WB_6 ($P < 0.01$) and W ($P < 0.05$) breads made using the sponge dough method.

Storage stability of vitamin B_6 was determined in the commercially prepared WB_6 bread and all purpose flour enriched with vitamin B_6. There was no significant change in vitamin B_6 levels in the bread stored under frozen and refrigerated conditions for seven and four weeks, respectively. However, a significant drop of 10% was observed in the vitamin B_6 content of the bread after three days of storage at room temperature ($P < 0.01$). Vitamin B_6 content of the WB_6 flour did not change when stored over a period of 26 weeks at room temperature.

Bioavailability of vitamin B_6 was studied in nine men, age 21-33 years. Each week one of the three types of bread, WHW (570 g), WB_6 (600 g) and W (600 g) was fed daily to each subject using a 3X3 Latin square design. The WHW, WB_6 and W bread supplied 1.20, 1.18 and 0.35 mg of vitamin B_6, respectively, while 0.38 mg was supplied by the constant diet. The daily vitamin B_6 intake was set at 1.5 mg, of which approximately 3/4 was supplied from WHW or WB_6 bread. During the period when W bread was consumed, the subjects also received an oral dose of 0.81 mg of vitamin B_6 in order to maintain a constant daily intake throughout the study. The predominant form of vitamin B_6 in the diets was found to be pyridoxine.

Twenty-four hour urines, daily fecal collections and fasting blood samples three days per week were analyzed for vitamin B_6 and its metabolites. using microbiological, chromatographic and fluorometric techniques.

The fecal vitamin B_6 level was significantly higher when WHW bread was fed as compared to when WB_6 or W bread was fed (P < 0.01). There was no significant difference in the urinary excretion of total and free vitamin B_6 in relation to the type of bread. The predominant form of vitamin B_6 in urine was found to be pyridoxal. The urinary 4-pyridoxic acid content was significantly lower when the diet was based on WHW bread as compared to WB_6 or W bread (P < 0.01).

The percentage of the daily intake of vitamin B_6 accounted for by the excretory products analyzed in this study was 91.6 when WHW bread was fed. The corresponding percentages when WB_6 and W breads were fed were 81.5 and 79.8, respectively. The plasma vitamin B_6 and pyridoxal phosphate levels were slightly lower when WHW bread was consumed as compared to WB_6 or W bread. These data suggest that vitamin B_6 was not as available from WHW bread as from WB_6 and W bread. The availability of vitamin B_6 from WB_6 and W bread as determined in the present study was similar.

For the populations who are dependent on refined wheat products, enrichment of flour with vitamin B_6 will be of advantage.

However, enrichment of refined wheat products cannot replace completely the benefits of consuming whole wheat products.

Vitamin B_6 Enrichment of Wheat Flour:

Stability and Bioavailability

by

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TO

MY MOTHER

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INTRODUCTION

Wheat products comprise about 30 percent of the cereals consumed by the populations around the world (1). Almost 19 percent of the calories and 20 percent of the proteins in the average world daily per capita intake come from wheat (2). Whole grain wheat is a rich source of B complex vitamins including B_6 (3, 4, 5). Concentration of the vitamins is highest in the aleurone layer and germ fraction. About 85 percent of vitamin B_6 is lost when wheat is converted to refined flour (6). White bread, which is lower in vitamin B_6 (40 μg/100g) than whole wheat bread (180 μg/100g), is consumed quite extensively (3). With the importance of bread in the diet of all people, it becomes important to consider the loss of vitamin B_6 as well as other nutrients during milling.

Following the discovery of certain vitamin deficiency diseases in the United States, efforts were initiated to meet the nutritional needs of the people (7). Early attempts to use vitamin supplements were unsuccessful as were those to pursuade people to use whole grain products. From this experience, the concept of enrichment programs was developed in the United States during the 1930s and

early 1940s. The first step toward this was the mandatory enrich-

ment of white flour with thiamin, riboflavin, niacin and iron which

came into effect from January 1, 1942 (8). According to the Food and

Nutrition Board of the National Academy of Sciences, there seems to

be evidence of potential risk of deficiency of vitamin A, vitamin B_6,

folacin, iron, calcium, magnesium, zinc, thiamin, riboflavin and

niacin among some groups of the population (9). Because of this,

the National Academy of Sciences in 1974 proposed the addition of

vitamin A, vitamin B_6, folic acid, magnesium and zinc along with

an increase in the amount of the other nutrients added to the cereal

products. The level of enrichment recommended for vitamin B_6

was 2 mg (as pyridoxine) per pound of refined flour.

Before any enrichment program is adopted, it becomes impor-

tant to test the stability of the nutrient under customary conditions

of storage and use. However stable a nutrient may be, the nutri-

tional value of a food is governed in part by its bioavailability in a

given food product. The availability of vitamin B_6 from foods may

be influenced by a number of factors, one of which is digestibility

of proteins (10). Proteins of plant origin are known to be digested

less completely than those of animal origin. However, in a study

on wheat flour as a source of protein for adult human subjects,

Bolourchi, Friedmann and Mickelsen (11) reported that the digesti-

bility of the protein in a diet containing animal products and one

containing wheat were almost the same, as evidenced by fecal nitrogen excretion.

Vitamin B_6 in nature exists mostly as coenzymes bound to proteins and the difference in digestibility may lead to a lower bio-availability of the vitamin from certain foods of plant origin compared to those of animal origin. Justification for assessing the bioavailability of vitamin B_6 from a cereal source is based on the dependency of a significant proportion of the world population on these foods for their nutrients, the current trend to replace animal products with plant sources because of food shortages (12), the high costs of food and the increasing number of vegetarians (13).

The information obtained from this study can be used to make recommendations on the enrichment of wheat flour and bread with vitamin B_6. Such data may also be utilized to arrive at a more accurate recommended dietary allowance (RDA) for this nutrient, since the present level of 2 mg for an adult is an estimation based on results obtained from studies in which the crystalline form of vitamin B_6 was given to correct biochemical disorders in subjects depleted of the vitamin (14).

REVIEW OF LITERATURE

Importance of Vitamin B$_6$

Vitamin B$_6$, as a distinct member of the B complex group,
was first recognized by György as early as 1934 (15). Since that time,
numerous metabolic functions for vitamin B$_6$ have been identified (16).
However, the mechanisms of these are not all well established.
Vitamin B$_6$ has been referred to by some authors as the "sleeping
giant of nutrition" (17).

The major form in which vitamin B$_6$ functions is as a coenzyme,
pyridoxal-5-phosphate (PLP). It acts in a wide spectrum of bio-
chemical reactions related primarily to the metabolism of amino acids
(18). Some of the reactions catalyzed by these enzymes involving
amino acids include transamination, racemization, decarboxylation,
dehydration and desulfhydration (19). The transaminases represent
a major group of PLP catalyzed enzymes which are responsible for
the transfer of the α-amino group of the amino acids to keto acids (20).
After transamination the metabolites may be converted to fats,
carbohydrates or other amino acids.

Pyridoxal phosphate dependent decarboxylation of tyrosine,
histidine, dihydroxy-phenylalanine (DOPA) and 5-hydroxy-tryptophan
results in the formation of amines of biological importance in human

biochemistry. For example, histamine and 5-hydroxy-tryptamine
or serotonin are required for the functional activities of nervous
tissue. Pyridoxal phosphate is also important as the coenzyme for
δ-amino levulinic acid synthetase which decarboxylates α-amino-β-
ketoadipic acid to δ-amino-levulinic acid. The latter compound is
an intermediate in the formation of porphyrin which is required for
hemoglobin synthesis (21).

Pyridoxal-5-phosphate is an essential constituent in glycogen
phosphorylase which catalyzes the breakdown of glycogen to glucose-
1-phosphate (22). It has been reported that PLP plays a role in lipid
metabolism, especially in the conversion of linoleic acid to the more
unsaturated arachidonic acid (23, 24). A recent study has shown
that arachidonic acid levels in liver decreased in rats made pyridoxine
deficient (25). The reason for this decrease was reported to be the
degradation of arachidonic acid rather than lowering of the conversion
of linoleate to arachidonate.

Vitamin B_6 also appears to play an important role in the
maintenance and functioning of the immune system, probably through
its influence on nucleic acid systhesis (26, 27). The effect of
vitamin B_6 deficiency on immune responses was discussed in a
recent review (28). However, the mechanisms involved in the sup-
pressive effect of the vitamin B_6 deficiency on the immune system
are not yet established.

A relationship between the vitamin B_6 dependent metabolism of tryptophan and the occurrence of tumors was shown by Yoshida, Brown and Bryan (29). Their data suggested that tryptophan metabolites may play a role in recurrence of bladder tumors in patients with abnormal tryptophan metabolism. The reason for this has been speculated to be due to either the products of tryptophan having some direct carcinogenic action or poor vitamin B_6 status, which leads to abnormal tryptophan metabolism, resulting in the development of tumors, probably through the influence of low vitamin B_6 on the immune response (30).

Although the exact mechanisms of vitamin B_6 metabolism are not clearly established, attempts have been made to study the probable pathways in animal tissues (31, 32).

Metabolism of Vitamin B_6

Vitamin B_6 is widely distributed in foods, both as the free and phosphorylated forms (5). The phosphorylated forms of the vitamin from the ingested food are probably hydrolyzed in the intestine by phosphatases (33, 34). In the free state, vitamin B_6 is absorbed in the upper intestinal tract. Booth and Brain (31) observed that there was virtually no absorption of vitamin B_6 through the stomach wall. A greater part of the pyridoxine that was administered orally to rats was absorbed in the jejunum, and a small

amount in the ileum. Their data supported the concept that absorption of pyridoxine was by passive diffusion rather than active transport.

Vitamin B_6 exists as a group of closely related compounds which include pyridoxine (PIN), pyridoxal (PAL) and pyridoxamine (PAM) (35). These three forms, which are interconvertible, can be transformed in vivo to their phosphorylated forms (36), as shown in Figure 1. The oxidation of PAL by aldehyde oxidase results in the direct formation of 4-pyridoxic acid (4-PA), the major urinary metabolite of vitamin B_6 (37).

A review on the interconversions of vitamin B_6 in mammalian tissues has been presented by McCoy and Colombini (32). Ingested PIN was reported to be either phosphorylated to pyridoxine phosphate (PNP) by the action of pyridoxal phosphokinase and adenosine triphosphate (ATP) or oxidized to PAL by pyridoxine oxidase. Pyridoxal is phosphorylated directly to PLP by pyridoxal phosphokinase. Pyridoxine phosphate can also be oxidized to PLP by pyridoxine phosphate oxidase (39). Brain, liver and kidney were reported to have contained one of the highest concentrations of pyridoxal phosphokinase while muscle had very low concentrations (40). This suggests that non-phosphorylated forms of vitamin B_6 are converted into phosphates mainly in the brain, liver and kidney (41).

COOH

HO CH₂OH

CH₃ H

4-Pyridoxic Acid

aldehyde oxidase

CH₂OH

HO CH₂OH   CHO   CH₂NH₂

CH₃ N H   Pyridoxine Oxidase   HO CH₂OH   Pyridoxine   HO CH₂OH
           (NADP)   CH₃ N H   Transaminase   CH₃ N H

                                            Pyridoxal phosphate
                                            Oxidase (FMN)

Pyridoxal Kinase +ATP   Phosphatase   Phosphatase Pyridoxal Kinase +ATP   Pyridoxal Kinase +ATP   Phosphatase

CH₂OH   CHO   CH₂NH₂

HO CH₂OPO₃H₂   Pyridoxine Phosphate   HO CH₂OPO₃H₂   Pyridoxine Phosphate   HO CH₂OPO₃H₂
CH₃ N H   oxidase (FMN)   CH₃ N H   oxidase (FMN)   CH₃ N H

Pyridoxine-5-   Pyridoxal-5-   Pyridoxamine-5-
phosphate   phosphate   phosphate

Figure 1. Metabolic interconversions of vitamin B_6 and the formation of 4-pyridoxic acid. (Adapted from 36 and 38). ATP, FMN, NADP - see Appendix 5.

The possible role of circulating erythrocytes in these inter-conversions was suggested by Hamfelt (42), and Yamada and Tsuji (43). Anderson et al. (44) proposed a pathway for the conversion of pyridoxine to the active form of vitamin B_6 in the red cells (Figure 2). These observations suggest that PIN is converted to PLP in red cells by a pathway previously demonstrated (41) to occur in other tissues, and that PLP is converted to PAL, which then enters the plasma from the red blood cell. Pyridoxal was reported to be the major transport form of the vitamin (44, 45). However, it still remains to be determined to what extent PAL in blood plasma enters other tissue cells for conversion to the active coenzyme, or the extent to which it may be converted to 4-PA. Schwartz and Kjeldgaard (37) reported that PAL could be oxidized in the presence of liver aldehyde oxidase to 4-PA. Several investigators have shown that 4-PA was the predominant metabolite of vitamin B_6 in urine when the vitamin was made available either through food or as supplements of PAL, PIN and PAM (46, 47, 48). Urinary excretion of 4-PA generally accounts for about 30-50% of the vitamin B_6 ingested (49).

The exact site and mode of conversion of PAL to 4-PA have not been fully established. Contractor and Shane identified 4-pyridoxic acid-5- phosphate (4-PAP) in urine and tissues of rats (50). This compound was found in rat tissues following an

intra-peritoneal injection of ^{14}C- tagged PIN. The same authors

observed a buildup of 4-PAP and some 4-PA after the appearance

of PLP in liver, kidney and brain of these rats (51). They observed

that 4-PA was more prevalent in blood than 4-PAP. These findings

led them to propose the following pathway for the catabolism of

PLP in animal tissue: PLP → 4-PAP → 4-PA.

```
        ┌─────────────────────────┐
        │       PYRIDOXINE         │
        └─────────────────────────┘
                     │
              (enters red cells)

              Pyridoxal Kinase
                     │
                     ▼
     ┌───────────────────────────────┐
     │     PYRIDOXINE PHOSPHATE       │
     └───────────────────────────────┘
                     │

                  oxidase
                     │
                     ▼
     ┌───────────────────────────────┐
     │     PYRIDOXAL PHOSPHATE        │
     └───────────────────────────────┘
                     │

                Phosphatase
                     │
                     ▼
        ┌─────────────────────────┐
        │       PYRIDOXAL          │
        └─────────────────────────┘
```

(from red cells to plasma)

Figure 2. Proposed pathway for the conversion of pyridoxine to
           pyridoxal in blood (44).

Urinary 4-PA has been determined in a few investigations as a measure of the metabolism of vitamin B_6. Wachstein suggested that the concentration of this metabolite in urine could be used as a measure of the quantity of vitamin B_6 metabolized in the body (52).

Boxer, Pruss and Goodhart reported that the human body can apparently convert 4-7 mg of vitamin B_6 per day into PLP (53). Higher intakes of the vitamin did not cause any further increase in the PLP content in the blood. According to Baker et al., the vitamin B_6 content stored in the liver was 5-20 μg/g, in muscle 2-6 μg/g and in brain 12-25 μg/g (54). The amount of vitamin B_6 in the whole body has been estimated at 40-150 mg (55). About half of the PLP in the body appeared to be bound to α-glycogen phosphorylase in the muscles (22). The daily turn over of vitamin B_6 was reported to be 2.2-4.4% with a 2-3% depletion of the body's reserves (55).

Human Requirements for Vitamin B_6

Although several attempts have been made to determine the vitamin B_6 requirements of man, data on this are still meager (56). Estimates of the state of vitamin B_6 nutriture in man have been based on the production or cure of clinical signs of the vitamin deficiency. The recommended dietary allowances of vitamin B_6 of normal people of various ages are shown in Table 1.

Table 1. Estimated recommended dietary allowances of vitamin B_6
for various age groups (mg/day).

Individuals	Age Group (yr)	Amount
Infants	0 - 0.5	0.3
	0.5 - 1.0	0.4
Children	1 - 3	0.6
	4 - 6	0.9
	7 - 10	1.2
Adolescents	11 - 14	1.6
	15 - 18	2.0
Adults	19 Upwards	

Source (14)

There has been much concern over the possible increase of

vitamin B_6 requirement of women during pregnancy, particularly

during the last trimester (57). It has been observed that most

pregnant women have decreased levels of vitamin B_6 in blood and

urine (58), lower blood transaminase activities (59) and increased

levels of tryptophan metabolites in urine, particularly following a

tryptophan load test (60).

The placenta seems to actively transport vitamin B_6 to the

fetus to maintain a five-fold concentration gradient favouring the

fetus (61). Because of this, there is a great tendency to develop

deficiency of vitamin B_6 during pregnancy. Studies on the require-

ment of vitamin B_6 during pregnancy have been covered in a recent

review (62). The RDA set by the National Academy of Sciences (14) for pregnant and lactating women is 2.5 mg/day. In a majority of women studied, daily oral doses of 5-10 mg of vitamin B_6 corrected the biochemical signs of deficiency of this vitamin (57). The benefits derived from the higher levels of intake are still not fully understood and require further study.

Increased levels of tryptophan metabolites in urine have also been observed in oral contraceptive users (63). However, the other indices of vitamin B_6 status, namely, blood transaminase activities, plasma PLP, blood and urinary vitamin B_6 levels and urinary 4-PA of the oral contraceptive users were not significantly different from the control group, the non-users of oral contraceptives. This suggests that there may not be an extra demand for vitamin B_6 in women using oral contraceptives.

The establishment of the RDA for vitamin B_6 is complicated by the fact that the requirement appears to be increased when high protein diets are consumed (64, 65). A daily intake of 1.25 mg of vitamin B_6 has been found to be adequate for healthy male subjects on a low protein diet providing 30 g protein per day. On a high protein intake of 100 g daily, 1.5 mg of vitamin B_6 appeared to be the daily minimum while 1.75 to 2 mg was considered optimum (66).

The relationship between vitamin B_6 and protein seems to be critical even at the stage of infancy. According to Filer and

Martinez (67), metabolic requirements for vitamin B_6 could be satisfied at 6 months of age if the vitamin is present in amounts of 20 $\mu g/g$ of dietary protein. The vitamin B_6 requirement of older infants and young children has been estimated to be 0.5 to 1.0 mg/ day (14).

Information on the vitamin B_6 requirement of infants, children and adolescents is limited. However, there has been some evidence to show that requirement increases with age (68). Hamfelt found that plasma pyridoxal phosphate (PLP) levels decreased with increasing age. It is possible that older persons have a reduced absorptive capacity for vitamin B_6 at physiological levels. The normal newborn infant has sufficient tissue stores of vitamin B_6 to meet his needs during the neonatal period (61). The concentration of vitamin B_6 in human milk is approximately 10-20 $\mu g/liter$ during the first month of lactation and gradually increases to 100 μg (56). The daily requirement of vitamin B_6 of infants can be met by adequate consumption of human milk. Clinical abnormalities have appeared in infants when the vitamin B_6 content failed to rise to a level of at least 60-80 $\mu g/liter$ of human milk (61).

In developing countries where marginal or deficient vitamin B_6 intakes are common, children may not develop clinical signs of deficiency (69). This is because the amount of protein in the diet is relatively low and vitamin B_6 requiring enzymes are usually

adapted to a lower level of activity. However, long term deficiency of vitamin B_6 may lead to detrimental effects on physical and mental development.

Data available to make an accurate estimation of vitamin B_6 requirements of older children and adolescents are insufficient. However, the RDA for this group has been set in the range of 1.5 to 2 mg/day (70). Although the vitamin B_6 requirement could usually be met by an adequate diet (5), there may be some individuals who might manifest clinical and biochemical signs of deficiency. This is due to an increased vitamin B_6 requirement or to a disturbance of the vitamin B_6 metabolism in these individuals.

Manifestations of Vitamin B_6 Deficiency

Symptoms of vitamin B_6 deficiency vary greatly with the species and age of the individual (35, 71). A variety of symptoms have been observed in both animals and in humans. Dermatitis or acrodynia is the characteristic sign of a vitamin B_6 deficiency in the rat. In addition to this, poor growth, muscular weakness, fatty livers, convulsive seizures, anemia and nerve degeneration are among the common deficiency symptoms.

The first evidence of vitamin B_6 deficiency in humans was reported by Spier, Bean and Ashe (72). Patients on poor diets who were suffering from weakness, irritability, nervousness, depression

and difficulty in walking were relieved of these symptoms within 24 hours of administration of 50 mg of vitamin B_6. Dietary deprivation of this vitamin in infants results in convulsive seizures, nervous irritability and mental retardation (73). This was evident in approximately 300 infants who received an autoclaved commercial liquid milk formula low in vitamin B_6 (74, 75). Addition of vitamin B_6 to the formula reversed some of the above symptoms. Another study has shown that infants placed on a vitamin B_6 deficient diet for several months ceased to gain weight (76). Their urinary vitamin B_6 and 4-PA were reduced to very low levels.

Symptons of vitamin B_6 deficiency in man have been obtained with human adults placed on diets low in this vitamin (71). Abnormalities of the tryptophan to niacin pathway as indicated by the excretion of xanthurenic acid have been observed in these subjects. Abnormally high excretion of xanthurenic acid and related metabolites of tryptophan were also found in pregnant women (52), as well as in women using oral contraceptives (63). Biochemically, vitamin B_6 deficiency is recognizable by the increased excretion of xanthurenic acid and other tryptophan metabolites in urine, especially following an oral dose of tryptophan (77, 78). This is referred to as the tryptophan load test, which has been a very useful procedure for detecting or in evaluating vitamin B_6 deficiency, particularly in clinical cases and in controlled research studies (79).

Miller and Linkswiler studied the effect of protein intake on the development of abnormal tryptophan metabolism by men during vitamin B_6 depletion (64). After 40 days of depletion, men given 54 g of protein and 0.16 mg of vitamin B_6 excreted 0-29% of the 2 g load of L-tryptophan as the metabolites. In contrast, men given 150 g of protein and 0.16 mg of vitamin B_6 excreted 29% of the tryptophan load as the metabolites after 14 days of depletion. However, with a daily vitamin B_6 intake of 1.5 mg the excretion of tryptophan metabolites was normal whether protein intake was 54 or 150 g. Canham et al. studied the rapidity of onset and the severity of the biochemical manifestations of vitamin B_6 deficiency with differing protein intake. They found a direct relationship between these two conditions (65).

The association of vitamin B_6 deficiency with a form of microcytic hypochromic anemia has been reported (80). A case study on pyridoxine-responsive anemia was reported by Horrigan (81). In a man, 54 years old, anemia which was followed for 18 years had been unaffected by folic acid, vitamin B_{12}, multivitamin preparations containing niacin, riboflavin and thiamin; but responded to 12.5 mg of pyridoxine hydrochloride (PIN-HCl) given by mouth daily for 5 days. Keyhani et al. studied erythropoeisis in pyridoxine-deficient mice and observed that the mice deprived of vitamin B_6 developed progressive anemia characterized by hypochromia, microcytosis,

reticulocytosis and erythroid hyperplasia of bone marrow and spleen (82).

The effect of pyridoxine deficiency on some aspects of carbohydrate metabolism in rats was investigated by Angel and Mellor (83). Vitamin B_6 deprivation decreased liver and muscle glycogen content and the activity of glycogen phosphorylase in both tissues. They concluded that alteration of amino acid metabolism due to vitamin B_6 deficiency may have limited the supply of carbon for gluconeogenesis, thereby restricting the availability of glucose for glycogenesis. Along the same line, Angel and Song studied the effect of pyridoxine deficiency on lipogenesis, and showed that there was decreased availability of glucose for lipogenesis in vitamin B_6 deprived rats (84). As mentioned earlier, vitamin B_6 plays a role in lipid metabolism. The effect of vitamin B_6 on the fatty acid composition of liver (25) and phospholipids (85) has been studied. DeLorme and Lupien observed a decrease in the proportion of arachidonic acid and an increase in the linoleic acid in the major phospholipids in the rats deprived of vitamin B_6 (85). A similar trend was observed by Dussault and Legape in the liver fatty acids (25). The exact role vitamin B_6 plays in these reactions is not clear.

Vitamin B_6 deficiency seems to be involved in a number of physiologic conditions. The formation of renal calculi (86) and

increased tendency for dental caries (87) are two examples.

Calhoun, Jennings and Bradley reported that rats receiving a vitamin

B_6 deficient diet and a sulfa drug (phthalylsulfathiazole) exhibited

hematuria associated with abnormally high oxalate excretion via

kidney (88). Pyridoxine administration prevented both conditions

and it was suggested that oxalate excretion in urine may be the

result of the improper utilization of the amino acid, glycine. The

first human study on this aspect of vitamin B_6 was done by Gershoff,

Mayer and Kulozycki in mongoloid and non-mongoloid children (89).

They observed a marked reduction in urinary excretion of oxalic

acid when pyridoxine was given.

Supplementary vitamin B_6 has been found to reduce dental

caries in experimental animals and in humans (90). Strean suggested

that a deficiency of vitamin B_6 in the diet might render teeth more

susceptible to dental caries and addition of this vitamin to the diet

could increase resistance probably through a change in oral flora

(91). He reported that it may be linked to the essentiality of vitamin

B_6 as a nutritional requirement for heterofermentative bacteria and

the non-essentiality of this vitamin for the homofermentative type.

Inborn errors of metabolism resulting in vitamin B_6 dependency

or change in the metabolism of this vitamin have been studied (92).

The symptoms of vitamin B_6 dependency are limited to the central

nervous system and include hyperirritability, convulsions and

electroencephalographic changes (93). It has been proposed that an inborn or acquired abnormality changes the binding capacity of the specific vitamin B_6 dependent enzymes to the cofactor. Symptoms of vitamin B_6 dependency have been controlled by administration of large doses of pyridoxine over and above the requirements of the individual in order to overcome the adverse binding kinetics.

There are a number of compounds which are structural analogs of vitamin B_6 group members which function biochemically as antagonists interfering with normal functions of the vitamin and resulting in manifestations of vitamin B_6 deficiency (94). Some commonly used drugs are known to promote vitamin B_6 deficiency through inhibition of coenzyme synthesis or by chemical inactivation of PLP, or by competitive displacement of PLP by the structural analogs (95). Among these drugs are isoniazid, D-penicillamine and deoxypyridoxine. Isonicotinic acid hydrazide (INH) or isoniazid is a common therapeutic agent used in tuberculosis therapy (96). Deoxypyridoxine, once used in the treatment of cancer, competes with PLP by displacing the coenzyme in a number of different enzymes (94). D-penicillamine is used in pathological conditions such as Wilson's disease, cystinuria, certain types of heavy metal intoxication and rheumatoid arthritis (97). Levy observed increased excretion of microbiologically active forms of vitamin B_6 during administration of high levels of vitamin B_6 antagonists (98).

Excretion of Vitamin B_6

Evaluation of vitamin excretion in relation to intake has been used as an approach to the assessment of requirements for B vitamins (99). Such balance studies are also valuable in assessing availability of nutrients (100). Earlier studies on vitamin B_6 metabolism in human subjects were reported by Denko et al. (101); Johnson, Hamilton and Mitchell (102); Rabinowitz and Snell (47). However, a complete balance study of vitamin B_6 and its known metabolites on human subjects was first undertaken by Linkswiler and Reynolds (48). Three levels of vitamin B_6 intake were evaluated in terms of the sum of fecal and urinary B_6 excretion as well as excretion of urinary 4-PA, the major urinary metabolite of vitamin B_6. They used nine human subjects and observed that vitamin B_6 elimination exceeded the amount given in the diet. The probable reason for this according to these authors was a synthesis of vitamin B_6 by intestinal flora.

With a basal diet containing 0.78 mg of vitamin B_6 per day, subjects studied by Linkswiler and Reynolds eliminated a total of 3.54 mg equivalent of the vitamin, of which about 75% was accounted for by 4-PA (48). The sum of fecal and urinary vitamin B_6 excreted was almost equal to or slightly in excess of the amount of vitamin B_6 ingested in the basal diet. The results of Denko et al. showed a

somewhat different trend (101). They observed an average daily excretion of 1.01 mg as urinary and fecal vitamin B_6 on a diet containing 1.76 mg of vitamin B_6 per day. Vitamin B_6 in urine and feces accounted for only 57% of the intake.

Yano and Fujita studied the synthesis of vitamin B_6 by intestinal bacteria as affected by cellulose (103). They used three types of diet, vegetable, meat and mixed. Filter paper served as the source of cellulose. They observed that about 90% of the sum of urinary vitamin B_6 and 4-PA was made up of 4-PA. Because of this, they measured only 4-PA as the urinary excretory produce of vitamin B_6. They observed an increase in the excretion of fecal and urinary vitamin B_6 when the subjects were fed a vegetable diet. On the basis of amount excreted in relation to the intake, they concluded that vitamin B_6 synthesized in the intestine increased markedly with the vegetable diet as compared to the meat diet in which a decrease in the above parameters was observed. With the addition of cellulose to either diet, there was an increase in the total vitamin B_6 excreted. Their findings support the idea that addition of cellulose to a diet increases the synthesis of vitamin B_6 by intestinal microflora. They also reported that the synthesized vitamin B_6 was absorbed into the system, as reflected by the increased excretion of 4-PA in the urine.

The methods of 4-PA determination used by Linkswiler and Reynolds (48) and Yano and Fujita (103) were those of Huff and Perlzweig (46) and Fujita and Fujino (104), respectively. Reddy, Reynolds and Price (105) and Woodring, Fisher and Storvick (106), in their contribution to the 4-PA methodology, pointed out the unreliability of the older methods, mainly due to the incomplete elimination of fluorescent substances other than 4-PA. Thus, the older methods would lead to an overestimation of 4-PA.

In a study on drug-induced vitamin B_6 deficiency, Levy observed that his subjects were in positive balance for vitamin B_6 (intake exceeding excretion) during control as well as the experimental period (98). He determined urinary and fecal 4-PA by the method of Woodring, Fisher and Storvick (106). Although the primary route of excretion of 4-PA was through the kidney (2 μM/24 hr), there was a small amount of 4-PA in feces (0.3 μM/24 hr).

Average values of urinary 4-PA for healthy women consuming self-selected diets under normal conditions have been reported to range from 3.8 to 6.9 μM/24 hr (106). Urinary excretion of 4-PA by men receiving a constant daily intake of 1.66 mg of vitamin B_6 was 1.01 $\pm$ 0.32 mg (5.52 $\pm$ 1.75 μM) per 24 hr (107). In subjects who received 1.57 mg vitamin B_6 from a constant diet of natural foods, the amount of 4-PA excreted ranged from 3.9 to 4.2 μM/24 hr (105). Contractor and Shane reported that men and women

excreted an average of 7.21 ± 4.26 and $6.61 \pm 4.64 \,\mu M$ of 4-PA/24

hr, respectively (58). The male and female subjects studied by

Mikac-Devic and Tomanic excreted 3.6 to 7.8 and 3.1 to 5.3 μM

4-PA per day, respectively (108). Based on such studies, Sauberlich,

Skala and Dowdy suggested 3-7 μM/day as normal range for urinary

excretion of 4-PA which would generally reflect about 30-50% of

the daily vitamin B_6 intake (49).

There seems to be a number of dietary factors that influence

the excretion of vitamin B_6 and its metabolites. The role of cellulose

was discussed by Yano and Fujita (103). In a review of the effect

of different carbohydrates on vitamin and amino acid requirements,

Harper and Elvehjem have discussed that when a less soluble carbo-

hydrate is substituted for a more soluble one in the diet, the require-

ments for most members of vitamin B complex and for essential

amino acids decrease (109). They reported that the effect of complex

carbohydrates in lowering the requirement for B vitamins was related

to changes in the intestinal microflora. Sarma, Snell and Elvehjem

observed a higher growth rate in rats when dextrin was used in

place of sucrose in a pyridoxine-deficient diet and concluded that it

was due to synthesis of vitamin B_6 by the intestinal microflora in

the presence of dextrin (110). With the diet containing dextrin they

also found an increase in the amount of urinary 4-PA. These findings

were similar to those of Yano and Fujita who studied the effect of

cellulose on the synthesis of vitamin B_6 by human intestinal bacteria (103).

The effect of ascorbic acid intake on the urinary excretion of 4-PA was studied by Selivanova (111); Selivanova, Agasin and Poljakova (112). On basal diets containing a mean value of 8.8 $\pm$ 0.6 mg of vitamin C per day, 38 men excreted an average of 40.6 μg of 4-PA/hr (range 0-161.9) in their urine collected before breakfast. After receiving a supplement of 100 mg of vitamin C daily for almost 50 days, they showed a significant increase in the urinary 4-PA, with an average of 234.6 μg/hr (range 66.4-456.5). The authors concluded that, as the urinary excretion of vitamin C increased as a result of increased vitamin C intake, the urinary 4-PA also increased.

Under normal conditions the level of urinary 4-PA has been recognized to reflect the intake of vitamin B_6 (47). In some cases of abnormal vitamin B_6 metabolism, urinary 4-PA drops considerably and cannot be used as an index of vitamin B_6 intake (93). Extensive studies have not been conducted to investigate the influence of graded intakes of vitamin B_6 on the excretion of 4-PA (49). Sauberlich et al. therefore feel that measurement of 4-PA, at present, cannot be recommended as a reliable index of evaluating vitamin B_6 nutrition especially in clinical cases (49). Levels of 4-PA, as with levels of urinary vitamin B_6, probably provide information on the

immediate dietary intake of the vitamin and may not reflect the body reserves (79).

Although the vitamin B_6 requirement of the human is dependent on the level of protein consumed, dietary protein seems to have little effect on the urinary excretion of vitamin B_6 (65). Vitamin B_6 in urine can be measured by several methods (113, 114). Of these, microbiological assays, using Saccharomyces carlsbergensis as the test organism, have been commonly employed.

The free forms of vitamin B_6 in urine can be measured by its ability to support growth of S. carlsbergensis (115). The bound or phosphorylated forms can be converted to the free form by acid hydrolysis or urine and the total vitamin B_6 measured by the same microbiological method.

Kelsay et al. studied the effect of vitamin B_6 intake on urinary excretion of vitamin B_6 in adult males (107). A low level of urinary vitamin B_6 was noticed in subjects when they received only 0.16 mg of the vitamin daily. Continuation of this level of intake for several days results in excretion levels of the free vitamin of 20-40 µg/24 hr, compared to 80-140 µg prior to depletion when the subjects received a daily supplement of 1.5 mg PIN. Donald et al. obtained somewhat similar results in young women (116). These subjects excreted a daily average of 61 µg free vitamin B_6 on a normal diet. When they were given a diet containing 0.34 mg of vitamin B_6 per

day for 43 days, their levels of urinary vitamin B_6 dropped to 15.1

µg. On increasing the daily intake to 0.94 mg, the urinary vitamin

B_6 rose to 34.1 µg per day, after 10 days. When the intake level

was further increased to 1.5 mg, the excretion of vitamin B_6 in

urine increased to 55.8 µg which was close to the original level of

61 µg/24 hr observed by Donald et al. With a supplement of 30 mg

PIN-HCl, urinary vitamin B_6 in the same subjects reached a level

as high as 75.5 µg/24 hr. In healthy female subjects on normal diets,

Kokkeler observed an excretion of 83 ± 60 and 136 ± 73 µg/24 hr,

as free and total vitamin B_6, respectively (117).

Sauberlich et al. reported a urinary excretion of 35-55 µg of

free vitamin B_6 per day, or 20 µg/g creatinine, as being normal for

individuals with a vitamin B_6 intake of approximately 1.5 mg daily

(49). Urinary B_6 levels less than 20 µg/g creatinine are considered

to reflect inadequate dietary intakes of the vitamin (79). In cases

where collection of a 24 hr urine is not practical, fasting urine

samples have been used. In such instances the results are generally

expressed per gram of creatinine.

Little information is available on the distribution of PAL,

PIN and PAM in urine. One reason for this may be the lack of

methods sensitive enough to determine these forms (107). The dif-

ferential microbiological assay of Rabinowitz and Snell resulted in

low recoveries of pyridoxine added to human urines (47). Fujita and

Fujino proposed a fluorometric method following chromatographic

separation of the forms and oxidation of these to 4-pyridoxic acid

(104). However, poor reproducibility of the method as well as

difficulty in converting the different forms into 4-pyridoxic acid and

the lactone have set limitations on this method.

Toepfer and Lehmann reported on a method for assay of the

three forms of vitamin B_6 in foods, in which chromatographic

separation (using Dowex 50 ion exchange resin) into different forms

was followed by microbiological assay using S. carlsbergensis (118).

Their method was later improved (119) and is established as the

AOAC method (120).

Kelsay, Baysal and Linkswiler observed that PAL was excreted

in the largest amount when their subjects were on self-selected

diets or when experimental diets were supplemented with 1.5 mg

PIN-HCl (107). When assayed by the method of Toepfer and Lehmann

(118), PAL accounted for approximately 65% and PAM about 30% of

the urinary vitamin B_6, with only a negligible amount excreted as

PIN. The percentage of PAL, PIN and PAM in urinary vitamin B_6

reported by Contractor and Shane was 13.69, 23.37 and 62.73,

respectively (58). They used a phosphocellulose column to separate

the free and phosphorylated forms of vitamin B_6 as well as 4-PA,

followed by fluorometric determination of the components. The

difference in the percentages of PAL, PIN and PAM reported by Contractor and Shane (58) and Kelsay et al. (107) may be due to the difference in the methods of separation and determination. Further studies using standardized techniques will help in obtaining a clear picture of the distribution of the three forms of vitamin B_6 in urine.

The percentage of dietary vitamin B_6 reflected in the urinary excretion of the vitamin is very small (98, 103). However, when evaluated together with other excretory products, this may help in assessing the availability of the vitamin ingested.

Studies on Bioavailability of Vitamin B_6 and Other Nutrients

There is a growing interest in assessing the biological availability of nutrients from foods. Several reports have appeared on iron with reference to wheat and enriched bread (121, 122, 123, 124), and other food products (125, 126, 127, 128, 129). Many of these studies employed response of blood hemoglobin, hemoglobin repletion and tissue iron levels to assess the bioavailability of iron.

The bioavailability of iron from sources commonly used in bread enrichment was studied in anemic rats fed enriched bread containing 20 ppm iron (121). Based on the amount of hemoglobin repleted in 30 days, the availability of iron from different sources as well as at different stages in breadmaking was determined. The authors reported that fermentation and baking steps caused some increase in the biological availability of iron, which could probably be due to a breakdown of phytic acid or to a denaturation of iron binding sites or both.

Miller used total hemoglobin, hematocrit, red blood cell count, ceruloplasmin, serum iron, iron and copper levels in tissues and carcasses to assess the utilization of iron from enriched white bread by normal and anemic rats (124). The anemic group was provided with iron by addition of either ferrous sulfate or iron-enriched bread to their diets. Bread appeared to stimulate the production of red blood cells to a greater extent even though iron was less available from bread than from ferrous sulfate for the synthesis of hemoglobin or for storage in solid tissues. One possible interpretation was that bread contained some component which specifically stimulated the production of erythrocytes from erythroblasts in anemic rats.

Bioavailability of magnesium from wheat flour and various organic and inorganic salts was investigated by Ranhotra, Loewe and

Puyat (100). They based their assessment on the apparent absorption,

using fecal excretion and tissue concentration of magnesium. Their

results demonstrated that magnesium was equally available from

all sources tested. de Muelenaere, Chen and Harper assessed the

availability of lysine in cereal products using growth and fecal

analysis methods (130). They found that lysine of corn and rice

protein was highly available. Bioavailability of sulfur amino acids

in corn protein was demonstrated by Sasse and Baker using slope-

ratio technique and a standard curve method (131).

Documented reports on the availability of vitamins are rather

limited. Pelletier and Keith studied the bioavailability of synthetic

and natural ascorbic acid, utilizing cumulative urinary excretion

and serum levels as indices for comparison (132). A similar tech-

nique was used by Tamura and Stokstad to study the availability of

food folate in man (133). The similarity in absorption of vitamin C

and folic acid from orange juice and a synthetic source was reported

by Nelson, Streiff and Cerda (134). They used the method of triple

lumen perfusion of the human small intestine to determine the rate

and amount of absorption of the vitamins.

In a recent study, using the same intestinal perfusion method,

Nelson, Lane and Cerda showed that the mean vitamin B_6 absorption

was significantly greater from a synthetic than from a natural source,

which was orange juice (135). They used a triple lumen tube with a

30 cm study segment to determine the uptake of vitamin B_6 in 15

normal subjects. The intestinal absorption was expressed as $\mu g/$

cm/hr. They also compared, in six subjects, the absorption of

vitamin B_6 from orange juice with that of the synthetic source with

and without added glucose. Although they observed some variation

in the absorption of water from these three sources, the difference

between orange juice and solution containing glucose was not

significant. Water absorption from the vitamin solution without

glucose was significantly lower than that of either of the other two.

Despite the fact that absorption of water was quite high from orange

juice, the mean percent absorption of total vitamin B_6 from this

source was significantly lower than that from the synthetic sources.

This indicates that factors which promote water transport in the

intestine may not necessarily increase the biological availability of

water soluble vitamins from food sources.

Bioavailability of vitamin B_6 from food sources has been studied

in animal models. Lantz found that cooking of pinto beans increased

the availability of vitamin B_6, suggesting that it may naturally exist

in a bound form which is released on cooking (136). Yen, Jensen

and Baker assessed the bioavailability of vitamin B_6 in corn and

soybean using day old chicks (137). A standard curve for vitamin B_6

response was prepared using diets containing increasing levels of

crystalline PIN-HCl, for which availability was assumed to be 100%.

They examined the growth patterns of the chicks as well as the plasma and serum glutamic oxalate transaminase (SGOT) activities. The former was found to be more sensitive than the latter, as an indicator of the availability of vitamin B_6 from these food sources. Their results suggested that corn was significantly lower in available vitamin B_6 content than soybean. Dry roasting of corn at 160°C significantly reduced the availability of the vitamin compared to roasting at 80 and 120°C. Similarly, autoclaved full fat soybean had a lower concentration of available vitamin B_6 than did the soy-meal.

Tissue transaminase activities and vitamin B_6 levels were studied by Thiele and Brin as indices of vitamin B_6 availability (138). They fed male rats for 14 days on a diet devoid of vitamin B_6, with and without daily supplements of 5 or 15 μg of PAL, PIN and PAM and observed that large amounts of each form gave higher concentrations of total vitamin B_6 and transaminase activities in liver, kidney, brain, muscle and heart of the rats that received vitamin B_6 compared to those that did not receive vitamin B_6. Their data also indicated that all the vitamers appeared to be equally available to the rats.

Vitamin B_6 in Foods

Vitamin B_6 in food occurs as a group of related compounds in

the free forms, PIN, PAL and PAM or chemically bound forms, PLP

and PMP (139). Distribution of PIN, PAL and PAM in some natural

products was first reported by Rabinowitz and Snell (140). Since

then several methods have been developed for the determination

of the three forms of vitamin B_6. Chemical procedures proposed

for the determination of vitamin B_6 in foods have mainly been based

on fluorometric methods (141, 142, 143). Microbiological methods

based on the growth of organisms are also widely used (144, 145).

Toepfer and Lehmann described a technique to separate PIN, PAL

and PAM in foods using ion exchange chromatography (118). The

components were then assayed by microbiological method.

Bioassay techniques have also been used to evaluate the

activity of vitamin B_6 from different foods (146, 137). A micro-

biological method has been widely used in analysis of food for

vitamin B_6. A semiautomated system for microbiological vitamin

assays has been attempted (147), and application of this in deter-

mining vitamin B_6 would considerably improve the efficiency of the

method.

Most of the data reported in literature on the vitamin B_6 con-

tent of foods have been obtained using microbiological methods.

Saccharomyces carlsbergensis has been generally used for the

determination of vitamin B_6 activity in foods (120). The bound forms

of the vitamin are inactive for this microorganism (10, 148). The

response of the microorganisms to vitamin B_6 requires prior hydrolysis of most products assayed. Different hydrolysis procedures have been discussed in the literature (10, 149). These procedures vary depending on whether a food product is of animal or plant origin (120). Plant products generally need to be autoclaved with 0.44 $\underline{N}$ HCl for 2 hr at 121°, whereas those of animal origin require autoclaving with 0.055 $\underline{N}$ HCl for 5 hr at the same temperature.

The forms PAL and PAM are predominant in foods of animal origin while the major form in plant products is PIN (150, 151). Although vitamin B_6 is widely distributed in foods, some foods are richer sources of this vitamin than others. Muscle meat, liver, legumes, whole grain cereals and especially the bran from cereal grains are among the best sources of vitamin B_6 (152). The vitamin B_6 content of foods and the distribution of the three forms are presented by Orr (5).

Most of the whole grain products contain 2-4 µg of vitamin B_6 per gm and are considered better sources of vitamin B_6 than refined products which contain less than 1 µg/g (4). Polansky and Toepfer reported that durum wheat contained more vitamin B_6 (4.3 µg/g) than hard or soft wheats (3.4 µg/g) (4). Whole wheat flour was analyzed in the collaborative study of vitamin B_6 methodology (145). Among the 13 laboratories that participated, the levels of vitamin

B_6 determined varied from 1.64 to 4.55 µg PIN-HCl per g, with an average of 3.17.

Pyridoxine accounts for about 2/3-3/4 of the total vitamin B_6 in wheat while PAL and PAM together form the remaining 1/3-1/4 (153). Vitamin contents of air-classified high and low protein flour fractions were determined by Jones, Fraser and Moran (154). Their data indicated that vitamin B_6 levels in the fine fractions were higher in hard than in soft flours. The same fractions were also reported to be proportionately higher or lower in protein, suggesting that vitamin B_6 may be bound to the proteins. Vitamin B_6 content of wheat bran has been reported to be quite high, around 13.7 µg/g whereas that of whole grain was 3.7. In the patent flour the level was as low as 0.5 µg/g (155). Harris reported that vitamin B_6 content of whole wheat is under genetic control (156). Because of this and of the different effects of various milling practices, the vitamin B_6 content of different flours at any one extraction rate may not be the same. The mechanical process of milling and separation of various components of wheat grain have a definite effect on vitamin B_6 level of the flour as indicated by the data of Polansky and Toepfer (155). The loss of vitamin B_6 during milling of wheat into refined flour was reported to be around 85% (6). To combat this, as well as for the reasons discussed later in this review of literature, the

National Academy of Sciences has proposed addition of vitamin B_6, along with some other nutrients, to white flour (9).

Cereal Enrichment Program

Cereals make an important contribution to the nutrients of the world's food supply. Cereal products are also considered suitable carriers for the addition of nutrients to the diet of many people (9). There is evidence of potential risk of deficiency of certain nutrients among some segments of the population such as low income groups and pregnant women. Because of this, the Food and Nutrition Board of the National Academy of Sciences has recommended broadening the cereal enrichment program that was started in early 1940s (8).

The nutrients recommended for enrichment of cereal products were selected mainly on the basis of their role in meeting the needs of significant groups of people, susceptible to nutritional deficiencies (157). The currently used nutrients and their levels as revised recently to be effective from 1977 (158), as well as those proposed by the National Academy of Sciences (9), are reported in Table 2.

Among the cereal products, about 30% of those consumed in the world and 82% of those in the U.S. are reported to be of wheat origin (1, 159). Breads made from wheat flour are consumed in almost every country of the world and would serve as a suitable carrier for added nutrients.

Table 2. Enrichment levels for cereal products.

Nutrient	Presently used[a] mg/lb	Proposed[b] mg/lb	mg/100 g
Thiamin	1.8	2.9	0.64
Riboflavin	1.1	1.8	0.40
Niacin	15	24.0	5.29
Iron	25	40.0	8.81
Calcium	600	900.0	198.20
Vitamin A	-	2.2	0.48
Vitamin B_6	-	2.0	0.44
Folic acid	-	0.3	0.07
Magnesium	-	200.0	44.10
Zinc	-	10.0	2.20

[a] Federal Register, 1976 (158)

[b] National Academy of Sciences, 1974 (9)

A Federal Standard of Identity for enrichment of white flour was first established and made effective on January 1, 1942 (8). This was amended in 1943 to provide the standards, to enrich flour with thiamin, niacin, riboflavin and iron with the option of including calcium and vitamin D (160). Recently the Standard of Identity of baked food was revised to establish new levels of enrichment (158).

The proposed level of enrichment for vitamin B$_6$ is 2 mg

(pyridoxine) per pound or 0.44 mg per 100 g of flour (9). Pyridoxine

hydrochloride (molecular weight 205.6) is the commonly available

synthetic form of the vitamin. It occurs as white platelets and is

readily soluble in water (35).

Although cereal enrichment program appears to be technically

feasible, it is recommended that studies be conducted to determine

the uniformity of distribution of the nutrients, their stability during

preparation and storage, the availability of the nutrients and the

consumer acceptance of the foods prepared from these cereals.

Stability of Vitamin B$_6$ and Other Nutrients during Food Processing

Much attention has been paid to vitamin B$_6$ studies in humans

in the recent years. This can be attributed to a number of factors,

one of which is the loss of vitamin B$_6$ during food processing (49).

Exposure to direct sunlight, diffused daylight or artificial light

causes inactivation of the three forms of vitamin B$_6$, with the

direct sunlight producing the greatest destruction (161). For this

reason, during its analysis in the laboratory, precautions are taken

to avoid exposure to light of the samples containing vitamin B$_6$

(149).

Of the three forms, PIN is quite stable in acid solutions, but rapid destruction by light and heat occurs at neutral or alkaline pH (162, 163). Pyridoxal and pyridoxamine are reported to be relatively less stable than pyridoxine (164).

About 90% of the vitamin B_6 in fresh milk was reported to be PAL, both free and as the phosphate (140). Heating and storage losses of this vitamin in milk and in infant formula were high (165). Added PIN-HCl was much more stable in infant formula than vitamin B_6 in milk under the same processing conditions. Spray-drying caused some loss of vitamin B_6, but less than that observed during sterilizing of liquid milk. Davies, Gregory and Henry reported a substantial loss of vitamin B_6 (45-70%) during processing of evaporated milk. Additional losses were observed when this milk was stored 6 months at room temperature (166).

The effect of the processing method on vitamin B_6 retention was reported by Everson et al. (167). Percent retention of this vitamin in bean, beef, and tomato juice when processed by conventional method was 86.7, 94.2 and 100, respectively. When processed by high temperature short time (HTST) method and canned aseptically, the corresponding retention figures were 93.0, 98.3 and 100%. In the conventional method, heat transfer is very slow particularly when the products are non-liquid. Since heat penetration into the product is through the periphery, the material close to

the edge gets more drastic heat than required to achieve sterility. The HTST method and aseptic canning are receiving increasing attention due to better retention of nutrients in the products processed this way (168).

Raab, Luh and Schweigert studied the effects of heat processing on the retention of vitamin B_6 in lima beans (169). Assuming the vitamin B_6 retention of dry beans at 100%, they observed 76-81% and 83-87% retentions of vitamin during water and steam blanching, respectively. Although steam blanching may have had improved retention, the results were not significantly different from those of water blanching.

Several reports have appeared on the vitamin retention of different meats during processing and storage. Vitamin B_6 is considered fairly stable during frozen storage (170). About 82% retention in beef liver and 77% in boned chicken were observed. Lushbough, Weichman and Schweigert reported 42-67% retention of vitamin B_6 in meat after cooking, with 1-13% in the drip (171). High loss of vitamin B_6 has been reported during heat processing and irradiation of boned chicken (170). However, the retained vitamin appeared to be stable on storage as long as 15 months at 24-27°C.

The amount of vitamin retained seems to vary with the type of meat and the method of cooking. Meyer, Mysinger and Wodarski did vitamin B_6 retention studies on oven-roasted beef loin and oven

braised beef round (172). The retention in the loin averaged 72% with 16% in the drip; round averaged 49% with 34% in the drip. Microwave heated chicken breasts retained more vitamin B_6 (on a drip weight-basis) than conventionally roasted meat (173). Bowers, Fryer and and Engler observed a similar pattern with turkey breasts and suggested that higher percentage of retention for microwave cooking may be due to greater loss of moisture by this method than by conventional heating (174). The same authors reported that on a cooked weight basis, conventional and microwave heated pork differed little in vitamin B_6 content (175). On a dried weight basis, however, they observed more retention of this vitamin in conventionally heated muscle.

Studies on some of the B vitamins in cereal products have been reported since the 1940s. Early work was done mainly on thiamin, niacin and riboflavin. Meckel and Anderson studied thiamin retention and composition of four types of U.S. army breads made from enriched white flour (176). Thiamin losses varied from 14 to 24% depending on the nature of the loaf and the treatment to which it was subjected. There was no loss of thiamin, riboflavin and niacin in the field type of loaf after storing at room temperature for as long as two weeks.

Stability of these three vitamins during fermentation, baking and storage of canned bread was investigated by Brenner, Dunlop and Wodicka (177). Riboflavin and niacin were retained almost completely during fermentation, baking and storage. Thiamin however, was decreased by approximately 15% during baking and 20-50% during 6 months storage at 72° and $100^{\circ}F$, respectively.

They further noted that protein quality of whole wheat bread was significantly superior to that of enriched white bread.

Loss of thiamin in toasting of breads made from whole wheat, enriched and unenriched white flour was studied by Downs and Meckel (178). The percent loss of thiamin in toast made from unenriched white flour was higher than either of the other two types. They thought that this apparent loss may have been due to a discrepancy introduced by the assay method and the difficulty in accurately measuring small concentrations.

Maleki and Daghir reported the effect of baking on retention of thiamin, niacin and riboflavin in both white and brown arabic bread (179). Their data suggested that loss of thiamin was greater in brown bread than in white bread; loss of riboflavin was similar in both types and there was only a negligible loss of niacin in the two breads. They also observed a higher retention of riboflavin in vitamin enriched samples compared to unenriched samples. Niacin was found to be stable in both enriched and unenriched samples.

Morgereidge studied the retention of vitamins in enriched bread under practical retail conditions to determine the effect of transparent versus semi-opaque wrapping material (180). He showed that regardless of the type of wrapper, there was no loss of thiamin, riboflavin and niacin when bread displays were subjected to normal intermittant illumination for periods up to five days.

A recent investigation on stability of native and added folic acid in flour during bread processing and storage was done by Keagy, Stokstad and Fellers (181). Native folic acid in flour was stable when stored at 84^OF with 86% retention after 12 months, but showed progressively increasing losses as the temperature increased. Retentions of 78% in 4-5 months at 100^OF and 62% in 1 month at 120^OF were reported. Synthetic pteroylglutamic acid added at levels of 1 or 5 μg/g of flour showed very small losses. Average baking losses of 11% for added and 31% for native folacin were observed. They also reported that folacin synthesis by yeast during fermentation made up for the loss of native folacin during bread processing. According to these investigators, fermentation time was a major factor in determining the final native folacin content of bread.

Good recoveries of PIN-HCl added to flour and baked into bread were reported by Hennessy et al. (182). They have presented vitamin B_6 values obtained by both fluorometric and microbiological methods. In the former method, only PIN responded, whereas all three forms of vitamin B_6 were accounted for in the microbiological method. However, the results obtained by these two methods agreed relatively well. This could result because about 3/4 of the vitamin in wheat is in the PIN form (153). Good stability of added vitamin B_6 to flour during baking and storage was reported in a recent study

by Cort et al. (183). They baked regular yeast-risen bread using flour fortified with vitamins including vitamin B_6. Vitamin B_6 was stable during baking and after 5 days of storage of bread at room temperature. The breads baked from vitamin-fortified flour stored four months at room temperature also had a vitamin B_6 level similar to that of the regular loaf baked from freshly enriched flour.

Stability of vitamin B_6 added to corn meal and macaroni was studied by Bunting (184). He observed excellent stability of the vitamin during storage and cooking. In storage 90-95% of the added plus natural vitamin B_6 was recovered from enriched corn meal, and 100% from enriched macaroni after one year at $100^{\circ}F$ and 50% relative humidity. During the baking of corn bread made with enriched and unenriched corn meal, almost 100% of the vitamin B_6 was retained. After macaroni was cooked, about 50% of the natural and added vitamin B_6 remained in the solids while 50% leached into the cooking water. It was suggested that these percentages should be presented on the packages of the uncooked products in order to fulfill the Federal Standards of Identity.

Dry roasting of corn to $160^{\circ}C$ significantly reduced the concentration of available vitamin B_6 compared to the samples in which the roasting temperature did not exceed 80 and $120^{\circ}C$ (137). Some loss of heat susceptible components of vitamin B_6 may have occurred when heated to the highest temperature of $160^{\circ}C$. Corn has 82-91%

PAL and PAM which are more susceptible to heat than PIN (153).
This may be the reason for greater loss of vitamin B_6 observed at
$160^{\circ}C$ by Yen, Jensen and Baker (137).

There seems to be a host of factors which determine the
stability of vitamin B_6 during food processing. The predominant
form of the vitamin and the temperature of processing appear to be
of particular importance.

MATERIALS AND METHODS

Stability

Preparation of Breads

Breads were prepared using whole wheat (WHW), white

enriched with vitamin B_6 (WB_6) and white (W) flour, under both

commercial and home conditions. Commercial breads were baked

on a large scale at a local bakery (Albertson's, Corvallis). These

breads were used in assessing the bioavailability of vitamin B_6 in

human subjects (discussed later). Brominated bakers patent flour[1]

was used in commercially prepared white breads, WB_6 and W, and

hundred percent whole wheat flour in the WHW bread. These breads

were prepared by a straight dough type method with formulae gen-

erally used in the bakery. Details are given in Appendix 1. Com-

mercially baked breads were machine slided and packaged in

polyethylene bags to protect against desiccation, packed in cardboard

boxes and stored in a freezer ($-40^{\circ}C$) until used.

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[1] Brominated bakers patent flour provided courtesy of Centennial Mills, Portland, Oregon.

Straight-dough and sponge-dough methods were compared under home conditions. All purpose flour[2] and 100% whole wheat flour[3] were used in home style baking. The flours were purchased from a retail market. Straight dough breads were prepared using the Finney and Barmore formula (185), with the slight modification of replacing 2% compressed yeast with 1% active dry yeast (on flour basis). Sponge dough breads were made by the approved method of American Association of Cereal Chemists (186) with the omission of yeast food. The quantities of the ingredients were kept at the same levels as in the case of straight dough formula. Details of the formula and procedures are given in Appendix 2. The major difference between these two methods was in the time of fermentation and proof. These times were 235 min and 330 min for the straight dough and sponge dough methods, respectively.

Crystalline pyridoxine-monohydrochloride[4] was used as the source of pyridoxine in the enrichment of WB_6 breads. The level of enrichment recommended by National Academy of Sciences (9), 2 mg

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[2] All purpose flour was unbleached and enriched with thiamin, riboflavin, niacin, and iron levels indicated in Appendix 1, product of Centennial Mills, Portland, Oregon.

[3] 100% whole wheat flour, product of Fisher Mills, Inc., Seattle, Washington.

[4] Pyridoxine monohydrochloride, Lot number 501655, Calbiochem, San Diego, California.

pyridoxine per pound of flour was used in the WB_6 breads prepared under home conditions. In the case of commercially prepared breads, WHW and W were baked first and their vitamin B_6 levels were determined before the level of enrichment for WB_6 bread was arrived at. A level of only 1.12 mg pyridoxine per pound of flour was used in the commercially prepared WB_6 bread. This amount was used to attain a level of vitamin B_6 in the commercial WB_6 bread close to that of WHW, such that the quantities of the breads given to the human subjects in the bioavailability study would be similar.

During the course of bread making, representative samples were taken in triplicate at three stages; after mixing all the ingredients (mix), at the end of proof just before baking (proof) and after baking (bread). These were analyzed for vitamin B_6 and moisture content. Samples of mix and proof taken for vitamin B_6 analysis were weighed and immediately frozen between two slabs of dry ice to stop fermentation and held frozen until hydrolyzed. In the case of bread, five slices were taken from different positions within the loaf. Crumbs were made from these slices using a Waring blender. Three representative samples of approximately one gram of the crumbs were weighed and hydrolyzed.

The samples were hydrolyzed by autoclaving in 0.44 $\underline{N}$ HCl for 2 hr at $121^{\circ}C$. These samples were assayed for vitamin B_6 using a microbiological method (120). The standard method was slightly

modified in the following manner. In place of 100 ml of acid

hydrolyzed casein solution, 10 g of vitamin free casamino acid[5] were

used in the preparation of the basal medium stock solution. The

inoculum of <u>Saccharomyces</u> <u>carlsbergensis</u> (also known as <u>S.</u> <u>uvarium</u>)

ATCC No. 9080 was prepared by incubating the cells in liquid

culture medium for 6 hr instead of 20 hr.

The method involves the growth of microorganisms. The

percent transmittance of the cell suspension was read on an Evelyn

Photoelectric Colorimeter[6] set at 660 nm. The unknown concen-

trations of vitamin B_6 were read against a standard curve prepared

with graded levels of standard pyridoxine hydrochloride. A range

of 0 to 5 ng of pyridoxine was used. Although a drawn standard

curve served as a check on the distribution of points, the unknown

concentrations were determined using a calculator[7] programmed to

interpret unequally spaced data.

Moisture contents of the mix, proof and bread samples were

determined in triplicate using a vacuum oven (120). Vitamin B_6

values of the samples were expressed on a dry weight basis. The

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[5] Vitamin free casamino acid, Difco Laboratories, Detroit, Michigan.

[6] Evelyn Photoelectric Colorimeter, Rubicon Company, Philadelphia, Pennsylvania.

[7] Programmable Calculator, 9810 A Model 10, Hewlett Packard, Loveland, Colorado.

means of mix and proof, proof and bread were tested for significance using Student's t test at 5 and 1% levels of probability.

Storage of Vitamin B_6 Enriched Bread

Stability of vitamin B_6 during storage of commercially prepared WB_6 bread was investigated. Several randomly selected loaves from the commercial batch were placed under three different storage conditions; three loaves at room temperature (25-$27^{o}C$) with moderate lighting, six loaves under refrigeration (4-$5^{o}C$) and nine loaves in a chest type freezer ($-5^{o}C$). The weight of each loaf was noted initially and at the time of sampling.

The bread storage study started on the same day the commercial WB_6 bread was baked. Zero day samples were obtained after the loaves had remained under respective storage conditions for three hours. Sample preparation and vitamin B_6 analysis were similar to that of bread described in the previous section. Breads stored at room temperature were analyzed at three day intervals over a period of one week. Those stored in the refrigerator and the freezer were sampled and analyzed at weekly intervals for four and eight weeks, respectively.

Storage of Vitamin B_6 Enriched Flour

Crystalline pyridoxine monohydrochloride was added to all

purpose[8] flour at the level of 2 mg pyridoxine per pound of flour.

An even distribution of the vitamin was attained by mixing the flour

in small quantities. Initial mixing was done by rotating the vitamin

and flour in a glass jar containing glass beads to facilitate thorough

mixing. This flour-vitamin mixture was transferred to a bowl of

a Kitchen Aid mixer equipped with a wire whip. As the mixing

continued, the rest of the flour was blended into the first mixture.

Mixing was continued for one hour after the addition of all the flour.

Cold towels were placed around the bowl to minimize the heat

produced due to mechanical action.

Flour was stored in amber-colored, tightly sealed glass jars.

These were placed under three storage conditions; room temperature

($25-27^{\circ}C$) under moderate lighting, cold storage ($5-7^{\circ}C$) and frozen

($-40^{\circ}C$). Triplicate samples of flour from each storage lot were

analyzed for vitamin B_6 after 0, 1, 4, 8, 12, 19 and 26 weeks.

Bioavailability

Subjects

Nine healthy college men, age 21 to 35 years, were recruited

by advertisement or personal contact. Each participant was

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[8] All purpose unbleached, enriched flour, Centennial Mills, Portland, Oregon.

interviewed initially for medical and diet histories. The protocol

of the study was outlined and their role in it was explained clearly.

Personal information, activity and the consent of the selected

subjects were obtained in writing on the forms given in Appendix 3.

The vital statistics of the subjects are presented in Table 3. This

study had been approved by the Human Subjects Committee at Oregon

State University.

Experimental Design

The study involved three variables--the breads; whole wheat

(WHW), white enriched with vitamin B_6 (WB_6) and white (W). The

experiment was based on a three 3 x 3 Latin square design which is

illustrated in Figure 3. The subjects were randomly assigned to

one of the nine columns.

Diet

The three week experimental period was preceded by six days

of adjustment during which time the diet was gradually changed from

one containing proteins of animal origin to one based primarily on

wheat protein.

The major food item in the experimental diet was bread. The

three types of bread, WHW, WB_6 and W required for the entire

study were prepared at a local bakery (Albertson's, Corvallis) and

Table 3. Vital statistics of the subjects.

Subject (No.)	Age (Years)	Starting Body Weight (kg)	Height (cm)
1	32	90	180
2	29	96	170
3	21	63	178
4	27	70	183
5	32	51	169
6	25	77	180
7	23	66	178
8	23	90	185
9	27	67	168
Range	21 - 32	51 - 96	168 - 185
Mean $\pm$ SD[1]	26.6 $\pm$ 3.9	74.4 $\pm$ 14.9	176.8 $\pm$ 6.3

[1] Mean $\pm$ Standard deviation

PERIOD									
I	WHW	WB_6	W	WHW	WB_6	W	WHW	WB_6	W
II	WB_6	W	WHW	W	WHW	WB_6	W	WHW	WB_6
III	W	WHW	WB_6	WB_6	W	WHW	WB_6	W	WHW
SUBJECT	8	2	6	4	3	7	9	5	1

Figure 3. Latin square design for three breads; whole wheat (WHW), white enriched with vitamin B_6 (WB_6) and white (W) over three experimental periods, I, II, and III, for nine subjects 1-9.

kept under frozen storage as described earlier, until they were thawed and served to the subjects.

The level of vitamin B_6 for the enrichment of white bread was selected after analyzing the whole wheat and white breads for their vitamin B_6 contents. Even though the proposed level of enrichment is 2 mg of pyridoxine per pound of flour (9), only a 1.12 mg level was used in order that the amount of vitamin B_6 in the WB_6 bread would be closer to that of whole wheat bread. The vitamin B_6 contents of the three types of bread as determined by microbiological assay using <u>Saccaromyces</u> <u>carlsbergensis</u> were 0.21, 0.20, and 0.06 mg of pyridoxine per 100 grams of WHW, WB_6 and W breads, respectively.

One reason for the selection of the level of vitamin B_6 intake was that 2/3 to 3/4 of vitamin B_6 would be contributed by the bread. Six hundred grams (22 slices) was selected as the daily quota of bread. This amount of WB_6 bread contained 1.2 mg of vitamin B_6. On this basis, 1.5 mg was set as the daily intake level of vitamin B_6 in this study, although the recommended dietary allowance (RDA) is 2 mg (14). According to Sauberlich (190), 1.5 mg of vitamin B_6 is a level that marginally meets the requirements of human adults. This level of intake usually prevents excretion of abnormal levels of tryptophan metabolites in the urine after a load test (49).

In order to maintain 1.2 mg of vitamin B_6 from bread, 570 g of WHW bread was used. Six hundred grams of W bread contained only 0.36 mg of vitamin B_6. In order to maintain the level of vitamin B_6 equivalent to that from bread, 0.84 mg of pyridoxine hydrochloride was supplied to the subjects in the form of an oral dose distributed equally among the three meals when they received W bread.

The daily quota of bread was consumed by all subjects with the exception of one (Subject No. 5), who was unable to consume the bulk of the entire quantity. His intake of bread was decreased to 2/3 of that of others on day six of the adjustment period. The oral dose of PIN-HCl was also reduced proportionately when he received W bread. His total daily intake of vitamin B_6 was 1.2 mg compared to the 1.5 mg given to the others.

The type of bread was the only variable in the study during the three experimental periods. The remaining diet, which was constant throughout the study, consisted of the food items given in Table 4. The composition is found in Appendix 4. This diet was adequate in all nutrients known to be required by man according to the RDA (14).

In addition to these foods, margarine, jelly, beverages, (coffee

tea, Tang[9], Lemonade[10], 7 -UP[11]), candy and sugar were also made

available to the subjects. A record of the daily consumption of these

items was maintained. They were encouraged to maintain their body

weights by regulating the amounts of these foods consumed. A three

meal regimen was followed. All meals were prepared and served

at the metabolic unit in the Department of Foods and Nutrition at

Oregon State University.

Table 4. Constant diet with partial composition.

Item	Amount g	K Cals	Protein g	Vitamin B_6 mg (PIN)
Orange juice	170	76	1.2	0.048
Cream of Wheat (cooked)	83	36	1.1	0.005
Milk	240	156	8.4	0.168
Peaches	130	104	0.5	0.025
Rice Casserole:				
Rice	25	91	1.7	0.042
Carrot	25	10	0.3	0.038
Celery	25	4	0.2	0.015
Olive	10	13	0.1	0.001
Onion (dehy.)	2	7	0.2	0.010
Tomato juice	34	65	0.3	0.065
Total		661	14.3	0.435

[9] Tang, General Foods Corporation, White Plains, New York.

[10] Lemonade, Wyler Foods, Borden, Inc., Northbrook, Illinois.

[11] 7 -UP, Bottled under the authority of 7 -UP Services Inc., St. Louis, Missouri.

Non perishable foods were purchased in bulk so that products from the same lot were used throughout the study. Perishable items such as milk were purchased on a weekly basis. Composites of the constant diet were prepared every week and analyzed for vitamin B_6 content by the microbiological method (120). Breads were also analyzed for vitamin B_6 at weekly intervals. The oral dose of vitamin B_6 was prepared each week by dissolving 28 mg of crystalline PIN-HCl in 500 ml of 2% acetic acid. In addition to the determination of total vitamin B_6 content of the constant diet and the breads, these samples were also analyzed for the amount of the three forms of the vitamin--pyridoxal, pyridoxine and pyridoxamine by the method of Toepfer and Polansky (119).

Metabolic Study

The adjustment period was helpful in acquainting the subjects with the routine of the study, so that they were well prepared for the experimental periods that followed. They were asked to maintain their normal activities during the study. Body weights were recorded each day.

At the beginning of each experimental period, the subjects were given a fecal marker of FDA Blue No. 1 dye in a gelatin capsule to mark the beginning of each dietary period. All the fecal specimens were collected in disposable cartons labelled with the subject's

initials, date and time of sample collection. Fecal content was weighed daily and stored frozen ($-20^{o}C$) until they were analyzed for total vitamin B_6.

Complete 24-hr urine collections were made throughout the study. All daily urine specimens were stored refrigerated at $4^{o}C$ under a layer of toluene. They were mixed and measured for total volume the following morning. About 15% of the total urine volume of each subject was stored frozen ($-20^{o}C$) in plastic containers until it was analyzed for creatinine, total and free vitamin B_6 and 4-pyridoxic acid.

On the 1st, 3rd and 5th days of each period, fasting blood samples were drawn from the antecubital veins of the subjects by a medical technician. Heparinized vacutainer tubes were used for this purpose. Hemoglobin concentrations and the hematocrits were determined. Plasma was separated and stored frozen ($-50^{o}C$). Erythrocyte transaminase activities and plasma vitamin B_6 as well as pyridoxal phosphate were determined by another investigator as a separate part of this study.

Among the various analyses done on the biological material, urinary and fecal vitamin B_6, and urinary 4-pyridoxic acid will be discussed in this thesis.

Analytical Methods

Prior to the assay of vitamin B_6 by microbiological method, the material to be assayed first requires hydrolysis. This step varies somewhat from material to material. Sample preparation and the hydrolysis procedures used with the diet, feces and urine samples will be discussed in the following sections.

Analysis of the Diet for Vitamin B_6. The diet composite was prepared by blending together a day's constant diet of known weight in a Waring blender. After addition of each item, the weight was noted and the composite was pureed for two minutes. Finally the total weight of the composite was noted and 1-2 gm of a well-homogenized sample was accurately weighed into a beaker. This was followed by hydrolysis on the same day to release the bound forms of vitamin B_6.

Samples of each bread were analyzed separately for vitamin B_6 content. Five representative slices of bread were taken from a randomly selected loaf. One half of each of these 5 slices was placed in a Waring blender and blended until it formed finely divided, well mixed bread crumbs (generally set at "puree" and blended for 3-4 minutes). A representative sample (1-2 g) of this was weighed and hydrolyzed.

The hydrolysis step for both the food composite and breads were carried out by autoclaving the sample with approximately 150 ml of 0.44 $\underline{N}$ HCl at 15 lbs pressure for 2 hours. After cooling to room temperature, the pH was adjusted to 4.5 with potassium hydroxide. The solution was then made up to a known volume and filtered through Whatman No. 40 filter paper. This filtrate was used in the assay of vitamin B_6 by microbiological method as discussed previously. The extracts of the food composite as well as of the three types of bread from one week were used in the separation of the three forms of vitamin B_6 by the procedure of Toepfer and Polansky (119). Each form was assayed by the microbiological method, using Saccaromyces carlsbergensis. A standard curve of each form of vitamin B_6 was used to determine the unknown concentrations of the corresponding form.

Fecal vitamin B_6. The fecal collections of each subject during one experimental period were pooled toegher in a large container. They were thawed and then mixed on a rotary shaker for 2-3 hours. The pooling of collections was done in order to minimize the number of samples that had to be analyzed for vitamin B_6.

A representative sample prepared in the above manner was subjected to hydrolysis, by autoclaving at 15 lb pressure with 0.44 $\underline{N}$ HCl for 2 hours. This was followed by adjustment of pH to 4.5,

making up to a known volume with redistilled water and filtering.

The filtrate was used in the microbiological assay.

Urinary Vitamin B_6. Total and free vitamin B_6 was determined in the urine samples collected on the 2nd, 4th, 6th and 7th days during each of the three experimental periods. The days, 7, 2 and 4 respectively represented the days immediately before the days on which fasting blood samples were drawn. Urine samples from days 1 and 6 of the adjustment period were also analyzed for total and free vitamin B_6.

Prior to the determination of total vitamin B_6 in urine, the samples were subjected to acid hydrolysis. Ten milliliters or 1% of the total volume of urine (whichever was smaller) was autoclaved with 50 ml of 0.1 N HCl at 15 lb pressure for 30 minutes. In the determination of free urinary vitamin B_6, 50 ml of redistilled water were added to the urine sample. No autoclaving was involved.

From this stage on, both samples were treated identically. The pH was adjusted to 4.5 using either KOH or HCl. The volume was made up to 100 ml with redistilled water and the mixture was filtered through Whatman No. 1 filter paper. This filtrate, after subsequent dilutions, was used in the microbiological assay for vitamin B_6.

A sample of urine was analyzed with every other assay to determine the variability of the method as well as to determine if there

was a change in the vitamin B_6 content of the urine during storage

of the sample. The recovery of a known amount of the vitamin B_6

added to a urine sample was determined with each assay.

The hydrolyzed extracts of the urines of five selected sub-

jects (1, 2, 4, 6 and 7) on the sixth day of adjustment and of each

of the experimental periods were used for determination of pyridoxal,

pyridoxine and pyridoxamine. Recoveries of the three forms when

chromatographed alone and when mixed with a sample of urine were

determined. The procedure described by Toepfer and Polansky

(119) was followed.

Urinary 4-Pyridoxic Acid. Samples of urine collected on days

2, 4, 6, and 7 of each of the experimental periods and days 1 and 6

of the adjustment period were analyzed for 4-PA. The method of

Reddy, Reynolds and Price was used (105). This involved ion

exchange chromatorgaphy to separate 4-PA from other fluorescent

compounds in the urine, followed by fluorometric determination. An

Aminco-Bowman spectrophotofluorometer[12] was used to read the

fluorescence.

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[12] Aminco Bowman Spectrophotofluorometer, American Instrument
Co., Inc., Silver Spring, Maryland.

Statistical Analysis

The data were statistically analyzed by the method of analysis of variance (ANOVA) using the system of statistical package for the social sciences (SPSS). The analysis was done by a statistician using the computer at Oregon State University. The pertinent data from ANOVA tables are given in Appendix 6 for all the variables analyzed.

RESULTS AND DISCUSSION

Stability

Stability of Vitamin B_6 During Bread Making

The total vitamin B_6 content of mix, proof and bread prepared
from WHW, WB_6 and W flour, under commercial and home baking
(straight dough and sponge dough methods) conditions is given in
Table 5. The means presented in Table 5 are graphically shown
in Figure 4. The difference in the vitamin B_6 content between mix
and proof, proof and bread were tested for significance at 5% and 1%
using Student's t test.

Among the three types of flour used, WHW and W showed a
decrease in the vitamin B_6 content during fermentation. This
decrease was observed in breads prepared under both commercial
and home conditions. In the case of WB_6 flour, however, there was
an increase of 2-8% in the vitamin B_6 content at the proof stage
as compared to that of mix in both home and commercially prepared
breads, of which the one prepared using the sponge dough method
was significant ($P < 0.05$). The increase is probably due to vitamin
B_6 synthesis accompanying yeast growth during fermentation. Keagy
et al. observed a similar increase in folacin content during fermenta-
tion, and attributed this increase to synthesis of folacin by yeast (181).

Table 5. Vitamin B_6 content of mix, proof and bread from WHW[a], WB_6 and W flour, prepared under commercial and home conditions (μg pyridoxine/100 g sample, dry basis).

	WHW				WB_6				W			
	R_1[b]	R_2	R_3	Mean $\pm$ SD[c]	R_1	R_2	R_3	Mean $\pm$ SD	R_1	R_2	R_3	Mean $\pm$ SD
COMMERCIAL												
Mix	348	351	348	349 $\pm$ 2	361	288	345	332 $\pm$ 38	88	92	95	92 $\pm$ 3
Proof	357	322	352	344 $\pm$ 18	358	315	385	353 $\pm$ 35	92	88	83	88 $\pm$ 4
Bread	320	297	299	305 $\pm$ 12	297	290	308	298 $\pm$ 9	78	78	80	79 $\pm$ 1
HOME MADE												
a) Straight-dough method												
Mix	340	353	374	356 $\pm$ 17	535	569	546	550 $\pm$ 17	86	89	88	88 $\pm$ 2
Proof	331	350	354	345 $\pm$ 12	564	564	560	563 $\pm$ 2	77	75	78	77 $\pm$ 2
Bread	322	321	333	325 $\pm$ 7	569	549	563	560 $\pm$ 10	76	75	71	74 $\pm$ 2
b) Sponge-dough method												
Mix	381	384	350	372 $\pm$ 19	621	609	626	619 $\pm$ 8	107	88	88	94 $\pm$ 11
Proof	370	365	372	369 $\pm$ 3	646	677	677	667 $\pm$ 18	82	81	82	81 $\pm$ 1
Bread	348	336	338	341 $\pm$ 7	594	595	579	590 $\pm$ 9	76	79	77	77 $\pm$ 2

a. WHW, WB_6 and W - see Appendix 5.
b. R_1, R_2 and R_3 represent the three replications taken from the same mix, proof or bread preparation.
c. Mean $\pm$ Standard Deviaion.

Figure 4. Graphic presentation of mean vitamin B_6 levels in mix, proof and bread.

The reason that such an increase was observed only in WB_6 proof in the present study may be because the synthetic form of the vitamin B_6 (PIN-HCl) used in the enrichment of white flour was readily available for yeast growth.

Although the vitamin B_6 content of WHW flour was fairly high, there was no increase in the vitamin B_6 after fermentation as was observed with WB_6 bread. A decrease in the vitamin B_6 levels after fermentation was observed for both WHW and W breads. These changes in vitamin B_6 content during fermentation were not significant.

As seen in Figure 4, the vitamin B_6 level of commercially prepared WB_6 bread was lower than that of home baked breads. This difference was due to the lower level of vitamin B_6 enrichment (1.12 mg PIN/lb of flour) of commercially prepared WB_6 bread compared to that of home baked bread which contained the level of enrichment (2 mg PIN/lb of flour) recommended by the NAS (9). The reason for the lower level of enrichment of commercial WB_6 bread was discussed in detail earlier.

The mean vitamin B_6 level observed at different stages in the commercial bread making process using WHW flour was slightly lower than the corresponding level observed under home baking conditions. These differences, however, were not significant. There was a higher level of vitamin B_6 at all stages in the three

types of bread prepared by the sponge-dough method compared to the straight dough method under home conditions. This could be attributed to the growth of yeast and probably synthesis of vitamin B_6 during the four hours of fermentation of the sponge containing 60% of the flour, water and all the yeast, before being mixed with the 'dough' ingredients (Appendix 2). The total fermentation time for sponge-dough method was 330 min compared to 235 min for the straight dough method.

Baking losses in the commercial breads ranged from 10-15% (mean 13%). Of these breads, the ones made from WHW and W flours had a significant baking loss ($P < 0.05$), compared to the one made from WB_6 flour. There was a 0.5 to 6% (mean 3.5%) loss due to baking of the bread made using straight dough method under home conditions, but this was not statistically significant. However, when sponge dough method was used, a significant baking loss of 5-12% was observed in WHW, WB_6 ($P < 0.01$) and W ($P < 0.05$) breads.

In the folacin stability study (181), Keagy et al. found an 8% baking loss of added folic acid when the straight dough method was used as compared to 14% using the sponge dough method. These losses were considered to be fairly low. The average baking loss observed by Keagy et al. for the native folacin was 31%. The baking losses of the added vitamin B_6 in the present study were 3.5 and 8%

for the straight dough and sponge dough breads, respectively,

compared to a 13% loss when baked under commercial conditions.

The comparatively greater loss of vitamin B_6 in the latter case,

may be due to exposure to light during baking, cooling and subsequent

handling of the bread. Under home baking conditions, precautions

were taken to minimize undue exposure of the dough and bread to

light. It is seen that both folacin (181) and vitamin B_6 (present

study) were lost to a greater extent when the sponge dough method

was used, compared to the straight dough method. A higher mean

level of vitamin B_6 was observed in the mix, proof and bread pre-

pared by sponge dough method as compared to straight method.

Perhaps, the vitamin B_6 synthesized by the yeast during the longer

fermentation period using the former method was more easily

destroyed by the baking process as compared to the added vitamin

B_6. Good stability of added pyridoxine in bread baked from enriched

white flour has been reported (182, 183). The greater stability of

pyridoxine, which is predominant in wheat and which is also the

form used for enrichment may have contributed to the low baking

losses observed during bread making.

Stability of added Vitamin B_6 during Bread Storage

Vitamin B_6 content of the bread stored frozen ($-5^{\circ}C$), under

refrigeration ($5^{\circ}C$) and at room temperature ($27^{\circ}C$), over varying

lengths of time is presented in Table 6. The mean vitamin B_6 level

of bread stored frozen over a period of seven weeks ranged from

186 to 210 $\mu g/100$ g. There was no trend observed in the distribution

of these means. Similarly no definite pattern was observed in the

vitamin B_6 levels of the bread stored under refrigeration. These

results indicate that there was no loss or gain of vitamin B_6 when

the WB_6 bread was stored under frozen conditions or in a refrig-

erator.

On the other hand, in the case of bread stored at room tem-

perature, vitamin B_6 analyses were done on the days 0 and 3.

Greenish and black types of mold, probably Penicillium and

Aspergillus species, respectively, were observed on the bread on

the fifth day of storage at room temperature. These breads did not

contain any preservative, therefore, it was not unexpected to find

mold growth after three to four days of storage at room temperature.

The temperature of the room where the bread was stored was

$27 \pm 2^{o}C$. The mean of vitamin B_6 level decreased from 192

on day 0 to 172 $\mu g/100$ g on day 3 of storage--a 10% loss at room

temperature. This change in the vitamin B_6 level was statistically

significant compared to the changes observed in the vitamin B_6

levels of the bread stored under frozen and refrigerated conditions.

In contrast to this observation, the results of Cort et al. indicated

that there was no loss of vitamin B_6 even after five days of storage

Table 6. Vitamin B_6 content of commercial WB_6 bread stored under three different conditions (μg pyridoxine/100 g).

Time of Analysis	Storage Conditions											
	Freezer (-5°C)				Refrigerator (5°C)				Room temperature (27°C)			
	R_1[a]	R_2	R_3	Mean ± SD[b]	R_1	R_2	R_3	Mean ± SD	R_1	R_2	R_3	Mean ± SD
day 0	221	204	204	210 ± 10	186	204	182	191 ± 12	190	195	191	192 ± 3
day 3	214	199	180	198 ± 17	187	174	181	181 ± 6	174	170	172	172 ± 2
week 1	190	194	190	192 ± 2	179	188	187	185 ± 5				
week 2	178	199	180	186 ± 11	178	185	182	181 ± 3				
week 3	197	192	189	193 ± 4	187	205	186	192 ± 11				
week 4	187	190	184	187 ± 3	183	190	181	185 ± 5				
week 5	193	187	202	194 ± 8								
week 6	207	194	190	197 ± 9								
week 7	196	195	187	193 ± 5								

a. R_1, R_2, and R_3 refer to the three replications taken from the same loaf of bread.

b. Mean ± Standard Deviation

at room temperature (183). The 10% storage loss of vitamin B_6 observed in the present study may be due to the temperature and/or exposure to light.

The results of this section of the study suggest that storage of bread under frozen conditions or in a refrigerator will retain vitamin B_6 better than storage at room temperature. Bread stored in the refrigerator tends to stale faster than the bread stored either at room temperature or under frozen conditions (188). However, as indicated by the results of this study, retention of vitamin B_6 is greater when stored in a refrigerator than when kept at room temperature.

Stability of Added Vitamin B_6 during Flour Storage

Vitamin B_6 levels of all purpose flour enriched with pyridoxine hydrochloride and stored under three different conditions over a period of 26 weeks (six months) are presented in Table 7. The means of the triplicate analysis indicate that there was practically no loss of vitamin B_6 of the WB_6 flour when stored at room temperature, in a cold room or under frozen conditions.

Although flour is generally not stored frozen, this condition was selected in order to establish a control for each analysis. Vitamin B_6 of the flour samples were determined at the times indicated in Table 7. The value determined for the flour stored in

Table 7. Vitamin B_6 content of WB_6 flour stored under three different conditions (μ g pyridoxine/100 g).

Time of Analysis	Storage Condition											
	Frozen (-45°C)				Cold storage (5°C)				Room temperature (27°C)			
	R_1[a]	R_2	R_3	Mean $\pm$ SD[b]	R_1	R_2	R_3	Mean $\pm$ SD	R_1	R_2	R_3	Mean $\pm$ SD
week 0	608	584	580	591 $\pm$ 15	563	606	597	589 $\pm$ 23	644	548	584	592 $\pm$ 48
week 1	581	544	575	566 $\pm$ 20	599	571	588	586 $\pm$ 14	544	550	577	557 $\pm$ 18
week 4	592	548	582	574 $\pm$ 23	529	539	559	542 $\pm$ 15	528	512	506	515 $\pm$ 12
week 8	587	544	649	594 $\pm$ 52	590	601	588	593 $\pm$ 7	588	605	578	590 $\pm$ 14
week 12	553	525	498	525 $\pm$ 28	584	563	586	578 $\pm$ 13	567	573	535	558 $\pm$ 21
week 19	556	602	594	584 $\pm$ 24	591	567	583	580 $\pm$ 13	594	607	572	591 $\pm$ 18
week 26	635	553	570	586 $\pm$ 44	628	557	537	574 $\pm$ 48	604	575	559	579 $\pm$ 23

a. R_1, R_2 and R_3 are the three replications taken from the same lot of flour.

b. Mean $\pm$ Standard Deviation.

the cold room and at room temperature were expressed as a percentage of the $-45^{\circ}C$ control flour analyzed at the same time. These percentages are given in Table 8.

Table 8. Vitamin B_6 of flour stored under different conditions expressed as a percentage of the $-45^{\circ}C$ control flour analyzed at the same time.

Time of Analysis (Wk)	0	1	4	8	12	19	26
Cold Storage ($5^{\circ}C$)	99.6	100.5	94.4	99.9	110.0	99.4	98.0
Room Temperature ($27^{\circ}C$)	100.2	98.3	89.7	99.4	106.3	101.2	98.9

There was no trend observed in the changes of the vitamin B_6 levels of the flour. This observation was similar to that of Keagy et al. (181), who tested the stability of the added folacin during flour storage. However, the native folacin of the flour in their study showed an exponential decay pattern. In the present study, the stability of native vitamin B_6 in flour was not tested.

From the results obtained on the stability of added vitamin B_6 to flour, under the conditions of the present study, one could conclude that there was good stability of vitamin B_6 in flour during storage. The stability observed of the added vitamin B_6 in both bread and flour may be due to the inherent stability of PIN, the form generally used in the enrichment with vitamin B_6.

<center>Bioavailability</center>

Subject Response to the Diets

The calorie content of the constant diet together with the bread was 2029 and 2272 K cal for whole wheat and white bread diets, respectively. In addition to this, the subjects were allowed to consume extra calories in the form of margarine, jelly, sugar, candy and beverages. When necessary, these extra calories were consumed to maintain body weight. However, some fluctuation in body weight was observed for some subjects during the study.

The initial weight and the weight at the end of each experimental period are given in Table 9. The average additional calories consumed per day by each subject during the three week experimental period are also listed. Data listed in Table 9 are presented without taking into account the order in which the bread was fed. Subjects 2, 4, 7 and 8 showed a reduction in weight during the experimental period as compared to their initial body weight. However, these reductions were not significant.

Of the above subjects, 2 and 8 consumed only 90 and 210 extra calories/day, respectively although they were encouraged to increase their calories in order to maintain weight. The RDA for men of the age category 21 to 33 years is 2700-3000 calories (9).

Table 9. Body weights and extra calories consumed per day.

Subject	1	2	3	4	5	6	7	8	9	Mean $\pm$ SD[a]
Body weight (kg)										
Initial	90.2	95.9	62.6	70.0	50.7	77.3	65.8	90.2	65.4	74.2 $\pm$ 15.2
End of period										
WHW[b]	89.1	92.3	64.2	68.5	51.6	75.7	64.4	87.1	65.8	73.2 $\pm$ 13.8
WB_6	90.0	93.7	61.6	68.5	51.9	76.6	64.4	86.2	66.9	73.3 $\pm$ 14.2
W	90.0	91.6	61.8	68.0	52.3	77.6	64.6	85.7	65.8	73.0 $\pm$ 13.8
Average extra calories/day (K-cal)	1364	90	1156	2357	1462	3829	1676	210	1542	1521 $\pm$ 1117

a. Mean $\pm$ Standard Deviation

b. WHW, WB_6 and W - see Appendix 5.

In spite of consuming approximately 2350 and 1675 extra calories per day, subjects 4 and 7, respectively, showed some loss in body weight. This was probably due to their physical activities. Subject 3, who was also physically active, showed a reduction of 1 Kg with WB_6 bread during the first experimental period, followed by a gain in weight of about 2.5 Kg with WHW diet and finally a weight loss. This same subject also complained of fatigue and was examined by a physician. He was administered an oral supplement of iron,[13] three times a day from day 4 of the first experimental period. Subject 5 gained 1-1.5 Kg of weight in spite of consuming only 3/4 of the daily quota of bread. This weight gain probably occurred because the calorie value of food eaten during the study was far more than what he had been previously consuming. In spite of consuming an average 3829 extra calories, No. 6 did not gain weight because of his heavy physical activity. In general, all the subjects were maintained in good physical condition.

Since each subject was his own control, the weights of all nine were averaged. The mean and standard deviation for the starting weight was 74.2 ± 15.2 Kg compared to 73.2 ± 13.8, 73.3 ± 14.2 and 73.0 ± 13.8 for WHW, WB_6 and W bread diets, respectively. The changes in the body weights during the three experimental

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[13] 5 g $FeSO_4$

periods were not significant (Appendix 6). The average weight at the start of the experiment was higher than the average weight at the end of each experimental period. This indicates that either the bread diets or lack of calories caused some reduction in weight of most of the subjects. However, there was no set pattern in weight reduction in relation to the type of bread.

Claims have been made that diets containing a substantial amoung of bread can cause a significant loss in body weight (189). This was found to be so in a study involving two groups of eight-men who were on diets based on two types of bread, a commercially pre- pared supermarket type and a specially prepared low fat, high fiber type for 8 weeks. The group receiving the latter type of bread lost more weight on an average (19.4 lb) compared to the other group (13.7 lb). These subjects received 12 slices of bread daily, and other foods of their choice. Bread provided bulk and reduced the appetite for many of the other foods.

In the present investigation, the subjects consumed 22 slices of bread daily along with the other food items listed in Appendix 4. Of the total calories for the constant diet together with the breads 67.5% and 71.0% were supplied by WHW and the white breads, respectively. About 80% of the protein in the diet was derived from the bread. In a study on wheat flour as a source of protein for adult human, Bolourchi et al. reported that subjects were held in nitrogen balance

when fed a diet in which white flour provided 90-95% of the daily

protein intake (11). They also demonstrated that a 2500 K-cal

diet high in bread would provide more than an adequate amount of all

the essential amino acids.

Dietary Intake of Vitamin B_6

Although the RDA for vitamin B_6 for human adults is 2 mg, a

level of 1.5 mg was selected as the daily intake, for reasons dis-

cussed earlier in this thesis. A level of 1.5 mg of vitamin B_6 was

reported as being marginally adequate for human adults (89, 190).

The requirement for vitamin B_6 depends on the level of protein intake

(64, 65); a daily intake of 1.5 mg of vitamin B_6 is considered a

minimum when the diet contains 100 g of protein (66). In the present

study, the daily intake of protein was 65-66 g, and the 1.5 mg intake

of vitamin B_6 was therefore adequate to meet the requirements of the

subjects.

The amount of vitamin B_6 contributed by the different components

of the diet as determined by the microbiological method (120) is

presented in Table 10.

The vitamin B_6 content (as pyridoxine) of the constant diet

(bread not included), when calculated using a food composition table

was 0.44 mg (5). The assayed values ranged from 0.37 to 0.39

(0.38 $\pm$ 0.01 mg). Such a difference between the calculated and

Table 10. Total vitamin B_6[1] level in the different components of a day's diet.

Variable	WHW	WB_6	W
Bread	1.20 ± 0.06	1.18 ± 0.05	0.35 ± 0.04
Constant diet	0.38 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
Oral dose	-	-	0.81 ± 0.04
TOTAL	1.58	1.56	1.54

[1] Vitamin B_6, expressed as mg of pyridoxine (means of at least three assays).

assayed values can be expected when dealing with foods, due to varietal differences and variation in cooking and handling. In addition, there is also a 10-15% variability inherent in the microbiological assay for vitamin B_6.

The amount of PAL, PIN and PAM in the components of the diet are given in Table 11. Also listed are the percentages of the total vitamin B_6 accounted for as the three forms. The amount of PAL, PIN and PAM in the diet components are presented graphically in Figure 5. The percentages of the three forms of vitamin B_6 as assayed in the diet components, and the amounts calculated from literature values (5) are presented in Table 12. In the case of the constant diet, the calculated amounts represent the levels of PAL, PIN and PAM in the three major food sources of vitamin B_6: orange juice, milk and tomato juice.

Pyridoxine was found to be the predominant form of vitamin B_6 in the total diet followed by pyridoxal and a smaller amount of pyridoxamine. The diets of plant origin are generally reported as being higher in PIN compared to PAL and PAM (151). Yano and Fujita determined that there was a large amount of PIN and PAL, but only a small proportion of PAM in the vegetable diet given to their subjects (103).

Table 11. Levels of the three forms of vitamin B_6 in components of the diet (mg)[a].

Forms of Vitamin B_6	Bread			Constant diet	Oral[c] dose
	WHW[b]	WB_6	W		
PAL	.240	.142	.125	.184	---
PIN	.850	.884	.095	.145	.810
PAM	.145	.151	.132	.050	---
Percent of the total Vitamin B_6 accounted for as three forms	80.34	81.23	84.38	83.78	

a. Amounts adjusted to 100% recovery
b. WHW, WB_6 and W, see Appendix 5
c. Assumed to be 100% PIN

Table 12. Assayed and calculated percentages of PAL, PIN and PAM of vitamin B_6 of the diet components.

Diet Component	Assayed			Calculated		
	PAL	PIN	PAM	PAL	PIN	PAM
WHW	20	70	10	18	67	15
WB_6	12	75	13	12*	78*	10*
W	36	27	37	31	25	44
Constant	49	38	13	45	32	22

* Calculated values for WB_6 bread were based on those of W bread and the amount of pyridoxine hydrochloride added in the enrichment.

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Figure 5. Three forms of vitamin B_6 in the diet components.

Fecal Excretion of Vitamin B_6
_____

The fresh weight and the vitamin B_6 content of the feces for the three experimental periods are given in Tables 13 and 14. In Table 13 the values are presented in the order in which the three types of breads were served, whereas the values in Table 14 were tabulated for each subject, without taking into account this order. The data in Table 14 shows that the fresh weight of feces of all subjects for WHW bread was considerably higher when compared to the corresponding values for the other two types of bread. The mean fecal weight for the WHW bread diet was 2015 ± 422 g/wk, compared to 1148 ± 415 and 1142 ± 448 for WB_6 and W breads, respectively.

The WHW bread diet was higher in fiber (11.08 g/day) compared to the diets based on the WB_6 and W breads (3.16 g/day). The markedly higher stool weight associated with WHW bread may be due to the higher fiber content and the ability of the fiber to hold water. Southgate et al. have shown that consuming a diet high in fiber causes an increase in the stool weight, both moisture and dry matter (191). They also reported that diets high in fiber tend to have a shorter transit time in the gut. This could cause excretion of some nutrients which therefore would not be available to the body.

Table 13. Vitamin B_6 content[a] and weight of feces[b] in subjects fed diets based on WHW^c, WB_6 and W breads.

Bread Type		WHW			WB_6			W		
Subject		4	8	9	2	3	5	1	6	7
Week 1	Weight	1710	1597	2415	1249	1600	885	961	2100	753
	Vitamin B_6	4.2	4.7	5.6	3.0	4.2	2.0	3.3	2.6	2.4
Subject		3	5	6	1	7	8	2	4	9
Week 2	Weight	2295	1406	2725	734	1124	811	1275	856	957
	Vitamin B_6	6.2	3.9	4.5	2.7	2.8	3.0	2.8	3.4	3.5
Subject		1	2	7	4	6	9	3	5	8
Week 3	Weight	1987	1858	2142	931	2006	997	1621	845	911
	Vitamin B_6	5.9	3.2	5.2	3.5	3.9	3.1	3.9	2.0	3.2
Overall mean $\pm$	Weight	2015 $\pm$ 421			1148 $\pm$ 415			1142 $\pm$ 448		
Standard deviation	Vitamin B_6	4.9 $\pm$ 0.9			3.1 $\pm$ 0.7			3.0 $\pm$ 0.6		

a. Fecal vitamin B_6 expressed as mg of pyridoxine per week.

b. Fecal weight in grams of fresh feces per week.

c. WHW, WB_6 and W see Appendix 5.

Table 14. Vitamin B_6 content[a] and weight of feces[b] during the experimental periods.

Subject	1	2	3	4	5	6	7	8	9	Mean $\pm$ SD[d]
WHW[c]										
weight	1987	1858	2295	1710	1406	2725	2142	1597	2415	2015 $\pm$ 422
Vitamin B_6	5.9	3.2	6.2	4.2	3.9	4.5	5.2	4.7	5.6	4.9 $\pm$ 0.9
W-B_6										
weight	734	1249	1600	931	885	2006	1124	811	997	1148 $\pm$ 415
Vitamin B_6	2.7	3.0	4.2	3.5	2.0	3.9	2.8	3.0	3.1	3.1 $\pm$ 0.7
W										
weight	961	1275	1621	856	845	2100	753	911	957	1142 $\pm$ 448
Vitamin B_6	3.3	2.8	3.9	3.4	2.0	2.6	2.4	3.2	3.5	3.0 + 0.6

a. Fecal vitamin B_6 expressed as milligrams of pyridoxine per week.

b. Fecal weight represents the fresh weight in grams per week.

c. WHW, WB_6 and W - see Appendix 5

d. Mean $\pm$ Standard Deviation.

In the present study, higher levels of fecal vitamin B_6 were

associated with higher fecal weights. This relationship can clearly

be seen in Figure 6. The mean fecal excretion of vitamin B_6 while

subjects were fed WHW bread diet was 4.9 ± 0.9 mg/wk. This level

of excretion was significantly greater ($P < 0.01$) than the 3.1 ± 0.7

and 3.0 ± 0.6 mg/wk, when they were fed WB_6 and W bread diets,

respectively (Appendix 6). However, on the basis of per gram of

feces, vitamin B_6 values were 2.5 ± 0.4, 2.9 ± 0.7 and 2.9 ± 0.9

μg for the diets containing WHW, WB_6 and W breads, respectively.

This shows that although the total vitamin B_6 excreted in feces was

higher when WHW bread was fed, the concentration of the vitamin in

feces was lower during this same period than during the periods

when WB_6 and W breads were fed. Data of Yano and Fujita indicated

a similar pattern (103). When the diet was changed from an

ordinary mixed type to a vegetable diet, the vitamin B_6 content of the

feces increased. Addition of cellulose to the vegetable diet resulted

in further intensification of this effect. The reason for a higher

level of fecal vitamin B_6 when WHW bread was given, could be

related to the vitamin B_6 in whole wheat being present as phosphate

forms. Some of these forms may escape the action of alkaline

phosphatase on the brush border of the intestine where the phospho-

rylated forms are hydrolyzed to the free forms which are then

available to the body. Fecal excretion of vitamin B_6 accounted for

Figure 6. Scatter diagram of fecal weight against fecal vitamin B_6 of the nine (1-9) subjects, when fed experimental diets based on ⬤ WHW, ⬤ WB and ◇ W breads.

an average of 45.2 ± 7.6 percent of the dietary intake of the vitamin

when WHW bread was fed. The corresponding percentages for the

WB_6 and W breads were 29.4 ± 5.0 and 28.6 ± 4.6, respectively.

These values were calculated taking into account the lower level of

vitamin B_6 intake (1.2 mg) of subject 5.

When expressed as a percentage of the intake of vitamin B_6, the

fecal excretion of the vitamin when the diets were based on WB_6 and

W breads gave values which were closer to those reported in litera-

ture. Denko et al. reported that 30.5 and 21.6 percent of the vitamin

B_6 intake was excreted in feces when their subjects were on diets

containing 1.05 and 1.76 mg of vitamin B_6, respectively (101). Levy

found 23 and 18 percent of the daily intake of 2 and 3 mg of vitamin

B_6 excreted in feces of his subjects (98).

Urinary Excretion of Vitamin B_6

Urinary excretion of total vitamin B_6 in the samples collected

on the 2nd, 4th, 6th and 7th days of the experimental periods and

day 1 and 6 of the adjustment period are presented in Table 15. The

order in which the three types of bread were served is also indicated.

The starting value for total urinary vitamin B_6 in seven out of nine

subjects was higher than their value on the last day of the adjustment

period. The total vitamin B_6 level in urine during the experimental

periods was lower in almost all the subjects compared to the level

Table 15. Urinary excretion of total vitamin B_6 (μ g/24 hr.).

	1	2	3	4	5	6	7	8	9	Overall Mean ± S.D.[b]
					Subject Number					
ADJUSTMENT PERIOD										
Day 1	155.0	195.3	107.6	208.8	153.2	306.3	152.6	175.8	167.4	180.2 ± 55.4
Day 6	128.6	146.1	114.8	152.6	114.3	208.6	156.1	173.8	124.3	146.6 ± 30.8
EXPERIMENTAL PERIODS										
Week 1	W	WB_6	WB_6	WHW	WB_6	W	W	WHW	WHW[a]	
Day 2	112.4	151.2	104.3	136.5	113.1	199.9	123.6	187.6	114.4	
Day 4	112.6	109.2	84.8	123.2	119.8	176.8	120.0	190.4	117.6	
Day 6	113.0	114.5	103.8	129.9	112.9	181.5	116.8	182.3	114.0	
Day 7	104.0	127.3	101.5	144.8	96.0	188.5	110.5	189.4	101.3	
Mean ± SD[b]	110.5 ±4.3	125.5 ±18.7	98.6 ±9.3	133.6 ±9.2	110.4 +10.1	186.7 ±10.0	117.7 ± 5.6	187.4 ±3.6	111.9 ±7.2	
Week 2	WB_6	W	WHW	W	WHW	WHW	WB_6	WB_6	W	
Day 2	110.7	162.4	102.9	128.5	99.7	162.5	111.9	178.2	109.3	
Day 4	108.2	153.1	100.8	151.4	91.1	172.9	104.3	172.7	118.0	
Day 6	101.1	145.7	87.4	120.3	88.5	166.6	88.2	163.9	120.0	
Day 7	117.9	148.6	95.0	134.8	86.3	176.9	110.2	163.9	99.0	
Mean ± SD[b]	109.5 +6.9	152.4 ±7.3	96.3 ±6.7	133.8 ±13.2	91.4 ± 5.9	169.7 ±6.4	103.6 ±10.8	169.7 ±7.0	111.6 ±9.6	
Week 3	WHW	WHW	W	WB_6	W	WB_6	WHW	W	WB_6	
Day 2	102.3	136.7	96.8	132.9	87.4	165.2	128.7	153.5	127.7	
Day 4	99.3	132.8	92.7	136.1	98.7	184.7	102.7	143.5	134.8	
Day 6	99.3	135.0	95.4	127.5	98.3	180.1	105.5	124.4	137.7	
Day 7	97.7	127.5	96.1	122.4	86.6	174.4	104.8	107.8	128.3	

Table 15. Continued

	Subject Number									Overall Mean $\pm$ S.D.[b]
	1	2	3	4	5	6	7	8	9	
Mean $\pm$ SD[b]	99.6 $\pm$ 1.9	133.0 $\pm$4.0	95.2 $\pm$1.8	129.7 $\pm$6.0	92.7 $\pm$ 6.6	176.1 $\pm$ 8.4	110.4 $\pm$12.2	132.3 $\pm$20.3	132.1 $\pm$ 4.9	
Means WHW	99.6	133.0	96.3	133.6	91.4	169.7	110.4	187.4	111.9	125.9 $\pm$ 32.7
WB$_6$	109.5	125.5	98.6	129.7	110.4	176.1	103.6	169.7	132.1	128.4 $\pm$ 28.0
W	110.5	152.4	95.2	133.8	92.7	186.7	117.7	132.3	111.6	125.9 $\pm$ 30.0

a. WHW, WB$_6$ and W see Appendix 5

b. Mean $\pm$ Standard Deviation.

observed for day 1 of the adjustment period. Since the urinary excretion of vitamin B_6 is an indication of the level of vitamin B_6 intake on a short term basis, the values observed in the present study suggest that the daily intake of vitamin B_6 by the subjects a day or two prior to the study was probably greater than the 1.5 mg level they received during the study.

Subject 6 excreted 306 μg of total vitamin B_6 in 24 hr urine, which was higher than the amount excreted by other subjects. The reason for this higher level is related to the subject being on a vitamin supplement up to two weeks before beginning the present study. Subject 1, 5 and 6 had lower levels of total vitamin B_6 in urine during the periods they received WHW bread compared to the values observed during the other two periods. Subject 8 showed a tendency toward a gradual decrease in his urinary vitamin B_6 as the study progressed, probably indicating a gradual adjustment to the level of vitamin B_6 intake. A somewhat similar trend was also observed in subjects 1 and 3.

Toward the end of the study, the urinary vitamin B_6 levels of all the subjects appeared to be closer together, with a range of 86 to 175 μg/24 hr compared to 107 to 307 μg/24 hr at the beginning of the study. This suggests that the subjects approached a common vitamin B_6 status as the study progressed. Although one objective of the adjustment period was to achieve such a common status,

the six days of adjustment did not seem to be sufficient.

The overall mean and standard deviation for total urinary vitamin B_6 was 125.9 ± 32.7, 128.4 ± 28.0 and 125.9 ± 29.8 μg/24 hr for the periods when WHW, WB_6 and W breads were served, respectively. The slightly higher value observed with WB_6 bread did not appear to be significant (Appendix 6).

The urinary excretion of free vitamin B_6 are given in Table 16. These figures also follow a pattern very similar to that of the total vitamin B_6 in urine. The overall means for the free vitamin were 92.1 ± 35.7, 93.7 ± 29.6 and 92.2 ± 33.2 μg/24 hr when WHW, WB_6 and W breads were fed, respectively. Sauberlich, Skala and Dowdy have reported that with a vitamin B_6 intake of 1.5 mg, about 35-55 μg of free vitamin B_6/24 hr is excreted in the urine (49). This range is lower than that in the present study. The total and free vitamin B_6 levels reported in the present investigation are more in agreement with the values reported by Kokkeler (117). The means and standard deviations reported in this latter study were 136 ± 73 and 83 ± 60 μg/24 hr for urinary total and free vitamin B_6, respectively.

The ratio of total to free urinary vitamin B_6 is presented in Table 17. The ratio for each subject remained constant, but there was considerable variation between subjects. According to Scriver and Cullen the total urinary vitamin B_6 excreted by the healthy subjects on regular diets was five times the amount of the free

Table 16. Urinary excretion of free vitamin B_6 ($\mu g/24$ hr.).

	Day	1	2	3	4	5	6	7	8	9	Overall Mean ± S.D.[b]
ADJUSTMENT PERIOD											
	1	100.2	154.5	64.5	181.0	116.6	268.2	122.8	157.6	97.4	140.3 ± 59.8
	6	79.9	118.2	65.4	117.5	75.0	175.5	122.3	155.9	67.7	108.6 ± 39.6
EXPERIMENTAL PERIODS											
Diet		W	WB_6	WB_6	WHW	WB_6	W	W	WHW	WHW[a]	
Week 1	2	70.5	120.7	63.0	119.3	76.8	165.5	88.7	169.5	60.2	
	4	71.8	85.3	50.4	107.9	82.0	152.5	79.7	170.0	66.0	
	6	72.6	101.3	59.2	102.7	74.0	150.0	80.4	143.8	61.7	
	7	69.2	107.8	62.3	113.0	65.9	163.9	79.3	163.3	57.6	
Mean ± S.D.[b]		71.0 ±1.5	103.8 ±14.7	58.8 ±5.8	110.7 ±7.1	74.7 ±6.7	158.0 ±7.8	82.0 ±4.5	161.6 ±12.3	61.4 ±3.5	
Diet		WB_6	W	WHW	W	WHW	WHW	WB_6	WB_6	W[a]	
Week 2	2	71.0	133.3	60.2	96.8	66.9	127.4	84.8	156.0	65.9	
	4	71.2	128.0	61.6	117.1	61.7	134.1	72.8	152.7	72.8	
	6	63.1	112.9	51.7	98.0	59.3	134.4	60.6	149.1	74.9	
	7	75.5	121.8	54.3	102.0	58.3	129.2	81.9	138.1	62.3	
Mean ± S.D.[b]		70.2 ±5.2	124.2 ±8.8	57.0 ±4.7	103.5 ±9.4	61.6 ±3.9	131.3 ±3.5	75.0 ±10.9	148.9 ±7.8	69.0 ±5.8	
Diet		WHW	WHW	W	WB_6	W	WB_6	WHW	W	WB_6[a]	
Week 3	2	66.0	105.2	54.4	101.7	56.6	135.6	96.0	130.5	76.8	
	4	64.9	103.0	55.3	101.8	69.4	138.6	74.6	121.1	85.0	
	6	64.5	102.3	56.5	97.4	61.9	126.7	75.3	99.5	83.7	
	7	64.4	94.4	56.3	98.2	60.2	124.0	70.8	87.4	79.6	

Table 16. Continued.

	Day	1	2	3	4	5	6	7	8	9	Overall Mean ± S.D.[b]
						Subject Number					
Mean ± S.D.[b]		65.0 ±0.7	101.2 ±4.7	56.6 ±1.0	99.8 ±2.3	62.0 ±5.4	131.2 ±7.0	79.2 ±11.4	109.6 ±19.7	81.3 ±3.8	
Means WHW		65.0	101.2	57.0	110.7	61.6	131.3	78.2	161.6	61.4	92.1 ± 35.7
WB$_6$		70.2	103.8	58.8	99.8	74.7	131.2	75.0	148.9	81.3	93.7 ± 29.6
W		71.0	124.2	56.6	103.5	62.0	158.0	82.0	109.6	69.0	92.8 ± 33.2

a. WHW, WB$_6$ and W see Appendix 5.

b. Mean ± Standard Deviation.

Table 17. Urinary vitamin B_6 expressed on different basis.

| | Diet | \multicolumn{9}{c}{Subject Number} | Overall Mean ± S.D.[a] |
		1	2	3	4	5	6	7	8	9	
Total vitamin B_6 μg/24 hr	WHW[b]	99.6	133.0	96.3	133.6	91.4	169.7	110.4	187.4	111.9	125.9 ± 32.7
	WB_6	109.5	125.5	98.6	129.7	110.4	176.1	103.6	169.7	132.1	128.4 ± 28.0
	W	110.5	152.4	95.2	133.8	92.7	186.7	117.7	132.3	111.6	125.9 ± 30.0
Free vitamin B_6 μg/24 hr.	WHW	65.0	101.2	57.0	110.7	61.6	131.3	78.2	161.6	61.4	92.1 ± 35.7
	WB_6	70.2	103.8	58.8	99.8	74.7	131.2	75.0	148.9	81.3	93.7 ± 29.6
	W	71.0	124.2	56.6	103.5	62.0	158.0	82.0	109.6	69.0	92.8 ± 33.2
Total/free	WHW	1.55	1.32	1.70	1.20	1.48	1.30	1.40	1.11	1.82	1.43 ± 0.23
	WB_6	1.57	1.21	1.68	1.30	1.48	1.34	1.39	1.14	1.62	1.41 ± 0.19
	W	1.56	1.23	1.72	1.29	1.50	1.18	1.43	1.21	1.62	1.42 ± 0.20
Total vitamin B_6 μg/g creatinine	WHW	50.7	83.9	73.6	89.3	72.2	101.9	75.1	108.8	64.0	79.9 ± 18.2
	WB_6	51.4	73.2	70.0	79.8	80.0	109.2	72.0	101.4	81.2	80.3 ± 16.3
	W	53.9	80.6	67.0	75.4	72.2	103.7	71.9	98.1	67.0	76.6 ± 15.6
Free vitamin B_6 μg/g creatinine	WHW	33.0	63.9	43.5	73.6	48.6	78.4	53.6	93.5	35.1	58.1 ± 20.7
	WB_6	35.5	60.4	41.7	61.5	54.1	81.5	52.0	89.1	50.0	58.4 ± 17.4
	W	34.6	65.6	39.1	58.4	48.3	87.8	50.1	81.0	41.5	56.3 ± 18.6
Total vitamin B_6 as % basal	WHW	77	91	84	87	80	81	70	108	90	86 ± 10
	WB_6	85	86	86	85	97	84	66	98	106	88 ± 11
	W	86	104	83	88	81	89	75	76	90	86 ± 9
Free vitamin B_6 as % basal	WHW	81	86	87	94	82	75	65	104	91	85 ± 11
	WB_6	88	88	90	85	100	75	61	96	120	89 ± 16
	W	89	105	85	88	83	90	67	70	102	86 ± 12

Table 17. Continued.

| | Diet | \multicolumn{9}{c}{Subject Number} | Overall Mean $\pm$ S.D.[a] |
		1	2	3	4	5	6	7	8	9	
Total vitamin B_6	WHW	6.3	8.4	6.1	8.4	9.7	10.7	7.0	11.8	7.0	8.4 + 2.0
as % intake	WB_6	7.0	8.1	6.3	8.3	9.5	11.3	6.7	10.9	8.5	8.5 $\pm$ 1.8
	W	7.2	9.9	6.2	8.7	8.0	12.1	7.6	8.6	7.2	8.4 $\pm$ 1.8
Free vitamin B_6	WHW	4.1	6.4	3.6	7.0	5.2	8.3	5.0	10.2	3.9	6.0 $\pm$ 2.2
as % intake	WB_6	4.5	6.7	3.8	6.4	6.4	8.4	4.8	9.6	5.2	6.2 $\pm$ 1.9
	W	4.6	8.1	3.7	6.7	5.4	10.3	5.3	7.1	4.5	6.2 $\pm$ 2.1

a. Mean $\pm$ Standard Deviation

b. WHW, WB_6 and W   See Appendix 5.

vitamin excreted (192). In the case of vitamin B_6 dependant subjects receiving 10-50 mg of pyridoxine daily, the total urinary vitamin B_6 excreted was only twice that of the free vitamin. The ratio of total to free urinary vitamin B_6 in the present study ranged from 1.11 to 1.90, which is less than that observed by Scriver and Cullen in their healthy subjects (192). They reported 0.61 and 0.12 mμ moles/Kg/hr for the total and free urinary vitamin B_6 excreted, respectively. When expressed on the same basis, the average total and free urinary excretion of vitamin B_6 in the present study are 0.42 and 0.31 mμ moles/Kg/hr, respectively. The ratio of total to free urinary excretion of vitamin B_6 observed by Kokkeler (117) in healthy female subjects ranged from 1.5 to 2.5, which is closer to the range observed in the present study, than the ratio reported by Scriver and Cullen. In the study done by the latter group, hydrolysis of urine was carried out by autoclaving 5 to 10 ml of urine in 180 ml of 0.11 $\underline{N}$ HCl at 20 psi for 5-7 hr in contrast to the 30 min autoclaving time used in the present investigation as well as in the study by Kokkeler. The difference in the ratios of total to free urinary excretion of vitamin B_6 in the above studies may be due to the difference in the autoclaving time. Further, Scriver and Cullen used urine samples collected between midnight and 8 am before breakfast while 24 hr urine collections were made in the present investigation. There was no marked difference in the ratios of total to free urinary vitamin B_6

observed with the three types of bread. However, two out of nine

subjects (No. 2 and 9) had substantially higher ratios with WHW

bread compared to those observed with the other two types of

bread.

Urinary excretion of vitamin B_6 can also be expressed on the

basis of creatinine. This is generally done in cases where a 24 hr

urine collection is impractical (49). The mean total and free urinary

vitamin B_6 values in the present study, expressed as μg/g of creat-

inine are given in Table 17. Expressing the data in this manner did

not indicate a significant difference among the breads. According

to Sauberlich et al., a urinary free vitamin B_6 level of 20 μg/g of

creatinine generally reflects a marginal level of vitamin B_6 intake

(49). In the present study, the free urinary vitamin B_6 level ranged

from a minimum of 33 to a maximum of 93 μg/g of creatinine, with

a mean value of 57 (Table 17). This indicates that the 1.5 mg

vitamin B_6 intake was sufficient.

Considering the value of the day 6 of the adjustment period as

'basal', the total and free urinary vitamin B_6 of the experimental

period were calculated as a percentage of the corresponding basal

value of each subject. The results of total and free vitamin B_6

expressed in this manner are presented in Table 17.

There were no marked differences in the total and free vitamin

B_6 values during the three experimental periods, even when the data

were expressed in several different ways as presented in Table 17. This suggests that the type of bread had no appreciable effect on the urinary excretion of vitamin B_6 in the nine subjects under the conditions of the present study.

The values obtained for the urine sample that was analyzed seven times with alternate assays ranged from 114 to 121 (117 ± 3) and 67 to 75 (71 ± 4) $\mu g/24$ hr, for the total and free vitamin B_6 contents, respectively. These results indicate that the urine sample was fairly stable when stored frozen and the variability of the micro-biological method from one assay to another was well within the expected range of 10 to 15 percent.

When the excretion of total vitamin B_6 was expressed as a percentage of the dietary intake of the vitamin B_6, values of 8.4 ± 2.0, 8.5 ± 1.8 and 8.4 ± 1.8 were obtained when subjects were fed diets based on WHW, WB_6 and W bread, respectively (Table 17). The corresponding percentage of free vitamin B_6 were 6.0 ± 2.2, 6.2 ± 1.9 and 6.2 ± 2.1. The lower level of vitamin B_6 intake (1.2 mg) of subject 5 was taken into consideration in calculating these percentages. The data suggests that only a small percentage of the dietary intake of vitamin B_6 was excreted in urine as vitamin B_6. According to Levy, only about 5 percent of the total intake of vitamin B_6 is accounted for in the sum of PAL, PIN and PAM excreted in the urine (98).

As mentioned previously, the predominant form of vitamin B_6 in the diets was PIN. However PAL represented the largest percentage of vitamin B_6 in the urine of the subjects. The amounts of PAL, PIN and PAM excreted in the urine on the day 7 of each period for five selected subjects are given in Table 18. When PAL, PIN and PAM were expressed as percentages of the sum of the three forms (Table 19), there was a significantly lower value of 54.52 ± 3.31 for PAL when the subjects were fed WHW bread as compared to 60.68 ± 7.52 and 63.13 ± 3.84, for WB_6 and W bread diets, respectively $(P < 0.01)$.

Kelsay, Baysal and Linkswiler have reported that PAL was excreted in the largest amount in the urine of subjects who received a supplement of 1.5 mg of PIN-HCl in addition to a self selected diet (107). In their study PAL accounted for 60-65% and PAM 30-35% of the urinary vitamin B_6, with only a negligible amount of PIN. These proportions have also been reported by Sauberlich, et al. as normal proportions of the three forms of vitamin B_6 in human urine (49). The results of Kelsay et al. (107) and those of the present study seem to be in close agreement although the percentages of PIN reported in the latter cannot be considered negligible. Somewhat different proportions of the three forms of vitamin B_6 in urine have been reported by Contractor ans Shane (58). They observed 13.69, 23.37 and 62.73% of PAL, PIN and PAM, respectively in the urines

Table 18. Pyridoxal (PAL), pyridoxine (PIN) and pyridoxamine (PAM) in the urines of five subjects (μ g/24 hr).

Subject	1	3	4	6	7
			ADJUSTMENT PERIOD		
Form of Vitamin B_6					
PAL	56.47	45.78	69.93	100.43	75.76
PIN	15.54	10.74	16.07	25.48	10.70
PAM	26.19	26.04	23.04	28.56	27.45
			EXPERIMENTAL PERIODS		
			WHW		
PAL	44.66	34.54	58.84	76.62	43.22
PIN	14.31	11.02	16.04	21.14	9.74
PAM	25.31	24.41	28.64	37.56	23.15
			WB_6		
PAL	46.88	41.05	58.01	83.54	50.37
PIN	12.39	13.70	12.96	17.75	5.17
PAM	24.08	27.96	19.47	24.88	19.45
			W		
PAL	47.91	45.21	59.83	114.33	50.13
PIN	12.10	8.18	14.60	24.31	6.48
PAM	18.60	22.84	23.67	28.98	19.11

WHW, WB_6 and W see Appendix 5.

Table 19. Urinary pyridoxal (PAL), pyridoxine (PIN) and pyridoxamine (PAM) as a percentage of the total vitamin B_6.

Form of Vitamin B_6	PAL	PIN	PAM
	Percentages in urine		
Adjustment period	61.5 ± 5.4[a]	13.8 ± 2.9	24.2 ± 4.8
Experimental periods			
WHW[b]	54.5 ± 3.3	15.3 ± 1.5	30.2 ± 2.9
WB_6	60.7 ± 7.5	13.3 ± 3.7	25.9 ± 5.6
W	63.1 ± 3.8	12.8 ± 3.0	24.1 ± 4.3
Literature values (107)	60-65	little or none	30-35
	Percent recoveries		
Present study			
Standard	83-99	95-102	82-90
Added to urine	91-112	91-124	98-103
Literature values (193)			
Standard	80-125	70-105	70-130
Added to urine	85-125	70-130	86-134

a. Mean $\pm$ Standard Deviation for five selected subjects.

b. WHW, WB_6 and W see Appendix 5.

analyzed by a method employing phosphocellulose columns. This latter method is different from the one employed in the present study as well as that used by Kelsay et al. (107).

The percent recoveries of PAL, PIN and PAM of the chromatographed standards compared against the appropriate unchromatographed standards are presented in Table 19. Additional checks were done by mixing known amounts of the standards with the urine samples. The ranges observed in the present study were closer to 100 percent than those reported by Kelsay (193).

Urinary excretion of PAL, PIN and PAM expressed as a percentage of total intake of vitamin B_6 from the diets based on WHW WB$_6$ and W breads are presented in Figure 7. The amount of urinary PAL, expressed as a percentage of the total intake of vitamin B_6, while consuming the WHW bread diet was 4.2 ± 1.6, compared to 4.8 ± 1.4 and 5.4 ± 2.5 for WB$_6$ and W bread diets, respectively.

The possible reason for the lower percentage of PAL in urine when the diet contained WHW bread may be because the fecal excretion of vitamin B_6 was comparatively higher with this type of bread and a smaller percentage was available to the body. The lower PAL concentration of urines associated with the WHW bread was also reflected by the urinary 4-PA levels, which will be discussed in the next section.

Figure 7. Urinary excretion of PAL, PIN and PAM as a percentage of total intake of vitamin B_6 (data for five subjects).

Urinary Excretion of 4-Pyridoxic Acid

The urinary excretion of 4-PA by the nine subjects during the adjustment and the experimental periods is presented in Table 20. The order in which the three types of bread were fed is also indicated. With the exception of subjects 4 and 8, all subjects excreted a lower level of urinary 4-PA when they received WHW bread as compared to when WB_6 or W bread was fed. The amount of urinary 4-PA excreted on the day 6 of the period when WHW bread was fed was significantly lower ($P < 0.01$) than the amount when WB_6 or W bread was fed. When the amount of 4-PA was expressed as a percentage of the basal level (Table 21), the values for days 6 ($P < 0.01$) and 7 ($P < 0.05$) of the period when WHW bread was consumed were significantly lower than the corresponding percentage for the period when WB_6 or W bread was consumed. The corresponding percentages for the days 2 and 4 were not significantly different for WHW bread as compared to the other two types, which indicates that the effect of the type of bread on the excretion of 4 PA was more significant toward the end of the period than at the beginning. The reason for this difference may be that there was some carry-over effect to the first few days from the previous week's diet. The mean of 4-PA for the days 4, 6 and 7 was 3.28 $\pm$ 0.96 μM/24 hr when WHW bread was consumed. This value was significantly lower than

Table 20. Urinary 4-pyridoxic acid (μM/24 hr.).

	Day	Subject Number 1	2	3	4	5	6	7	8	9	Overall Mean ± S.D.
ADJUSTMENT PERIOD											
	1	6.07	5.12	3.98	6.26	3.79	7.78	4.17	6.83	3.04	5.23 ± 1.60
	6	4.45	3.84	3.44	5.06	2.43	6.88	4.25	6.68	2.63	4.41 ± 1.58
EXPERIMENTAL PERIODS											
Diet		W	WB_6	WB_6	WHW	WB_6	W	W	WHW	WHW[a]	
Week 1	2	4.43	5.46	3.60	4.74	2.47	6.07	4.36	5.88	1.90	
	4	4.48	3.35	3.14	4.44	3.04	5.10	3.93	5.38	2.19	
	6	4.43	4.17	4.17	3.79	2.08	4.80	3.79	4.36	1.90	
	7	3.85	4.84	3.88	4.02	2.31	4.66	4.39	5.38	1.69	
Mean ± S.D.[b]		4.30	4.46	3.70	4.25	2.48	5.16	4.12	5.25	1.92	
		±0.3	±0.9	±0.4	±0.4	±0.4	±0.6	±0.3	±0.6	±0.2	
Diet		WB_6	W	WHW	W	WHW	WHW	WB_6	WB_6	W	
Week 2	2	4.43	5.88	3.60	3.79	2.08	3.98	4.17	5.69	1.99	
	4	4.32	4.84	2.77	4.14	2.47	3.22	3.99	5.38	2.59	
	6	4.20	5.12	2.71	3.60	1.71	3.15	3.79	5.12	3.04	
	7	4.77	4.20	2.94	3.57	2.11	3.04	4.11	4.56	3.39	
Mean ± S.D.		4.43	5.01	3.00	3.78	2.09	3.45	4.02	5.19	2.75	
		±0.2	±0.7	±0.4	±0.3	±0.3	±0.4	±0.2	±0.5	±0.6	
Diet		WHW	WHW	W	WB_6	W	WB_6	WHW	W	WB_6	
Week 3	2	3.51	3.22	2.85	4.17	2.28	3.39	4.78	4.64	3.60	
	4	3.85	3.44	2.95	4.14	2.30	3.79	3.52	4.59	3.23	
	6	3.69	3.04	3.04	3.60	2.66	3.85	3.60	4.55	2.85	
	7	3.34	2.98	3.32	3.58	3.08	3.99	3.73	4.17	2.79	

Table 20. Continued

	Day	1	2	3	4	5	6	7	8	9	Overall Mean $\pm$ S.D.
					Subject Number						
Mean $\pm$ S.D.		3.60 ± 0.2	3.17 ± 0.2	3.04 ± 0.2	3.87 ± 0.3	2.58 ± 0.4	3.74 ± 0.3	3.91 ± 0.6	4.49 ± 0.2	3.12 ± 0.4	
Mean WHW		3.60	3.17	3.00	4.25	2.09	3.45	3.91	5.25	1.92	3.40 $\pm$ 1.03
WB_6		4.43	4.46	3.70	3.87	2.48	3.74	4.02	5.19	3.12	3.89 $\pm$ 0.79
W		4.30	5.01	3.04	3.78	2.58	5.16	4.12	4.17	2.75	3.91 $\pm$ 0.95

a. WHW, WB_6 and W   see Appendix 5.

b. Mean $\pm$ Standard Deviation.

Table 21. Urinary 4-pyridoxic acid expressed as a percentage of the basal level.

Experimental Periods	Day	Subject Number									Overall Mean ± S.D.
		1	2	3	4	5	6	7	8	9	
Week 1		W	WB_6	WB_6	WHW	WB_6	W	W	WHW	WHW[a]	
	2	100	142	105	94	102	88	102	88	72	
	4	101	87	91	88	125	74	92	80	83	
	6	100	108	121	75	86	70	89	65	72	
	7	86	126	113	79	95	68	103	80	64	
Mean ± SD[b]		96	116	107	84	102	75	97	78	73	
		±7	±24	±13	±8	±17	±9	±7	±10	±8	
Week 2		WB_6	W	WHW	W	WHW	WHW	WB_6	WB_6	W	
	2	100	153	105	75	86	58	98	85	76	
	4	97	126	80	82	102	47	94	80	98	
	6	94	133	79	71	70	48	89	77	116	
	7	107	109	85	70	87	44	97	68	129	
Mean ± SD		100	130	87	75	86	49	94	78	105	
		±6	±18	±12	±5	±13	+6	±4	±7	±23	
Week 3		WHW	WHW	W	WB_6	W	WB_6	WHW	W	WB_6	
	2	79	84	83	82	94	48	112	69	137	
	4	86	90	86	82	95	55	83	69	123	
	6	83	79	88	72	109	56	85	68	108	
	7	75	78	96	71	127	58	88	62	106	
Mean ± SD		81	82	88	76	106	54	92	67	118	
		±5	±5	±6	±6	±16	±4	±14	±3	±14	

Table 21. Continued

Experimental Periods		Subject Number									Overall Mean $\pm$ S.D.
		1	2	3	4	5	6	7	8	9	
Mean	WHW	81	82	87	84	86	49	92	78	73	79 ± 13
	WB_6	100	116	107	76	102	54	94	78	118	94 ± 21
	W	96	130	88	75	106	75	97	67	105	93 ± 20

a. WHW, WB_6 and W see Appendix 5.

b. Mean $\pm$ Standard Deviation.

3.82 $\pm$ 0.75 and 3.87 $\pm$ 0.78, the mean of 4-PA for days 4, 6 and 7

when WB_6 and W bread were fed, respectively. Although the mean

of 4-PA for days 2,4, 6 and 7 was lower when WHW bread was fed

than when WB_6 or W bread was fed, this difference was not

significant.

The amount of 4-PA reflects, in part, the vitamin B_6 that is

metabolized in the body (49). The above results suggest that the

quantity of vitamin B_6 while the subjects received WHW bread was

lower than the amount metabolized from either of WB_6 or W breads.

In the metabolic interconversions of vitamin B_6 (Figure 1), it is

seen that PAL is the only form that is oxidized to 4-PA by the

action of aldehyde oxidase. From the results of urinary excretion

of the three forms vitamin B_6, it was clear that the concentration

of PAL in the urines of the subjects while consuming WHW bread was

less than that during the other two periods. This indicates that the

factor or factors which contribute to the lower level of urinary PAL

may also have contributed to the lower levels of urinary 4-PA in

subjects receiving WHW bread.

From the results discussed so far, it is possible to infer that

availability of vitamin B_6 from WHW bread, the natural source, was

lower than that from the other two types of bread in which vitamin B_6

was provided mainly as the synthetic form, PIN-HCl, either

incorporated into bread or in WB_6, or given as an oral supplement

with W bread. A study of the absorption of vitamin B_6 from a natural

and a synthetic source was done by Nelson et al. (135). Their data

suggest that absorption of vitamin B_6 was significantly lower from

the natural source, orange juice, than from the synthetic solution.

The lower availability of vitamin B_6 from WHW bread seen in the

present study may be because vitamin B_6 is either bound to other

constituents in the whole wheat flour and was not all released during

digestion or that digestibility of WHW bread was lower due to the

presence of more fiber (9. 1 g) compared to WB_6 or W (1. 2 g) bread.

The higher level of excretion of vitamin B_6 in feces when the sub-

jects were fed WHW bread (4.9 mg/wk) as compared to when WB_6

(3. 1 mg/wk) or W (3. 0 mg/wk) bread was fed, support the above

idea.

Subjects 4 and 8 excreted a higher amount of 4-PA when WHW

bread was fed than when WB_6 or W bread was fed. These subjects

were two of three who received WHW bread the first week after the

adjustment period. During the six day adjustment period, all the

subjects consumed gradually increasing amounts of whole wheat

bread as part of the adjustment diet. This may have contributed

to the adaptation of the digestive system in digesting WHW bread for

these subjects receiving this type of bread immediately after the

adjustment period. However, subject 9, who also received WHW

bread the first week following the adjustment period, excreted lower

levels of 4-PA during this same period than during the period when WB_6 or W bread was fed. The reason for this difference between subjects 4 and 8 and subject 9 is not clear. The urinary 4-PA values of subject 9 were, in general, lower than those of the other subjects, which may suggest that he had an altered metabolism of vitamin B_6.

The 4-PA level observed on the day 1 of the adjustment period was higher than that of the day 6 of the same period. The higher initial 4-PA value indicates that the level of vitamin B_6 the subjects had been consuming prior to the study was greater than 1.5 mg. This trend was also indicated by the urinary excretion of vitamin B_6 (Table 15). The diet consumed by the subjects the day before the first day of the study was not evaluated for vitamin B_6 content. Had the vitamin B_6 levels consumed on that day been known, a more definite statement could be made in relation to the higher excretion of urinary vitamin B_6 and 4-PA levels.

It has been reported that 20-50% of the ingested vitamin B_6 is converted to 4-PA in the adult while the remainder is excreted via other routes (49). In the present study (Table 22), 37.2 ± 10.1 percent of the dietary intake of vitamin B_6 was accounted for as urinary 4-PA when the subjects received WHW bread. The corres-ponding percentages for WB_6 and W breads were 43.3 ± 6.9 and 44.0 ± 9.1, respectively. These values show that a higher percentage of

Table 22. Urinary 4-Pyridoxic acid excreted during the three experimental periods, expressed as percentage of vitamin B_6 intake.

Experimental Period	Subject Number									Overall mean ± S.D.
	1	2	3	4	5	6	7	8	9	
WHW	38.4	33.8	32.0	45.3	29.9	36.8	41.7	56.0	20.5	37.2 ± 10.1
WB_6	48.2	48.5	40.2	42.1	36.0	40.7	43.7	56.4	33.9	43.3 ± 6.9
W	47.2	55.0	33.4	41.5	37.8	56.7	45.2	49.3	30.2	44.0 ± 9.1

WHW, WB_6 and W - see Appendix 5.

the dietary intake was metabolized into 4-PA when the subjects were

fed WB_6 and W bread compared to WHW bread assuming the same

amount of vitamin B_6 was absorbed from all three sources.

Urinary excretion of 4-PA in humans has been found to be

dependent on body weight (194). Young found a negative relationship

between body weight and urinary 4-PA and reported that the lighter

subjects may have had a smaller body pool of vitamin B_6, thereby

making more vitamin B_6 available for conversion to the metabolite.

The body weight versus μM of 4-PA/24 hr of urine determined in the

present study is shown in Figure 8. Even if the data on subject 5

are disregarded, due to his lower intake of vitamin B_6, there seems

to be a positive relationship in that heavier subjects excrete com-

paratively more urinary 4-PA than the lighter ones. This indicates

that more vitamin B_6 has been converted to urinary 4-PA in persons

with a greater body weight. The larger body size and probably larger

size of organs (e.g., liver) of such persons may lead to increased

capacity to metabolize more vitamin B_6 than persons of smaller

body size. Further research is needed before a definite statement

can be made with regard to the relationship between body weight and

excretion.

Vitamin B_6 Balance

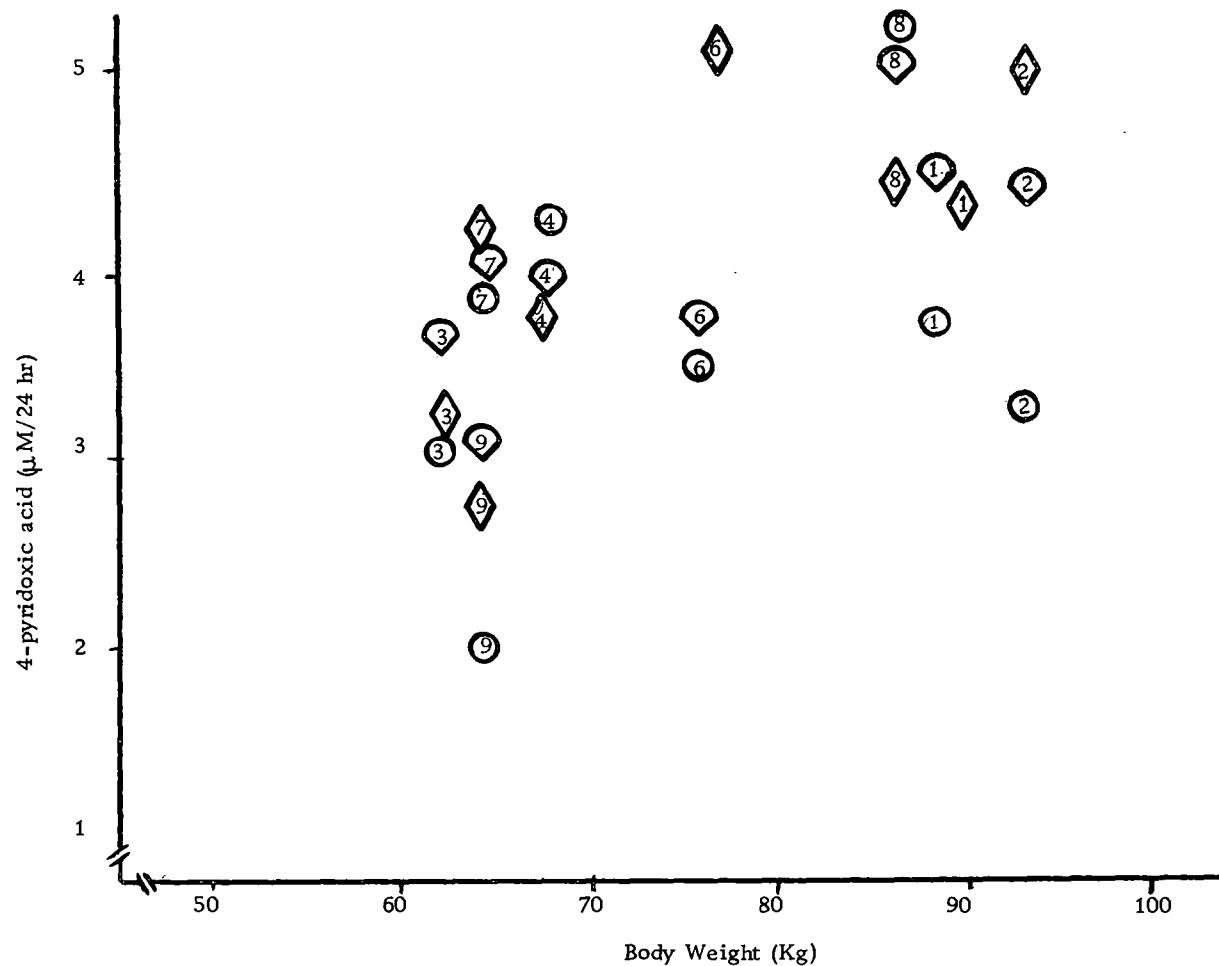The data on the intake, excretion and balance of vitamin B_6

Figure 8. Mean urinary excretion of 4-PA versus body weight during the experimental periods; ◯ WHW, ◗ WB_6 and ◇ W, for the nine (1-9) subjects. (Data for No. 5 not included because of his lower intake of vitamin B_6.)

during the three experimental periods is presented in Table 23.

The daily intake of vitamin B_6 for all the subjects except No. 5

was 9.37, 9.20 and 9.11 μM for WHW, WB_6 and W bread diets,

respectively, while the corresponding intake of subject No. 5 was

6.99, 6.88 and 6.82 μM, respectively. For all subjects, the sum of

the excretion of urinary and fecal vitamin B_6 and urinary 4-PA was

8.26 $\pm$ 1.32, 7.29 $\pm$ 1.12 and 7.20 $\pm$ 1.13 μM/24 hr, for WHW,

WB_6 and W bread diets, respectively. Of these excretory products,

total urinary vitamin B_6 and 4-PA levels reflect the amount of

vitamin B_6 that was absorbed. In the present study, this amount

was 4.14, 4.65, and 4.65 μM/day when subjects were fed WHW,

WB_6 and W bread, respectively. These amounts represent 45, 52

and 52 percent of the daily intake of vitamin B_6 for the WHW, WB_6

and W bread diets, respectively. Assuming that all the vitamin B_6

has been accounted for, there was a positive balance for vitamin B_6

with the three types of bread, since the intake of vitamin B_6 exceeded

the excretion as measured in this study. The same relationship

was true of the means observed for the individual subjects for all

the experimental periods, with the exception of No. 8, in whom the

average sum of daily excretion exceeded the intake of vitamin B_6

by 0.96 μM, during the period he received WHW bread. The overall

mean and standard deviation for the balance was 0.84 $\pm$ 0.99,

Table 23. Intake, excretion and balance of vitamin B_6 during the three experimental periods ($\mu \cdot M/24$ hr.).

	Diet					Subject Number					Overall Mean ± SD
		1	2	3	4	5	6	7	8	9	
Intake	WHW	9.37	9.37	9.37	9.37	6.99	9.37	9.37	9.37	9.37	9.10 ± 0.79
	WB_6	9.20	9.20	9.20	9.20	6.88	9.20	9.20	9.20	9.20	8.94 ± 0.77
	W	9.11	9.11	9.11	9.11	6.82	9.11	9.11	9.11	9.11	8.86 ± 0.76
Excretion Fecal B_6	WHW	4.98	3.04	5.24	3.55	3.29	3.80	4.39	3.97	4.73	4.11 ± 0.77
	WB_6	2.28	2.53	3.55	2.96	1.69	3.29	2.36	2.53	2.62	2.64 ± 0.58
	W	2.79	2.36	3.29	2.87	1.69	2.20	2.03	2.70	2.96	2.43 ± 0.54
Urinary B_6	WHW	0.59	0.79	0.57	0.79	0.54	1.00	0.65	1.11	0.66	0.74 ± 0.20
	WB_6	0.65	0.74	0.58	0.77	0.65	1.04	0.61	1.00	0.78	0.76 ± 0.16
	W	0.65	0.90	0.56	0.79	0.55	1.10	0.67	0.78	0.66	0.74 ± 0.18
Urinary 4-PA	WHW	3.60	3.17	3.00	4.25	2.09	3.45	3.91	5.25	1.92	3.40 ± 1.03
	WH_6	4.43	4.46	3.70	3.87	2.48	3.74	4.02	5.19	3.12	3.89 ± 0.79
	W	4.30	5.01	3.04	3.78	2.58	5.16	4.12	4.49	2.75	3.91 ± 0.95
Sum of Excretion	WHW	9.17	7.00	8.81	8.59	5.92	8.25	8.95	10.33	7.31	8.26 ± 1.32
	WB_6	7.36	7.73	7.83	7.60	4.82	8.07	6.99	8.72	6.52	7.29 ± 1.12
	W	7.74	8.27	6.89	7.44	4.82	8.46	6.82	7.97	6.37	7.20 ± 1.13
Balance	WHW	0.20	2.37	0.56	0.78	1.07	1.12	0.42	-0.96	2.06	0.84 ± 0.99
	WB_6	1.84	1.47	1.37	1.60	2.06	1.13	2.21	0.48	2.68	1.65 ± 0.64
	W	1.37	0.87	2.22	1.67	2.00	0.65	2.29	1.14	2.74	1.66 ± 0.71

1.65 ± 0.64 and 1.66 ± 0.71 µM/day when the subjects were fed

WHW, WB_6 and W bread diets, respectively.

A positive vitamin B_6 balance was observed by Levy in his

subjects when a vitamin B_6 intake of 2-3 mg was considered in

relation to the urinary and fecal excretion of PAL, PIN, PAM and

4-PA (98). These excretory products accounted for approximately

40% of the vitamin B_6 intake. On the average, 60% of the intake was

unaccounted for in their excretory products, and it is unlikely that

the subjects Levy studied retained that much, unless they were

deficient in vitamin B_6. The percentage unaccounted for by Levy

was much higher than the 10 to 20 percent unaccounted for in the

present study, where the average intake of vitamin B_6 was 1.5 mg.

In contrast to the above investigations, the studies done by

Linkswiler and Reynolds (48) and Yano and Fujita (103) revealed that

excretion of vitamin B_6 and 4-PA by humans accounted for more

vitamin B_6 than consumed. They considered this as evidence of the

synthesis of vitamin B_6 by the microflora present in the human

intestine. With a vitamin B_6 intake of 0.5 to 1.2 mg in nine sub-

jects, Linkswiler and Reynolds observed the total excretion of

vitamin B_6 and 4-PA. to be 3.2 to 3.8 mg. Of this, 1.6 to 2.8 mg

was accounted for as urinary 4-PA. The method of 4-PA deter-

mination used by Linkswiler and Reynolds (48) was that of Huff and

Perlzweig (46). This method has since been found to be unreliable

due to the incomplete elimination of fluorescent substances other than 4-PA. This may have led to an overestimation of urinary 4-PA. The method used by Yano and Fujita (103) for the determination of 4-PA was that of Fujita and Fujino (104), which had a drawback similar to that of Huff and Perlzweig, and therefore would result in an overestimation of 4-PA in urine. Therefore, those studies involving 4-PA determined by the older methods (48, 103), need to be viewed with caution.

The mean intake, excretion and the balance of vitamin B_6 when the subjects were fed diets based on WHW, WB_6 and W bread are illustrated in Figure 9. Of the 10 to 20 percent of the vitamin B_6 intake that was not accounted for by the excretory products in the present study, some may have entered the body pool of vitamin B_6, while some may have been excreted via routes not covered in this investigation; for example, sweat and saliva. Vitamin B_6 content of sweat and saliva has been reported as 0.04-0.07 and 0.1-1.7 µg/100 ml, respectively (195). Urinary 4-PA is known to be the major metabolite of vitamin B_6 (49). However, there could be some minor metabolites or intermediary products which are excreted.

A further explanation for the unaccounted vitamin B_6 could be attributed to the microbiological assay of vitamin B_6. The major form of vitamin B_6 of the diet in the present study was PIN. In the determination of total vitamin B_6, the standard curve generally used, is of PIN. The growth of S. carlsbergensis in the presence of PAM has been reported as 80-90 percent of that of PIN and PAL.
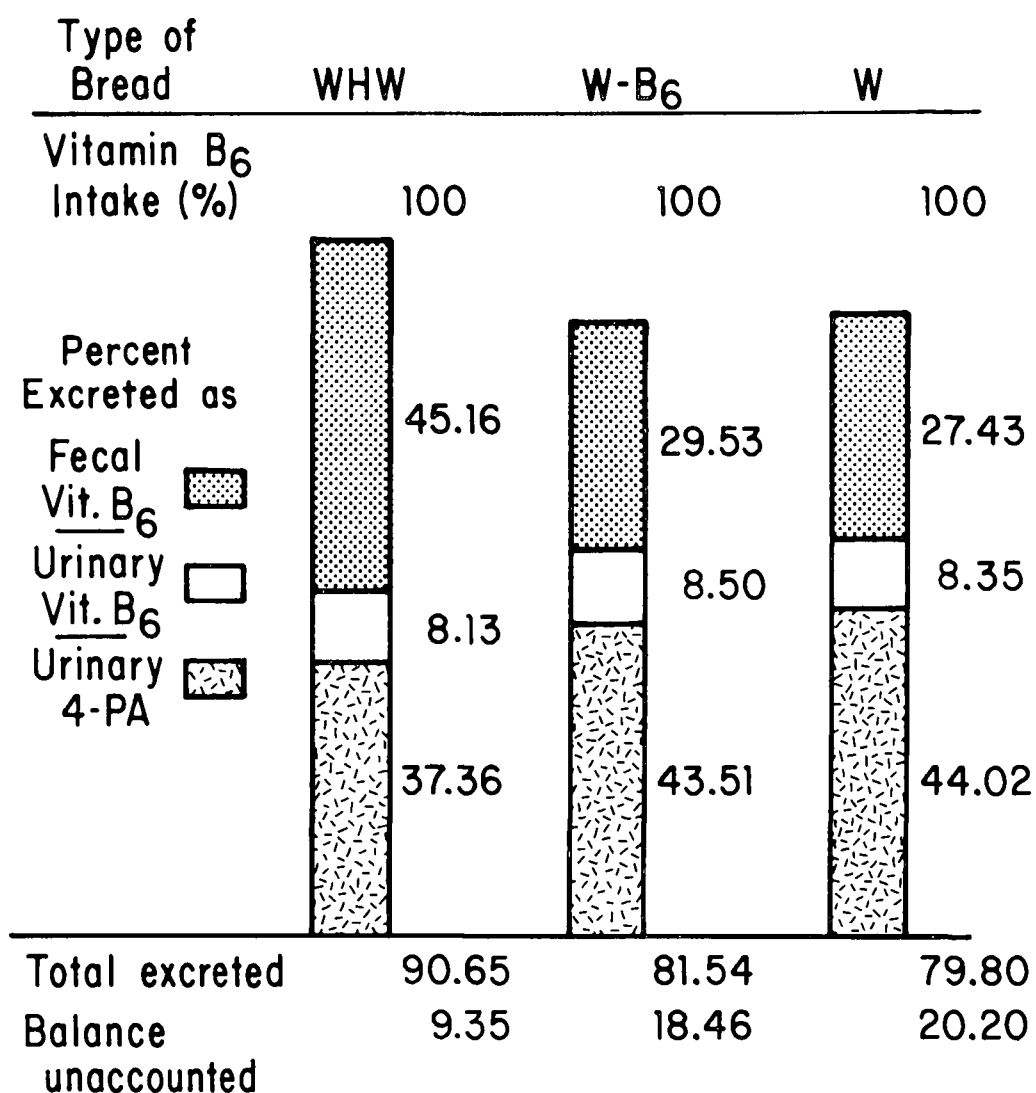
Figure 9. Daily intake, excretion and balance of vitamin B_6 of subjects fed diets based on WHW, WB_6 and W bread.

According to Shane and Snell, the uptake of PIN by S. carlsbergensis exhibits twin pH optima of 3. 5 and 6, PAL being transported primarily by the pH 3. 5 system and PAM by the pH 6 system whereas PIN is transported rather effectively by both systems (196). These observations may explain the reason for PAM being less active than PIN and PAL in supporting yeast growth, as most of the assays are conducted near pH 4. 5.

Yano and Fujita reported that PAM was the predominant form of vitamin B_6 in feces even when the diet contained mostly PIN and PAL (103). Assuming the observation of Yano and Fujita was true for the present study as well, the use of PIN standard to determine fecal vitamin B_6 may have given a value lower than the actual vitamin B_6 content in feces. The major form of urinary vitamin B_6 was determined to be PAL. This has also been reported by Kelsay et al. (107) and Sauberlich et al. (79). Therefore, if a PAL standard had been used instead of PIN for the standard curve to determine the vitamin B_6 excreted in urine, the total vitamin B_6 would have been lower than the levels reported in the present study. Using the appropriate form or a combination of forms in correct proportions would eliminate the errors introduced by the different response of the microorganism to the three forms of vitamin B_6.

As mentioned earlier, the body pool of vitamin B_6 could retain some of the ingested vitamin. Vitamin B_6 status is generally

reflected by the levels of vitamin B_6 in blood and the coenzyme activity (197). The vitamin B_6 parameters in the blood drawn from the subjects in the present study will be available in detail in a separate thesis (198). The mean and standard deviation of plasma vitamin B_6 were 7.5 ± 1.9, 7.9 ± 1.9 and 8.0 ± 1.8 ng/ml when the diet was based on WHW, WB_6 and W bread, respectively. The plasma pyridoxal phosphate (PLP) levels showed a mean and standard deviation of 7.8 ± 2.9, 8.3 ± 3.2 and 8.5 ± 2.9 ng/ml when the subjects were fed WHW, WB_6 and W bread, respectively. However, these differences in the plasma vitamin B_6 and PLP levels in relation to the type of bread were not significant.

Based on the results of the present investigation of the bio-availability of vitamin B_6 from WHW, WB_6 and W bread, the following observations are summarized:

1. There was significantly more vitamin B_6 excreted in feces when the subjects were fed WHW bread than when they were fed WB_6 or W bread.

2. The urinary vitamin B_6 content was not affected by the type of bread consumed.

3. There was a significant decrease of PAL in the urinary vitamin B_6 when the diet was based on WHW bread compared to WB_6 or W bread.

4.   The urinary 4-PA levels were significantly lower when the subjects received WHW bread than when they received WB_6 or W bread.

5.   The percentage of vitamin B_6 intake accounted for, in the excretory products was more when WHW bread was consumed than when WB_6 or W bread was consumed.

RECOMMENDATIONS

The results of this study support the feasibility of enrichment of wheat flour with vitamin B_6, as far as stability and bioavailability are concerned. Enrichment of white flour and bread with vitamin B_6 will have definite advantages for populations that depend on an appreciable amount of refined cereal products in the diet.

According to the results of the present study, the added vitamin B_6 was stable during storage of flour at cold and at room temperature, and during storage of bread under frozen and refrigerated conditions. Both native and added vitamin B_6 was fairly stable during bread making. As far as stability is concerned, enrichment of white flour with vitamin B_6 (PIN-HCl) would pose no problem.

Generally, countries where wheat is not produced, especially those in the tropical belt, import mostly refined flour as opposed to whole wheat flour because of better storage properties of the former. Enrichment of refined flour with vitamin B_6 will be of advantage to consumers of this product in such countries, most of which also have low per capita consumption of animal products, a major source of vitamin B_6 in the developed countries.

Although the results of the present study indicated that vitamin B_6 was less available from whole wheat bread compared to white bread enriched with vitamin B_6 or white bread plus the oral

supplement of vitamin B_6, there are advantages of consuming whole wheat bread which cannot be replaced by vitamin B_6 enriched white bread. One such advantage is the presence of fiber. Concern for fiber in the diet has been emphasized (199). A diet containing whole wheat bread as opposed to white bread will contribute fiber, especially to the typical U.S. diet which tends to be generally low in this constituent. In addition, as compared to white bread, whole wheat bread is also a rich source of several nutrients, especially the B vitamins and some minerals (3).

Although 20-22 slices of bread were consumed daily by the subjects in the present study, 4-6 slices (approximately 125 g) is a reasonable amount an average person would consume per day along with other food items. Some 0.3 mg of vitamin B_6 would be contributed by 125 g of WHW bread whereas the same quantity of WB_6 bread would contribute 0.4 mg. In an average daily diet containing 2 mg of vitamin B_6, the amount of vitamin B_6 contributed by bread will not make a substantial difference in relation to the type of bread consumed, i.e. WHW or WB_6. In diets which are generally low in fiber, WHW bread will supply some fiber in contrast to white bread. Consumption of WHW bread should be encouraged among the public. Perhaps, the enrichment of flour with vitamin B_6 might lessen the shift from refined wheat products to whole wheat products.

The present study was done in nine male subjects in a narrow age range (21-33 years). The results of this study do not lend to making a final decision as to whether refined cereal products should or should not be enriched with vitamin B_6. A study of nutritional status on a more representative population with respect to vitamin B_6 enriched bread will be necessary before a definite recommendation can be made.

Further, the feasibility of distribution of synthetic vitamin B_6 in the flour along with the enrichment mixture used at present needs to be evaluated. Since most of the commercial breads use preservatives to prolong shelf life, the stability of added vitamin B_6 in the presence of such preservatives needs to be studied.

SUMMARY

The objectives of the present study were two fold:

A. To determine the stability of vitamin B_6 in wheat flour during bread making and storage.

B. To investigate the bioavailability of vitamin B_6 from bread, in human subjects.

Three variables; whole wheat flour (WHW), white flour (W) and white flour enriched with vitamin B_6 (WB_6) were tested.

The WB_6 dough prepared using the sponge dough method showed a significant increase in vitamin B_6 content at the end of fermentation compared to that at the beginning ($P < 0.05$). The changes observed during fermentation in vitamin B_6 content of the remaining types of dough were not statistically significant. The WHW and W breads baked under commercial conditions had a significant loss ($P < 0.05$). Under home conditions, the WHW and WB_6 bread prepared using the sponge dough procedure resulted in a baking loss significant at the 1% level while that of W bread was significant at the 5% level compared to the baking loss observed in breads made using straight dough method which was not significant. The baking losses determined in the present study were 3.5 and 8 percent for the breads made using straight and sponge dough methods, respectively, and 13 percent for breads made under commercial conditions.

The WB_6 bread stored under frozen and refrigerated conditions showed good stability of vitamin B_6. However, the WB_6 bread stored under room temperature had a significant loss of 10 percent in the vitamin B_6 content after three days ($P < 0.01$). Mold growth was observed on this bread after five days of storage at room temperature. The level of vitamin B_6 of WB_6 flour stored in the cold room and at room temperature did not decrease over a period of 26 weeks, suggesting that added vitamin B_6 was stable during storage of flour. The results of the first part of the study indicate good stability of vitamin B_6 in wheat flour during bread making and storage.

Bioavailability of vitamin B_6 from the three types of commercial bread was studied in nine men, age 21-33 years. The level of vitamin B_6 intake was 1.5 mg, of which 1.2 was supplied from the bread. The major form of vitamin B_6 in the diets was PIN.

Fecal excretion of vitamin B_6 was 4.9 ± 0.9 mg/wk when WHW bread was fed. This level of vitamin B_6 was significantly ($P < 0.01$) higher than the levels of 3.1 ± 0.7 and 3.0 ± 0.6 mg/wk obtained when WB_6 and W breads were fed, respectively. The greater fecal excretion of vitamin B_6 during the period when WHW bread was fed was associated with a greater fecal weight. This was due to the high fiber content of the diet based on WHW bread. The percentage of daily intake of vitamin B_6 excreted in feces was 45.2 when WHW

bread was given, compared to 29.5 and 27.4 when WB_6 and W breads were given, respectively.

There was no significant difference in the urinary excretion of total and free vitamin B_6 in relation to the type of bread fed. Approximately 8 percent of the daily intake of vitamin B_6 was excreted in the urine as total vitamin B_6. The predominant form of urinary vitamin B_6 was PAL. Pyridoxal accounted for 54.5 percent of urinary vitamin B_6 when WHW bread was fed. This percentage was significantly lower ($P < 0.01$) than the 60.7 percent and 63.1 percent of the urinary vitamin B_6 as PAL when WB_6 and W bread were fed, respectively.

The mean of urinary 4-PA of days 4, 6 and 7 of each week was 3.28 ± 1.0, 3.82 ± 0.8 and 3.87 ± 0.8 $\mu M/24$ hr when the diets were based on WHW, WB_6 and W bread, respectively. The excretion of 4-PA in subjects receiving WHW bread was lower, with the exception of two of nine subjects. When expressed as a percentage of vitamin B_6 intake, 37.2, 43.3 and 44.0 percent was excreted as 4-PA when the diets were based on WHW, WB_6 and W bread, respectively. The lower values observed for the period when WHW bread was fed suggests that the amount of vitamin B_6 absorbed and/or metabolized during the same period was lower than that with WB_6 or W bread.

When the excretory products of vitamin B_6 analyzed in the
present study are summed, 91.6 percent of the vitamin B_6 intake
was accounted for when WHW bread was fed, compared to 81.5 and
79.8 percent for WB_6 and W bread, respectively. Plasma levels of
vitamin B_6 and pyridoxal phosphate were found to be slightly lower
during the period WHW bread was consumed, compared to the
periods WB_6 and W bread were consumed.

These data suggest that vitamin B_6 was not as available from
WHW bread as from WB_6 or W bread. Under the conditions of the
present study, the availability of vitamin B_6 from WB_6 bread and
W bread plus the synthetic vitamin B_6 was similar.

In conclusion, the enrichment of refined flour with vitamin
B_6 will have advantages for populations who are dependent on
refined wheat products. However, such an enrichment cannot
replace completely the benefits of consuming whole wheat products.
Therefore, consumption of whole wheat products should be encouraged
among the public.

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APPENDICES

APPENDIX 1

Commercial Bread Making
Whole Wheat Bread

Formula:

	Pounds
100% whole wheat flour	100
water	64
yeast (compressed)	8
honey	8
lard	6
gluten	4
powdered milk	3
salt	2.25

Procedure: The ingredients were all mixed in a Hobart mixer for about 30 min. using a dough hook attachment. The dough was allowed to remain in the mixing bowl for 15-20 min. after which 1 lb 10 oz pieces were scaled and rounded into balls. These were placed on a table coated with flour. After about 30 min. each dough ball was passed through a roller to flatten and shape into a cylinder. The dough was then panned and proofed in a proofing oven (100° F) for one hour. Baking was done for 30 min. in an oven preheated to 415° F and equipped with rotary shelves.

White breads (WB$_6$, W)

Formula:

	Pounds
Bakers white flour	75
White bread base[1]	25
Water	54
Yeast (compressed)	2.5
Enrichment mixture[2]	5.25 grams

Note: For WB$_6$ bread, 105 mg of pyridoxine hydrochloride was added to the water.

Procedure: Same as for WHW bread.

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1.   White bread base contained wheat flour (bleached and bromated) enriched (niacin, iron, thiamine and riboflavin), shortening with mone and diglycerides-dextrose, whey solids, salt, soy or flour and dough conditioners. Product of Centennial Mills, Portland, Oregon.

2.   Enrichment mix: As mg/lb flour, thiamine 2.9, riboflavin 1.8, niacin 24.0 and iron 13.0 to 16.5; courtesy of Centennial Mills, Portland, Oregon.

APPENDIX 2

Home Baking Methods

Straight Dough Method (185).

		Grams
Formula:	Flour	400
	Sugar	24
	Shortening	12
	Salt	6
	Yeast (active dry)	4
	Water*	250 ml $\pm$

* In the case of W-B_6 bread, 2.14 mg pyridoxine-hydrochloride was dissolved in the water.

Procedure: Yeast was dissolved in about 25 ml of water warmed to about 40-46°C. Flour, sugar and salt were mixed in a bowl of a Kitchen Aid mixer equipped with a dough hook. Yeast suspension and the remaining water were mixed in at speed two for one minute. The sides of the bowl were scraped and shortening was added. Mixing was continued at speed two for four minutes. Dough was then placed in a greased bowl covered with a plastic wrap and allowed to ferment for three hours at 30° C. Raised dough was punched down, shaped into a loaf and proofed in a lightly greased pan for 55 min. at 30°C. Bread was baked for 25 min. in an oven preheated to 425°F.

Sponge-dough Method (186).

Formula:

		Grams
Sponge:	Flour	240
	Yeast (active dry)	4
	Water*	150 ml.
Dough:	Flour	60
	Sugar	24
	Shortening	12
	Salt	6
	Water*	100

* In the case of W-B_6 bread, 2.14 mg of pyridoxine hydrochloride was dissolved in the total amount of water.

Procedure: The ingredients for sponge were mixed for two minutes at speed two, in a bowl of a Kitchen Aid mixer with a wire whip attachment. The sponge was allowed to ferment for four hours at 30°C. The dough ingredients were mixed at speed two for three minutes in a separate bowl using a dough hook.

Fermented sponge was added to the dough and continued to mix at the same speed for four minutes. (Samples of mix for analysis were taken at this stage.) The sponge and dough mixture was allowed to ferment for 30 min. The raised dough was punched down, panned and proofed for 60 min. at 30°C. Baking was done for 25 min. in an oven preheated to 425° F.

APPENDIX 3

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 vitamin B_6 and F. D. and C Blue No. 1 (fecal marker) when
required, as well as have blood drawn and collect timed urine and
fecal specimens. In addition, I will consume only the foods and
beverages that are allowed on the diet. In return for these services,
the Department of Foods and Nutrition will pay me $2.00 per day. 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 except the one
that I will receive in this study.

Signed _____

Witness _____

Date _____

WHEAT STUDY

Spring 1976

Name _____ Telephone No. _____

Local address _____

Date of birth _____Height_____Weight_____

Do you have any physical or metabolic defects? _____ If yes, please describe.

Are you taking any drugs, medications or vitamins or other dietary supplements?_____ If yes, please list (include brand names). Indicate length of use.

Do you have any food allergies? _____ If yes, please describe.

Describe briefly your daily physical activities.

Do you smoke? _____ How long? _____

What is your class and work schedule?

Thank You.

APPENDIX 4

Diet and the composition (187)

Item (unit)	Amount g	Calories K Cals	Protein g	Fat g	Carbohydrate g	Fiber g	Calcium mg	Phosphorus mg	Iron mg	Sodium mg	Potassium mg
Orange juice	170	76	1.2	0.2	18.2	tr.	15	27	0.2	2	316
Cream of Wheat	83	36	1.1	0.1	7.4	tr.	50	55	4.2	158	8
Milk	240	156	8.4	8.4	11.8	0	283	223	tr.	120	346
Peaches	130	101	0.5	0.1	26.1	0.52	5	16	0.4	3	169
Pears	130	99	0.3	0.3	25.5	0.78	6	9	0.3	1	109
Rice	25	91	1.7	0.1	20.3	0.05	6	24	0.2	1	23
Carrot	25	10	0.3	tr.	2.4	0.25	9	9	0.2	12	85
Celery	25	4	0.2	tr.	1.0	0.15	10	7	0.1	32	85
Olive	10	13	0.1	1.4	0.03	0.02	8	2	0.2	81	3
Onion	2	7	0.2	tr.	1.6	0.09	3	5	0.1	2	28
Tomato Juice	34	65	0.3	tr.	1.5	0.07	2	6	0.3	68	78
Sub Total		658	14.2	10.6	116.1	1.96	399	383	6.2	479	1250
Breads											
WHW	570	1370	51.7	14.8	271.9	9.12	478	1444	13.1	2887	1456
WB$_6$ W	600	1614	52.1	19.2	302.4	1.20	419	521	14.4	3032	509
Total with:											
WHW		2029[d]	66	25.4	388.0	11.08	877	1827	19.3	3366	2706
WB$_6$ W		2272	66	29.8	418.5	3.16	818	903	20.6	3511	1759
RDA[c]		2700-3000	54-56				800	800	10		

a. Vitamin B$_6$ values (5).   b. Vitamin B$_6$ values of breads are figures obtained by microbiological assay.   c. Recommended dietary allowances

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Item (unit)	Vitamin A IU	Thiamin mg	Riboflavin mg	Niacin mg	Vitamin C mg	Vitamin B_6[a] mg
Orange juice	340	0.15	0.02	0.51	76.5	0.476
Cream of Wheat	0	0.04	0.02	0.30	0	0.005
Milk	336	0.07	0.41	0.24	2.4	0.168
Peaches	559	0.01	0.03	0.78	3.9	0.025
Pears	tr.	0.01	0.26	0.13	1.3	0.018
Rice	---	0.02	0.01	0.40	---	0.042
Carrot	2750	0.02	0.01	0.15	0.2	0.038
Celery	60	0.01	0.01	0.08	2.2	0.015
Olive	6	tr.	tr.	---	---	0.001
Onion	4	0.01	tr.	0.03	70	0.010
Tomato juice	272	0.01	0.01	0.27	5.4	0.065
Sub total	4327	0.35	0.77	2.88	92.7	0.435
Breads						
WHW	tr.	1.71	0.57	15.92	tr.	1.20[b]
WB_6	tr.	1.50	1.04	13.77	tr.	1.18
W						0.35
Total with:						
WHW	4327	2.06	1.34	18.80	92.7	1.64
WB_6	4327	1.85	1.81	16.65	92.7	1.61
W						0.78
RDA[c]	5000	1.4-1.5	1.6-1.8	18-20	45	2.00

tr - trace   d. Extra calories to maintain body weight were available in the form of margarine, jelly, candy, sugar and beverages.

APPENDIX 5

List of Abbreviations

ATP	Adenosine triphosphate
FMN	Flavin mononucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PAL	Pyridoxal
PIN	Pyridoxine
PAM	Pyridoxamine
PLP	Pyridoxal phosphate
PNP	Pyridoxine phosphate
PMP	Pyridoxamine phosphate
PIN-HCl	Pyridoxine hydrochloride
4-PA	4-Pyridoxic acid
WHW	Whole wheat flour/bread
WB_6	White flour/bread enriched with vitamin B_6
W	White flour/bread

APPENDIX 6

Data taken from ANOVA Tables

			Mean Square					F			
Variable	Specific	Unit	Main Effects	Subject	Week	Bread	Residual	Main Effects	Subject	Week	Bread
1. Body weight	end of week	Kg	386.382	579.422	441	.163	.711	543.229	814.631	.620	.230
2. Creatinine	average	g/24 hr	.108	.146	.032	.028	.010	11.157	15.159	3.365	2.939
3. Fecal B_6	total/week	mg/wk	2.354	1.070	.151	9.694	.297	7.918	3.600	.509	32.600**
4. 4-PA	Day 2	μM/24 hr	2.454	3.304	1.181	.327	.681	3.601	4.848	1.733	.480
5. 4-PA	Day 4	μM/24 hr	1.505	2.077	.295	.429	.253	5.938	8.194	1.163	1.692
6. 4-PA	Day 6	μM/24 hr	1.548	1.875	.192	1.595	.214	7.244	8.776	.897	7.465**
7. 4-PA	Day 7	μM/24 hr	1.275	1.518	.457	1.121	.327	3.899	4.642	1.397	3.430
8. 4-PA	Day 2	% of Basal	691.633	859.116	299.056	414.278	433.898	1.594	1.980	.689	.955
9. 4-PA	Day 4	% of Basal	507.689	664.496	88.401	299.749	158.748	3.198	4.186	.557	1.888
10. 4-PA	Day 6	% of Basal	704.568	758.925	42.423	1149.287	175.666	4.011	4.320	.241	6.542**
11. 4-PA	Day 7	% of Basal	758.393	862.161	85.676	1016.038	255.153	2.972	3.379	.336	3.982*
12. 4-PA	Ave all 4	μM/24 hr	1.591	2.082	.472	.745	.223	7.124	9.324	2.115	3.336
13. 4-PA	Ave last 3	μM/24 hr	1.376	1.746	.301	.972	.163	8.427	10.691	1.842	5.954*
14. Total U B_6	Day 2	μg/24 hr	1763.061	2548.462	364.641	19.877	130.953	13.463	19.461	2.785	.152
15. Total U B_6	Day 4	μg/24 hr	1758.665	2612.935	63.005	37.246	242.515	7.252	10.774	.260	.154
16. Total U B_6	Day 6	μg/24 hr	1551.494	2267.076	227.774	12.888	230.027	6.745	9.856	.990	.056
17. Total U B_6	Day 7	μg/24 hr	1809.181	2570.668	413.613	128.801	279.775	6.449	9.188	1.478	.460
18. Total U B_6	Day 2	% of Basal	155.148	189.647	147.561	24.740	51.408	3.018	3.689	2.870	.481
19. Total U B_6	Day 4	% of Basal	167.401	238.809	25.114	24.056	114.457	1.463	2.086	.219	.210
20. Total U B_6	Day 6	% of Basal	183.303	244.040	106.014	17.646	98.865	1.854	2.468	1.072	.178
21. Total U B_6	Day 7	% of Basal	125.434	136.563	137.727	68.625	111.545	1.125	1.224	1.235	.615

Variable	Specific	Unit	Mean Square					F			
			Main Effects	Subject	Week	Bread	Residual	Main Effects	Subject	Week	Bread
22. Total U B$_6$	Ave all 4	μg/24 hr	1656.966	2435.012	183.658	18.094	171.589	9.657	14.191	1.070	.105
23. Total U B$_6$	Ave last 3	μg/24 hr	1649.867	2435.662	139.003	17.551	216.930	7.606	11.228	.641	.081
24. Free U B$_6$	Ave all 4	μg/24 hr	2077.730	3050.537	258.221	6.010	154.198	13.474	19.783	1.675	.039
25. Free U B$_6$	Ave last 3	μg/24 hr	1976.796	2902.517	244.468	6.240	171.927	11.498	16.882	1.422	.036
Degrees of Freedom			12	8	2	2	14				

* Significant at 0.05 level.
** Significant at 0.01 level.