

FOLACIN AND VITAMIN B₆ STATUS OF YOUNG WOMEN
INGESTING NAS/NRC FORTIFIED BREAD

by

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INTRODUCTION

Fortification of foods with essential micronutrients is one of the great accomplishments of nutritional science and its benefits to mankind in this century are immeasurable (Aykroyd 1970). The virtual disappearance of pellagra and rickets and drastic reduction of iodine deficiency goiter in the United States are beneficial results of fortification programs (Mertz 1977). Not all efforts have been so successful; for example, the elimination of iron deficiency anemia by iron fortification. Yet the potential benefits are enormous if the experience gained from past errors and increasing knowledge of human nutrient requirements are applied to fortification programs.

Cereal-grain products are an appropriate food source for fortification because of their broad usage and suitability as a carrier. In the U.S., cereal products are consumed daily by almost everyone. They constitute approximately 25% of the average energy intake of the U.S. population (USDA 1972). Bread and other cereal products are relatively economical food sources within the purchasing power of most segments of the population. Because cereal products are broadly consumed, increases in their nutritive value could increase the nutritional health of a vast number of people.

In the 1930's, pellagra, beriberi and riboflavin deficiencies were common throughout the U.S. Many young men inducted into military service during World War II showed signs of physical deficiencies, many of which were related to malnutrition (Sebrell 1966). In 1940, the National Research Council (NRC) of the National Academy of Sciences (NAS) endorsed a program

favoring the addition of thiamin, riboflavin, niacin and iron to flour. In 1941, the Food and Drug Administration (FDA) set a standard of identity for enriched flour which became effective in 1942 (Federal Register 1941). These regulations were amended to their present form in 1943 and require that thiamin, riboflavin, niacin and iron be added to flour, with calcium and vitamin D optional (Federal Register 1943).

Knowledge of human nutrient requirements, nutrient interactions and biological availability has greatly increased since the enactment of the 1943 enrichment program. As preferences influencing food intake and the intake of certain nutrients have changed, fortification measures also need to be changed. Methods for assessing the nutritional status of population groups have improved and nutrition surveys have been effective in defining nutrient consumption. In light of these developments, the Food and Nutrition Board (FNB) of the NRC thoroughly reviewed the existing enrichment program during the early 1970's. They concluded that there was evidence of potential risk of dietary deficiency of vitamin A, thiamin, riboflavin, niacin, vitamin B₆, folacin, calcium, iron, magnesium and zinc among certain segments of the American population. They proposed an expanded fortification policy for cereal-grains to include those ten nutrients (NAS/NRC 1974).

Thiamin, riboflavin and niacin have been used to enrich cereal products for forty years. With the NAS/NRC fortification proposal, cereal-grains would be fortified with two additional B-vitamins--folacin and vitamin B₆. This study was undertaken to determine the bioavailability of folacin and vitamin B₆ in bread fortified according to the 1974 proposed NAS/NRC policy by means of a human feeding study.

REVIEW OF LITERATURE

Fortification of Cereal-grain Products

Proposed NAS/NRC fortification policy. In 1974, the FNB of the NRC proposed an expanded fortification policy for cereal grain products. In addition to the enrichment nutrients (thiamin, riboflavin, niacin and iron), this proposal recommended the fortification of cereal-based products with six nutrients--vitamin A, folacin, vitamin B₆, calcium, magnesium and zinc. This proposal was based on the broad usage of cereal-grain products in the U.S., the suitability of cereal grain products as a carrier of fortification nutrients and the nutritional needs of significant segments of the population. The ten nutrients and levels suggested for fortification are given in Table I. The originally proposed level of vitamin A of 2.2 mg/lb was lowered to 1.3 mg/lb in 1976 (Hepburn 1976).

TABLE I
NUTRIENTS AND LEVELS RECOMMENDED BY NAS/NRC^a

Nutrient	Fortification Standard (mg/lb)	Nutrient	Fortification Standard (mg/lb)
Vitamin A ^b	2.2	Folic acid	0.3
Thiamin	2.9	Iron	40.0
Riboflavin	1.8	Calcium	900.0
Niacin	24.0	Magnesium	200.0
Vitamin B ₆	2.0	Zinc	10.0

^aTaken from NAS/NRC (1974).

^bRetinol equivalent.

The addition of iron to cereal products for the purpose of increasing the iron intake of the population is a more complex situation than was

originally envisioned. The NAS/NRC recommendation to triple the iron content in flour and bread (from 13-16.5 mg/lb to 40 mg/lb) was based in part on the previous substantial role of enriched cereal-grain products in providing dietary iron (NAS/NRC 1974). Evidence that benefits from iron enrichment programs have not been realized was attributed to insufficient or unavailable forms of iron in the fortified food (NAS/NRC 1974).

The FNB has stated that micronutrients used for fortification must be "physiologically available from the food" (NAS/NRC 1974). Although progress has been made in knowledge of biological availability, the problem of iron is complicated because other dietary constituents and the physiological state of the subject affect iron absorption (Anon. 1975). In 1978 the FDA withdrew their support for increased iron based on insufficient evidence of its effectiveness in decreasing iron-deficiency anemia and inadequate studies showing the safety of increased iron levels in individuals where iron overload and hemochromatosis posed potential problems (Federal Register 1978).

Assessment of need for additional nutrients. Approximately forty nutrients have been proven essential in human nutrition. The NRC has established recommended daily dietary allowances for seventeen of these nutrients and estimated safe and adequate dietary intakes for twelve other nutrients (NAS/NRC 1980). These guidelines were used in the determination of need for cereal fortification based on the adequacy of nutrient intakes as reported by recent food consumption surveys and comprehensive nutritional surveys.

The U.S. Department of Agriculture has assessed household food consumption six times since 1936. The 1965 survey (USDA 1972) included 24-hr dietary recalls of 14,519 individuals in the U.S. Analysis of

dietary recalls showed that calcium and iron were the nutrients most often taken at levels below the recommended allowances. The diets of children under 3 yrs of age were about 50% below recommended amounts of iron; the diets of adolescent girls and women were more than 30% below recommendations. Adolescent girls, women and older men had diets low in calcium, riboflavin and vitamin A. All males had diets below the recommendations for magnesium. Estimated levels of vitamin B₆ were considerably below recommended allowances for several sex-age groups, especially for females 9 yrs and over who were below the recommendations by 14 to 45%. The 1977-78 household food consumption survey (USDA 1980) reported the food and nutrient intake of 9,620 individuals during one day. All sex-age groups over 2 yrs of age had less than 100% of the RDA for vitamin B₆ and magnesium, with women's intakes lower than those of men. Of all the sex-age groups, calcium intakes of females 12 yrs and over were the lowest compared to their Recommended Dietary Allowances (RDA).

The first comprehensive attempt to assess the nutritional status of the American people was the Ten-State Nutrition Survey (USDHEW 1972). This study, conducted from 1968 to 1970, included a 24-hr dietary recall along with physical, anthropological and biochemical data to evaluate nutritional status. Iron-deficiency anemia, as evidenced by a high prevalence of low hemoglobin levels within the population surveyed, was widespread. The major problem with vitamin A nutriture was found among Spanish-Americans in low-income-ratio states. Young people in all populations had a high prevalence of low vitamin A values. Riboflavin appeared to be marginal among black and Spanish-American ethnic groups and among young people of all ethnic groups.

The first Health and Nutrition Examination Survey (HANES) was undertaken by the Department of Health, Education and Welfare during 1971

to 1974 (USDHEW 1974). During 1971 to 1972, data on dietary intake and biochemical tests were obtained from 10,126 persons aged 1-74 yrs. Iron was the nutrient most often found below the standard in population groups. Negro females, 18 to 44 yrs, had calcium intakes approximately 20% below the standard and white females of the same ages in the lower income group had vitamin A intakes 18% below the standard.

The nutritional importance of several trace elements, magnesium and zinc, has received increased recognition as information concerning their metabolic role has increased. The RDA (NAS/NRC 1980) for magnesium for adult men and women has been established at 350 mg and 300 mg, respectively. White (1969) found that the magnesium content of the total mixed diet of high school and college women in the U.S. was significantly less than the 300 mg/day estimated to be required for balance. In a recent study, Greger et al (1978) provided adolescent females menus similar to those they generally consumed. The level of magnesium in the diet was insufficient for most of the girls to maintain positive magnesium balance.

The recommended dietary zinc allowance for adult men and women is 15 mg/day (NAS/NRC 1980), provided the diet does not consist predominantly of unrefined cereals high in phytate. The first reports of human zinc deficiency were related to the syndrome of adolescent nutritional dwarfism and evolved from studies in Egypt and Iran (Prasad 1976). Observations by Hambidge et al (1972) suggested that zinc-responsive growth failure may not be limited to Middle East countries. Their Denver study revealed that some middle and upper income children with poor growth had anorexia, hypogeusia and low zinc hair levels. When given zinc supplements, these children demonstrated improved taste, appetite and growth. Based on usual dietary availability of zinc, Sandstead (1973) suggested that some infants,

pregnant women, teenage and college women, and institutionalized individuals have a marginal to deficient intake of zinc.

Feasibility of fortification proposal. In determining the technical feasibility of the 1974 NAS proposal, various aspects of fortification were taken into consideration. The recommended levels were intended to include total nutrient concentrations of wheat flour; therefore the average natural content of the nutrients had to be established. Ranum (1980) reported on the base-line data and suggested levels of the nutrients to be added to bring the content up to proposed fortification standards. The natural nutrient level and nutrient addition rates are given in Table II.

TABLE II
SUGGESTED LEVELS OF NUTRIENT ADDITION TO WHEAT FLOUR
FOR MEETING PROPOSED U.S. FORTIFICATION STANDARDS^a

Nutrient	Fortification Standard (mg/lb)	Natural Level (mg/lb)	Addition Level (mg/lb)
Thiamin	2.9	0.6 ± 0.2	2.65
Riboflavin	1.8	0.2 ± 0.1	1.8
Niacin	24.0	5.4 ± 1.4	21.0
Folacin	0.3	0.075 ± 0.020	0.26
Pyridoxine	2.0	0.18 ± 0.07	2.0
Vitamin A	4,333 IU/lb	0	5,000 IU/lb
Iron	13-16.5	5.1 ± 1.7	11.0
Calcium	900	62 ± 11	880.0
Zinc	10	3.5 ± 0.5	7.5
Magnesium	200	116 ± 23	110.0

^aTaken from Ranum (1980).

Several studies have shown that the dispersibility and stability of the vitamins added to fortify the flour were excellent. Pilot mill tests showed that the proposed nutrients were distributed uniformly when added continuously to the mill stream (Vetter 1978). The vitamin A in fortified flour was found to be stable in simulated storage and transportation conditions. Parrish et al (1980a) reported that vitamin A was stable in flour at warehouse temperature for up to six months with no tendency to

segregate in handling or shipping in bags. Vitamin A, pyridoxine and folic acid were stable in fortified flour after six months at room temperature and up to twelve weeks at 45°C (Cort et al 1976). Data from additional research indicated the vitamins were stable in the presence of added iron, zinc, magnesium and calcium in bread (Cort et al 1976, Emodi and Scialpi 1980, Parrish et al 1980b).

Results concerning the organoleptic effect of added zinc and magnesium in bread were not consistent. Ranhotra et al (1976b) noted that certain forms of magnesium adversely affected flavor and quality; whereas Emodi et al (1980) reported that the nutrients added at the proposed fortification levels did not have an adverse effect on crumb color, grain texture or flavor. Bhalla (1978) found that zinc oxide added to enriched bread ingredients adversely affected the crumb texture and appearance and the addition of all the fortification nutrients resulted in a burnt top crust flavor.

Bioavailability of fortification nutrients. Some research concerning the bioavailability of the micronutrients has been conducted. The results of a four-week rat feeding trial indicated that vitamin A in stored flour has the same biopotency as when originally added and was available for rat growth and body storage (Liu and Parrish 1979). Ranhotra et al (1976a) observed that magnesium from wheat flour was fully available to weanling rats and concluded that the slight differences in bioavailability observed among the organic and inorganic magnesium salts was of limited physiological significance. Foley (1979) concluded that the calcium of both calcium carbonate and calcium sulfate were biologically available for utilization in rat growth. Fortification of cornmeal with all ten proposed nutrients resulted in better rat growth, feed intake and feed efficiency than enrichment of cornmeal by present standards (Balogun 1978).

Recent research was conducted concerning the human utilization of vitamins present in cereal products. Urinary excretions of niacin, N¹-methyl nicotinamide, pantothenic acid, riboflavin, thiamin, vitamin B₆ and vitamin B₁₂ of 12 men were studied by Edwards et al (1971). When part of a wheat diet was replaced equinutritiously by pinto beans, rice or peanut butter, the excretion of the vitamins remained constant which suggested that utilization of the vitamins in the wheat diet was not affected by the nitrogen substitute.

As part of an effort to prevent folate deficiency by fortification of staple foods, Colman and coworkers (1975a) investigated folic acid absorption from fortified cereal products. Maize, rice and bread fortified with pteroylglutamic acid (PGA) increased serum folate concentrations in subjects, with the increment being approximately one third to one half of the increment observed in subjects receiving an aqueous solution of PGA. In a related study, Colman et al (1975b) observed an increase in serum folate levels in pregnant women if they received a folacin supplement either as fortified maize meal or as tablets; blood folate levels of the control group without supplements decreased. Further studies on the usefulness of bread as a vehicle for folacin fortification were carried out because of its importance as food in Western countries. Subjects in late pregnancy were given bread fortified to contain a daily dose of 900 µg folic acid (Margo et al 1975). The women showed a significant rise in red cell folate concentration similar to that observed in women receiving a daily dose of 300 µg folic acid in tablet form.

The differences in biological availability of various mineral elements have been researched extensively in the past decade. The absorption of iron is affected by various physiological factors and factors affecting the availability of iron from its source. The food source of iron, the phytate

content of foods, and other dietary factors such as protein, amino acids, ascorbic acid and carbohydrates have been suggested as having an effect on iron absorption (Ranhotra et al 1979, Cook et al 1973, Waddell 1974).

Less is known about the biological availability of zinc. In general, zinc in plant products is less readily absorbed than zinc in animal products (Halstead et al 1974). Other factors affecting the bioavailability of zinc are phytate (Oberleas et al 1966), other chelating agents (Vohra and Kratzer 1964) and the zinc status of the individual (Prasad 1966).

Folacin

In 1941 a substance that promoted bacterial growth was isolated from spinach leaves and named folic acid from the Latin folium, "leaf." Folic acid was found to be an effective treatment for certain types of dietary anemias and a cure for megaloblastic anemia. It has been suggested that there are more derivatives of folic acid than there are of any other vitamin.

Chemical composition. Folacin or pteroylglutamic acid is a pteridine derivative linked through a methylene bridge to a molecule of para-amino-benzoyl-glutamic acid (Krumdieck 1976). For humans, folic acid is the nutritionally essential precursor of a large family of compounds that serve as coenzymes for one-carbon transfer reactions. The active form of the vitamin is tetrahydrofolic acid (THFA). Folic acid is reduced to THFA in the presence of NADPH and ascorbic acid (Pike et al 1975).

The conversion of folacin to the folate coenzymes requires modification in three parts of the molecule (Krumdieck 1976): 1) the reduction of the pteridine ring, 2) the lengthening of the peptide chain by addition of several glutamyl residues, and 3) the addition of a one-carbon fragment. Since each modification has several possible forms, a very large number of folates occur in nature.

Absorption. Dietary folates which occur as polyglutamates are not absorbed intact. They are absorbed as monoglutamates; during absorption all except one unit of glutamic acid is removed (Chanarin 1979). The cleavage site seems to be the interior of the intestinal epithelial cells where the conjugase(s) necessary for cleavage occur within the lysosomal particles (Krumdieck 1976). Some data show that the polyglutamate is less well utilized than the monoglutamate. Absorption of the 150 to 300 μ g folate in the average Western diet ranges from 30 to 70% (Chanarin 1979).

The mechanism by which folates are absorbed ensures that only 5-methyltetrahydrofolate is delivered to the portal blood (Perry and Chanarin 1970). Folacin is removed from the serum by the tissues. Cells contain an enzyme capable of building up polyglutamate derivatives from the reduced folate monoglutamate. These polyglutamate compounds are incorporated in the folate coenzymes essential in purine and pyrimidine synthesis and amino acid metabolism (Hoffbrand et al 1977).

Dietary folate. Compounds exhibiting folic acid activity are widely distributed in nature; they are present in many animal and plant tissues and are abundant in green leafy vegetables, yeast and liver. Data on the content of folacin derivatives in various foods are incomplete due to the rapid changes in C_1 moiety during food preparation and analysis, the state of oxidation, and number of glutamyl residues in the peptide side chain (Baugh and Krumdieck 1971). In addition, as much as 50 to 95% of the folate activity may be destroyed by cooking or processing the food (Hoffbrand et al 1977).

Most of the folic acid in foods is present as polyglutamates, containing 3 to 7 glutamate residues. Microbiological assays have been used for quantitating folacin activity because of their sensitivity; however, none of the assay organisms can utilize pteroyl polyglutamates

with more than three glutamyl residues (Rodriguez 1978). Therefore it is necessary to degrade higher polyglutamates by enzymatic digestion before utilization by the organism. Due to differences in results obtained by different assay methods, the variability of the microbiological assay itself and the low concentrations and instability of folate compounds in food, there are discrepancies in the published values of the folacin content of food.

Folate deficiency. The current RDA for folacin is 400 μg for adults (NAS/NRC 1980). Folate deficiency due to inadequate intake is thought to be the most common hypovitaminosis; it affects indigent people in developing countries and elderly people in affluent societies (Blakley 1969). Due to the high folate requirement of growing tissues, any condition leading to increased rates of cell multiplication will result in higher requirements. Physiological states particularly vulnerable to the development of deficiency are pregnancy, lactation, early infancy and adolescence (Rodriguez 1978). Anemias of various origins, malignancy, and infections result in higher folate needs. Malabsorption of folate derivatives in tropical sprue, parasitic infestations and intestinal disease are often associated with megaloblastic anemia (Blakley 1969). Ethanol and anti-convulsants are substances that interfere with normal folate absorption (Lane et al 1976); whereas inadequate utilization of folates is most often due to drugs and oral contraceptives that interfere with folate metabolism (Bertino 1971, Prasad et al 1976).

More cases of folacin deficiency have been reported in pregnant women than any other population group (Rodriguez 1978). Elsborg and Rosenquist (1979) suggested that in order to meet the excess requirement for folates during pregnancy a woman would require increased intake during pregnancy or reserves built up prior to pregnancy. Several factors limit

the latter possibility. Adolescent girls (potential mothers) have greater nutritional requirements in relation to body size than do adult women (Heald 1975). Folate intakes by teenage girls are often insufficient to secure a high folate depot level before pregnancy (Brisson et al 1974, Elsborg and Rosenquist 1979). Another complicating factor is oral contraceptive usage. Blood folate levels were reported to be significantly lower in oral contraceptive users than the values of non-users (Shonjan et al 1969). More recently the residual effect of oral contraceptive agents on blood folacin levels during pregnancy was reported (Martinez and Roe 1977).

Vitamin B₆

Vitamin B₆ or pyridoxine is the collective name given to the 2-methyl pyridine derivatives which include an alcohol, pyridoxol, found especially in plants, an aldehyde, pyridoxal, and an amine, pyridoxamine. The aldehyde and amine are the most prevalent forms in animal tissue and the most easily absorbed (Guthrie 1979).

Absorption and metabolism. Dietary B₆ is absorbed in the upper part of the small intestine and is concentrated initially in the liver where different forms are interconverted and phosphorylated to yield pyridoxal phosphate (Anderson 1980). Pyridoxal phosphate circulates in the plasma bound to albumin and passes to the tissue stores. Removal from albumin and dephosphorylation are required before transport into the cell where rephosphorylation occurs. A major metabolite of vitamin B₆ is 4-pyridoxic acid, which is oxidized from the aldehyde form to pyridoxic acid phosphate which is hydrolyzed to pyridoxic acid and excreted in the urine (Pike and Brown 1975).

The major metabolic function of vitamin B₆ is as a coenzyme. The principal active form of the vitamin is pyridoxal phosphate, which acts as a cofactor for an exceptionally large number of different types of enzymatic reactions involved in various aspects of amino acid metabolism. These include decarboxylation, deamination, transamination, transmethylation and transsulfuration (Gershoff 1976). Pyridoxine also is involved in the metabolism of other vitamins--in the synthesis of niacin, along with vitamin E in fat metabolism, with ascorbic acid in tyrosine metabolism (Tryfiates 1980). Vitamin B₆ is essential in the formation of porphyrin and the heme portion of the hemoglobin molecule, in the enzyme phosphorylase which converts glycogen to glucose-1-phosphate, in the maintenance of lymphoid tissues and immunological function, and in the synthesis of neurotransmitters (Gershoff 1976, Robson and Schwartz 1980, Kelsall 1969).

Human requirement. Vitamin B₆ is a water-soluble vitamin and is found principally in muscle meats, liver, vegetables, whole grain cereals and egg yolks (Guthrie 1979). Because of the association between protein metabolism and vitamin B₆, requirements for the vitamin are proportionate to protein intake. The daily recommended allowance is approximately 20 µg/g protein or 2 mg/day for the adult woman (NAS/NRC 1980). There is an increased requirement of the vitamin to support the growth needs of pregnancy, lactation and childhood.

Vitamin B₆ deficiency. A wide variety of conditions give rise to biochemical vitamin B₆ deficiency which is manifest clinically by low vitamin B₆ blood levels and abnormal enzyme function tests. A deficient intake may result from abnormal diet, age or alcoholism. Hampton et al (1977) reported that significant sections of the elderly population consumed less than 50% of the RDA for the vitamin. Biochemical deficiency is common in old age and has been reported in some 20% of subjects above age 65

(Chrisley and Driskell 1979, Hoorn et al 1975). A major cause of B₆ deficiency is increased physiological requirements for the vitamin during growth, pregnancy and lactation (Ritchey et al 1978, Roepke and Kirksey 1979), and women taking oral contraceptives (Miller et al 1978).

Abnormal pyridoxine metabolism has been reported in patients on certain drugs, in alcoholics, in patients with liver disease, in pre-eclampsia and in asthma (Rosalki 1979). Drug therapy with isoniazid, cycloserine or penicillamine may give rise to a deficiency, usually as a result of complexing the drug to the vitamin to yield an inactive metabolite (Gershoff 1976).

Two studies have been conducted to specifically study vitamin B₆ needs of "normal" young women (Donald et al 1971, Shin and Linkswiler 1974). These requirement studies suggested that 1.5 mg of vitamin B₆ daily was adequate. Recent food consumption surveys showed that vitamin B₆ was one of the nutrients most often below recommendations (2.0 mg daily) for young women (USDA 1972, USDA 1981). Driskell and coworkers (1976) reported that of 119 young women studied, approximately one-third did not consume 50% of the RDA for the vitamin and the vast majority did not consume 100% of the RDA. Despite low dietary B₆, very few subjects had low coenzyme stimulation levels which are indicative of a vitamin B₆ deficiency. Kirksey et al (1978) observed 127 adolescent females. Approximately one-half consumed less than two-thirds of the RDA for pyridoxine; 31% of the subjects showed inadequacy of the vitamin based on coenzyme stimulation levels.

MATERIALS AND METHODS

Preparation and Analysis of Yeast Bread

Flour preparation. Unenriched all-purpose flour was purchased from a commercial source by the Department of Grain Science and Industry, Kansas State University.

Flour enriched to current standards (Federal Register 1943) was prepared by adding Flour Enrichment Vital Mix 102¹ to unenriched flour. The amount of each micronutrient added to the flour is shown in Table III. One-half ounce enrichment mix was dispersed in 4-5 lbs flour and mixed with the remainder of the 200 lbs flour in a rectangular wooden flour tumbler blender for 15 min.

Fortified flour was prepared by adding 60g of FNB Vital Mix 125¹ to 200 lbs unenriched flour as described above. Iron (Glidden Electrolytic, reduced) was added to the flour at the level originally proposed by the NAS/NRC recommendation. Calcium was obtained from the U.S. Gypsum Company and was added to each batch of dough with the sponge ingredients. The fortified flour met the proposed levels for fortification (NAS/NRC 1974) and the nutrient addition level is shown in Table III.

Bread baking and storage. All bread (one pound loaves) was baked weekly in the Baking Laboratory in the Department of Grain Science and Industry. Table IV shows the ingredients used for each batch of bread. White breads were prepared using the standard sponge dough procedure. The procedure for bread baking is shown in the Appendix (p. 43).

¹ Provided by Watson Foods Company, Inc., 57-01 32nd Avenue, Woodside, N.Y. 11377.

TABLE III
LEVEL OF NUTRIENT ADDITION TO WHEAT FLOUR

Nutrient	Enriched ^a (mg/100 lb)	Fortified ^b (mg/100 lb)
Vitamin A, palmitate	-	500,000 IU/100 lb
Thiamin, mononitrate	271.5	280
Riboflavin	175	180
Niacin	2,100	2,100
Pyridoxine HCl	-	200
Folic acid	-	26
Iron, reduced	1,200	3,547 ^c
Calcium, calcium sulfate ^d	-	210,000
Magnesium, magnesium oxide	-	10,800
Zinc, zinc oxide	-	720

^aFlour Enrichment Vital Mix 102, Watson Foods Company.

^bFNB Vital Mix 125, Watson Foods Company.

^cFNB Vital Mix 125 contained 1,200 mg/100 lbs. An additional 2,347 mg/100 were added.

^dAdded to sponge in bread baking process.

TABLE IV
STANDARD SPONGE DOUGH FORMULA FOR WHITE BREADS

Ingredients	Sponge (g)	Dough (g)	Total (g)
Flour	6719	2906	9625
Water	3768 ^a	1952 ^b	5720
Yeast	192.5		192.5
Salt		192.5	192.5
Sugar		590	590
Shortening		192.5	192.5
NFDM ^c		192.5	192.5
Malted barley flour	19.3		19.3
Arkady yeast food	24.1		24.1
SSL ^d	24.1		24.1

^aWater temperature 60°F.

^bWater temperature 45°F.

^cNonfat dry milk.

^dSodium stearoyl-2-lactylate.

After baking, the bread was sliced, wrapped in polyethylene bags and kept in frozen storage. Sufficient bread for two days of the feeding study was withheld from the freezer and consumed fresh.

Analysis of folacin. The extraction of folates was conducted according to the Association of Vitamin Chemists (1966). The quantitative microbiological method for determining folacin was that of the AOAC (1980),

modified slightly by using prepared Difco folic acid casei medium (code 0822-15-9) as the basal media. Lactobacillus casei ATCC 7469 was the organism chosen because it utilizes the oxidized and reduced folate forms as well as the methyl derivative (Malin 1975). Chicken pancreas conjugase was used to degrade pteroyl polyglutamates with more than three glutamyl residues, thus making the higher polyglutamates available for utilization by the organism (Krumdiek 1976). This method measured "total" folate, which included the "free" folate (unconjugated) as well as the polyglutamates containing more than three glutamic acid residues. The procedure used to prepare the bread samples for assay is given in the Appendix (p. 44).

Each bread sample was treated with conjugase in duplicate, five dilutions of each extract prepared and determined in duplicate. Folacin activity was estimated from plotted standard curves and is shown in Table V along with other estimates of bread nutrients.

TABLE V
CONTRIBUTION OF BREAD TO DAILY NUTRIENT INTAKE^a

Nutrient	Enriched		Fortified	
	Slice (25 g)	Daily (200 g)	Slice (25 g)	Daily (200 g)
Energy (kcal)	70	560	70	560
Protein (g)	2	16	2	16
Vitamin A (IU)	trace	trace	313	2560
Thiamin (mg)	0.14	1.12	0.13	1.04
Riboflavin (mg)	0.13	1.00	0.11	0.90
Niacin (mg)	0.8	6.4	0.8	6.4
Vitamin B ₆ (mg)	0.015	0.120	0.09	0.73
Folacin (μg)	10	80	40	320
Calcium (mg)	10.3	82.6	28.7	229.6
Iron (mg)	1.08	8.64	1.96	15.58
Magnesium (mg)	5.53	44.24	8.85	70.80
Zinc (mg)	0.27	2.14	0.46	3.66

^aEstimates of energy, protein, niacin and enriched vitamin A values were based on data in USDA Home and Garden Bulletin No. 72 (1978). Enriched folate levels were based on data in Perloff and Butrum (1977). Fortified vitamin A and folate levels, thiamin, riboflavin, vitamin B₆, calcium, iron, magnesium and zinc concentrations were determined by direct analysis.

Analysis of vitamin B₆. Total vitamin B₆ content of the bread was determined by the official AOAC method (1980) based on the growth response of the yeast Saccharomyces uvarum ATCC 9080. S. uvarum was used as the test organism because pyridoxine, pyridoxal and pyridoxamine are essentially equally active on a molar basis for this yeast (Sauberlich 1967). No attempt was made to separate the three forms of vitamin B₆.

Because the yeast grows only on the free forms of vitamin B₆, biological samples must be hydrolyzed prior to assay to free the vitamin of phosphates and protein (Sauberlich 1967). Bread samples were acid hydrolyzed according to the Association of Vitamin Chemists (1966) shown in the Appendix (p.45). Each bread sample was hydrolyzed in duplicate, four dilutions of each sample were prepared and determined in duplicate. The content of apparent B₆ in each assay tube was estimated from plotted standard curves. These data were employed to calculate bread vitamin B₆ concentration shown in Table V.

Human Feeding Study

Experimental plan. Twelve young women served as subjects in this study. Baseline data for each subject was obtained during 4 consecutive days preceding the study while the women consumed their usual meals. The baseline study period was followed by an 8-week controlled metabolic diet. The 12 subjects received a basal diet composed of common foods based on a 6-day menu cycle. Six young women ingesting NAS/NRC fortified bread served as the experimental group and another six, ingesting bread enriched according to present standards, served as controls. Both the experimental and control subjects consumed 200g of bread daily.

Subjects. Female subjects from a college-age population were recruited by advertisement and by personal contacts. A Medical Inventory and a Dietary History were completed by each subject (Appendix, p.46) to

eliminate persons with special health or dietary problems. The protocol of the experiment was approved by the University Committee on Research Involving Human Subjects. Informed consent was obtained from each participating subject (Appendix, p. 50).

The subjects were grouped into pairs on the basis of age and weight. The pairs were then randomly assigned to a numerical diet group.

Subjects ranged in age from 20 to 27 yrs with the mean ages of 23 and 22 for the experimental and control subjects, respectively. Body weight of the experimental subjects ranged from 51.8 kg to 74.5 kg with a mean of 63.4 whereas control subjects ranged from 55.5 kg to 71.8 kg with a mean of 63.5 kg. All subjects were in good health and did not use oral contraceptives nor smoke.

Diets. The complete diet was composed of the basal diet and enriched or fortified bread. The 6-day menu cycle is given in the Appendix (p. 51). The daily nutrient content of the basal diet is shown in the Appendix (p. 53). The same amount of bread (200 g) was eaten each day, but on two days equivalent substitutions were consumed.² The average nutrient content of the experimental and control diet is given in Table VI. The complete diets provided a mean calculated energy content of 1821 kcal and a protein content of 84 g daily. Of the ten fortification nutrients, the experimental diet satisfied all of the nutrient needs, except magnesium, of females, age 25 to 50, according to the RDA (NAS/NRC 1980). The women consuming the control diet were below their RDA for folacin, calcium, magnesium and zinc.

Subjects continued their normal activities as students of the University, but obtained all their food from the kitchen of the Metabolic

²Eleven g of bread crumbs (enriched or fortified) were substituted for 12 g of bread in the beefburgers (Day 2). Fifty-five g of bread dough (enriched or fortified) were substituted for 50 g of bread in the pizza crust (Day 3).

TABLE VI
AVERAGE DAILY NUTRIENT CONTENT OF DIETS^a

Nutrient	Basal Diet	Enriched Bread	Fortified Bread	Basal Diet + Enriched Bread	Basal Diet + Fortified Bread	RDA ^b
Energy (kcal)	1261 ± 38 ^c	560	560	1821	1821	2000
Protein (g)	68 ± 6	16	16	84	84	44
Vitamin A (IU)	6948 ± 2029	--	2506	6948	9454	4000
Thiamin (mg)	0.40 ± 0.06	1.12	1.04	1.52	1.44	1.00
Riboflavin (mg)	2.21 ± 0.26	1.00	0.90	3.21	3.11	1.20
Niacin (mg)	12.3 ± 2.4	6.4	6.4	18.7	18.7	13.0
Folacin (μg)	208 ± 57	80	320	288	528	400
Vitamin B ₆ (mg)	1.4 ± 0.4	0.6	3.6	2.0	5.0	2.0
Calcium (mg)	606 ± 80	83	230	689	836	800
Iron (mg)	11.0 ± 1.6	8.6	15.7	19.6	26.7	18.0
Magnesium (mg)	160 ± 24	44	71	204	231	300
Zinc (mg)	12.5 ± 1.5	2.1	3.7	14.6	16.2	15.0

^aAll values were obtained from direct analysis, except energy, protein, niacin and vitamin A (basal diet and enriched bread) which were based on data in USDA Home and Garden Bulletin No. 72 (1978) and enriched bread folate levels which were based on data in Perloff and Butrum (1977).

^bFor females aged 23 to 50 yr (NAS/NRC 1980).

^cMeans ± SD for 6-day menu cycle.

Suite of the Department of Foods and Nutrition. Each day three meals were eaten in the Metabolic Suite and a prepared sandwich was taken with each subject after the evening meal and consumed between 9-10 p.m. Subjects were required to eat all of the food presented to them at each meal. Instant tea and coffee were provided ad libitum, but no other foods or drinks were permitted.

All staples and canned foods were purchased from local supermarkets prior to the study and fresh produce, meat and dairy products were purchased weekly during the study. Meals were prepared by usual cooking procedures. All food and drinks were weighed to the nearest gram. Careful detail was taken in the preparation of casseroles, stews, ground beef dishes and pizza. Ingredients were weighed separately and cooked as individual servings. The milk, cookies, cole slaw, tuna salad (for sandwiches) and scrambled eggs were prepared for the whole group of subjects and then divided according to serving size.

Each subject weighed daily before breakfast. Jelly, gum drops, and whole milk (instead of skim milk) were supplied to provide energy, without providing additional vitamins and minerals, for the maintenance of initial body weight. Caloric supplements were begun during week 4 and given to 5 subjects during the course of the study.

Analysis of folate and vitamin B₆ in basal diet. Folate and vitamin B₆ assays were carried out on six 24 hr food composites. Food was prepared for analysis by measuring duplicate quarter portions of the prepared foods. Milk was added in the dry powder form and bread was analyzed separately. A measured amount of water was added to the aliquot and the sample was homogenized and stored in small plastic bags at -15°C until analyzed. The food samples also were analyzed for total folate (L. casei activity after

treatment with conjugase from chicken pancreas) and total vitamin B₆ by the same methods used for bread analyses.

Biological Determination of Nutritional Status

Tissue sample collection. Blood samples were obtained preceding the study, at the midpoint and on the last day. All blood samples were drawn prior to breakfast, after a 12 hr fast. A 10 ml venous blood sample was drawn, allowed to clot at room temperature and spun down. Ascorbic acid (5 mg/ml serum) was added to the serum which was frozen at -15°C until assayed.

Four 24 hr urine collections were made just prior to the start of the feeding study. Three 24 hr collections were made during weeks 4 and 8. Samples were collected in gallon plastic jugs containing toluene. Volume, creatinine and pyridoxine determinations were made on the fresh samples.

Serum folate. Sera collected during the study were prepared for the folate assay by the method suggested by Difco Laboratories (Appendix, p. 54). Folate activity was determined using L. casei according to the AOAC method (1980). The assay was performed on duplicate samples with four dilutions of each sample in duplicate.

Urinary excretion of vitamin B₆. Free urinary vitamin B₆ was measured because it correlates closely with the level of intake of the vitamin (Sauberlich et al 1974) and with total vitamin B₆ (Woodring and Storvick 1970). Urine was sampled in duplicate, four dilutions of each sample prepared and apparent vitamin B₆ in each tube determined in duplicate. Urine was analyzed for creatinine using the picrate method (ICNND 1963).

During the three urine collection periods, all urine samples, except those reported to be incomplete, were analyzed for free vitamin B₆ and creatinine. After the creatinine determinations were made, the urinary B₆

values for the two days of the period with the most nearly constant creatinine values were averaged and analyzed statistically.

Statistical Analysis

After baseline data on folacin and urinary B₆ levels had been collected, 24 days were allowed for adjustment to the diet. Statistical analyses were performed on the data collected during weeks 4 and 8. Significant differences were determined by analysis of variance, using the split plot design, to examine the separate and interactive effects of diet and time as measured by the F test. The relationship between the calculated and analyzed values of dietary folate and vitamin B₆ were measured by using Pearson's correlation coefficient.

RESULTS

Serum Folate

Serum folate values of each participant are shown in Table VII. The values for serum folate showed wide variation; initial values were 8.8 to 24.2 ng/ml with means of 12.5 and 16.3 ng/ml for the experimental and control groups, respectively. Normal serum folate levels are 5 to 17 ng/ml using *L. casei* as the assay organism (Goldsmith 1975). Levels of less than 3 ng/ml are suggestive of deficiency.

TABLE VII
INDIVIDUAL SERUM FOLATE VALUES (ng/ml)

Control Diet				Experimental Diet			
Subject Number	Base-line	Week 4	Week 8	Subject Number	Base-line	Week 4	Week 8
1	13.6	12.6	7.5	3	10.1	14.7	11.4
2	10.7	9.3	8.7	4	17.1	14.0	9.4
5	24.2	16.7	14.4	8	8.8	8.5	7.5
7	21.6	15.2	9.0	9	12.6	12.9	13.0
10	12.5	15.1	14.6	11	16.6	13.2	13.3
12	14.9	17.8	13.0	13	9.5	12.3	15.3
Mean	16.3	14.5	11.2	Mean	12.5	12.6	11.6

Serum folate levels reflect recent dietary intakes of the vitamin (Sauberlich et al 1974). In this study (Table VIII), serum folate concentrations were not significantly different due to diet (experimental vs control), but were different ($P < 0.05$) due to time (week 4 vs week 8). Serum folate levels for the experimental and control groups declined significantly from week 4 to week 8. Baseline values indicated that the control subjects had a higher dietary folate intake prior to than during the study.

TABLE VIII
ANALYSIS OF VARIANCE FOR SERUM FOLATE

Source of Variation	DF	Mean Squares	F-ratio
Treatments	3		
Diet	1	2.94	0.23 ^{ab}
Time	1	26.46	7.94 ^{cd}
Diet x Time	1	7.94	2.38 ^{cb}
Error A: Subject (Diet)	10	12.76	
Error B: Subject x Time (Diet)	10	3.33	

^aUsed Error A.

^b $p > 0.10$.

^cUsed Error B.

^d $p < 0.05$.

Several factors should be taken into consideration when evaluating the effect of the diet on the serum folate levels. First, the subjects in this study were healthy young women with high initial serum folate concentrations. Secondly, estimates of minimum human folate requirements range from less than 8 μg daily (Velez et al 1966) to 100 μg daily (Fleming et al 1963, Herbert et al 1962). Although the control diet contained approximately half as much folate as the experimental diet and 70% of the RDA for young women, the average mean intake of 288 μg daily was considerably greater than the estimates of minimum requirement. Both of these factors are reflected by the mean serum folate levels of the experimental and control groups. The serum levels of the control group, which were very high at the onset of the study, decreased more than the experimental group; since, however, the levels of both groups dropped during the study, the change was attributed to time effects rather than dietary effects.

Urinary Excretion of Vitamin B₆

Normal urinary levels of free vitamin B₆, based on S. uvarum assay, are greater than 20 $\mu\text{g/g}$ creatinine or 35 $\mu\text{g/day}$ (Sauberlich et al 1972). The preferred measurement for the urinary excretion of vitamin B₆ is made on a 24 hr collection of urine (Baker et al 1966); however, precise 24 hr

urine collections are difficult to obtain in most clinical cases and nutrition surveys. As an alternative, random fasting or morning urine collections have been utilized and the urinary excretion of vitamin B₆ has been expressed in terms of per gram creatinine (Sauberlich et al 1972, US DHEW 1972). Sauberlich (1970) reported that random morning samples or 6 hr collections were reasonably indicative of total 24 hr excretion of vitamin B₆. As shown in Tables IX and X, the individual values for urinary excretion of vitamin B₆/g creatinine and vitamin B₆/24 hr followed the same pattern.

TABLE IX
URINARY LEVELS OF FREE VITAMIN B₆^a (μg/g creatinine)

Control Diet				Experimental Diet			
Subject Number	Base-line	Week 4	Week 8	Subject Number	Base-line	Week 4	Week 8
1	30.4	32.1	36.4	3	76.8	115.8	103.9
2	31.8	38.7	46.3	4	52.4	47.3	58.1
5	40.3	53.8	54.8	8	41.2	52.6	47.6
7	63.4	53.2	48.2	9	60.3	63.0	74.8
10	24.8	36.1	35.1	11	28.8	61.5	65.2
12	37.2	39.7	42.6	13	47.0	70.9	99.8
Mean	38.7	42.3	43.8	Mean	51.1	68.7	74.9

^aAverage of two 24 hr composites.

TABLE X
URINARY LEVELS OF FREE VITAMIN B₆^a (μg/24 hr)

Control Diet				Experimental Diet			
Subject Number	Base-line	Week 4	Week 8	Subject Number	Base-line	Week 4	Week 8
1	40.2	49.0	53.3	3	121.3	185.9	161.8
2	39.5	51.3	73.9	4	82.2	71.0	86.7
5	55.9	70.7	70.9	8	72.7	85.2	79.3
7	103.5	77.8	68.6	9	92.6	101.7	106.5
10	29.0	44.5	44.8	11	47.1	80.0	95.3
12	68.6	52.3	51.9	13	61.1	114.8	132.2
Mean	56.2	57.6	60.6	Mean	79.4	104.7	110.1

^aAverage of two 24 hr composites.

Free urinary vitamin B₆ concentrations were significantly higher ($P < 0.01$) for persons consuming the fortified bread than for those consuming the enriched bread (Tables XI and XII). These results are consistent with those of Baker et al (1964) who reported that the urinary excretion of free vitamin B₆ increased and correlated closely with the level of intake of the vitamin.

TABLE XI
ANALYSIS OF VARIANCE FOR VITAMIN B₆ ($\mu\text{g/g creatinine}$)

Source of Variation	DF	Mean Squares	F-ratio
Treatments	3		
Diet	1	4973.76	8.66 ^{ab}
Time	1	86.26	1.56 ^{cd}
Diet x Time	1	33.84	0.61 ^{cd}
Error A: Subject (Diet)	10	574.29	
Error B: Subject x Time (Diet)	10	55.40	

^aBased on Error A.

^b $P < 0.01$.

^cBased on Error B.

^d $P > 0.10$.

TABLE XII
ANALYSIS OF VARIANCE FOR VITAMIN B₆ ($\mu\text{g/24 hr}$)

Source of Variation	DF	Mean Squares	F-ratio
Treatments	3		
Diet	1	14016.67	9.97 ^{ab}
Time	1	108.38	0.95 ^{cd}
Diet x Time	1	9.38	0.08 ^{cd}
Error A: Subject (Diet)	10	1405.41	
Error B: Subject x Time (Diet)	10	114.15	

^aBased on Error A.

^b $P < 0.01$.

^cBased on Error B.

^d $P > 0.10$.

There was a sharp increase in urinary vitamin B₆ excretion levels of subjects ingesting fortified bread in week 4 as compared to their baseline levels. By this time the subjects should have adjusted to their increased vitamin B₆ intake and reached a plateau in the urinary excretion of the

vitamin. As shown by the analysis of variance, no statistical differences due to time were noted from week 4 to week 8. These results are consistent with those of Woodring and Storvick (1970) who reported that free vitamin B₆ values in the urine increased significantly almost immediately following the ingestion of a supplementary dose of pyridoxine hydrochloride and remained at the higher levels until the end of the period of supplementation. Pike and Brown (1975) suggested that urinary excretion levels of water-soluble vitamins may be interpreted as a rough estimate of cellular function; the relationship between intake and excretion is fairly linear, except at levels lower than the tissue saturation point.

Diet

As shown in Table XIII, the total vitamin B₆ values obtained from the six 24 hr food composites by analysis were found to be significantly correlated ($P < 0.05$) with the published values (Orr 1969, Haskell 1978); whereas the correlation between analyzed and calculated folate values (Perloff and Butrum 1977) was not significant. Apparently folate

TABLE XIII
COMPARISON OF ASSAYED AND CALCULATED FOLATE AND VITAMIN B₆ CONTENT
OF SIX 24 HR FOOD COMPOSITES

Day	Total folate		Total vitamin B ₆	
	Assayed values ($\mu\text{g/day}$)	Calculated values ^a ($\mu\text{g/day}$)	Assayed values (mg/day)	Calculated values ^b (mg/day)
1	379.6	209.7	1.435	1.202
2	291.7	111.0	0.804	0.958
3	229.2	219.9	1.295	1.390
4	540.1	286.6	1.539	2.199
5	234.6	223.1	1.604	1.478
6	525.9	200.7	1.888	2.500
Mean \pm SD	366.9 \pm 139.5	208.5 \pm 56.6	1.428 \pm 0.36	1.621 \pm 0.60
Pearson correlation coefficient	0.4082 ^c		0.8116 ^d	

^aBased on data in Perloff and Butrum (1977).

^c $P > 0.10$

^bBased on data in Orr (1969).

^d $P < 0.05$

concentrations of food stuffs are subject to greater variability than pyridoxine concentrations and the assay methods are not as well-defined. The folate content of individual foods usually falls within a wide range depending on geographical location, storage and methods of food preparation. The assayed folate values were considerably higher than the calculated values; these findings are consistent with those of Pietarinen et al (1977) who reported assayed values about 45% higher than calculated ones.

Diets were generally well-tolerated and acceptable to all subjects. Some problems were reported in regard to transit time and satiety. Subjects who reported abdominal discomfort or constipation were given psyllium hydrophilic mucilloid (Metamucil). During the first weeks of the study, some women reported feeling excessively hungry between meals; these reports diminished as caloric supplements were given to maintain body weight.

Discussion

Serum folate levels of the young women showed no differences due to the ingestion of bread fortified with folacin. The urinary excretion of free vitamin B₆ was significantly higher for the women consuming fortified bread, which indicated that urinary excretion of vitamin B₆ was reflective of a dietary intake in a range higher than tissue saturation level. Based on the results of this study, there seems to be no evidence that bread fortified with folacin and vitamin B₆ was beneficial in the diets of healthy young women. It should be noted that the women involved in this study had high initial serum folate and urinary vitamin B₆ levels and none were in an overt physiological state that increased the requirement for the vitamins.

The results of this study are based on two commonly employed microbiological methods in assessing nutritional status. The urinary level of vitamin B₆ is a reflection of the subject's recent dietary intake.

Assessment of vitamin B₆ nutriture by use of erythrocyte transaminase measurements has been used to indicate levels of vitamin B₆ deficiency (Woodring and Storvick, 1970) but we were unable to obtain reproducible measurements with this procedure. The measurement of serum folate, the procedure most commonly used to evaluate folacin nutritional status, also reflects dietary intake but is a poor indicator of the degree of folate deficiency. Present methods for determining folates in biological materials remain imprecise and laborious. Considering the existence of folacin hypovitaminosis and the discrepancies of published values of the folacin content of food, it is evident that improved folate analytical methods need to be developed.

SUMMARY

An expanded fortification proposal for cereal-grain products by the National Research Council (NRC) of the National Academy of Sciences (NAS) prompted the investigation of the relationship between the ingestion of bread fortified with folacin and vitamin B₆ and the concentration of these vitamins in the serum and urine, respectively. Twelve normal women, aged 20-27 years, served as subjects in the 8-week controlled feeding study.

The subjects consumed a basal diet, which was composed of common foods and based on a 6-day menu cycle, and 200 g of bread daily. The experimental subjects ate bread fortified according to the 1974 NAS/NRC proposal. Their average daily dietary intakes of vitamin B₆ and folacin were 5.0 mg and 528 µg, respectively. The control subjects, who ate bread enriched according to present standards, ingested an average of 2.0 mg of vitamin B₆ and 288 µg of folacin daily.

Blood and urine samples were collected during the week preceding the study, at the midpoint (week 4) and at the end (week 8) of the study. Serum folates were measured microbiologically using Lactobacillus casei as the test organism. The urinary levels of free vitamin B₆ were obtained by a microbiological assay method using Saccharomyces uvarum as the assay organism.

Serum folate concentrations were not significantly different due to diet (experimental vs control); but were different ($P < 0.05$) due to time (week 4 vs week 8). Serum folate levels for the experimental and control groups declined significantly from week 4 to week 8. Urinary vitamin B₆

concentrations were significantly higher ($P < 0.01$) for persons consuming fortified bread than for those consuming the enriched bread, which indicated that the urinary excretion of vitamin B₆ was reflective of a dietary intake in a range higher than tissue saturation level. Based on the results of this study, there seems to be no evidence that bread fortified with folacin and vitamin B₆ was beneficial in the diets of healthy young women.

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APPENDIX

STANDARD SPONGE DOUGH PROCEDURE FOR WHITE BREADS

1. Ingredients were weighed separately for the sponge and dough and, except for water, yeast and shortening were emptied into separate containers.
2. Sponge ingredients were placed in the mixing bowl.
3. The sponge was mixed for 3 min at Speed 1. The sponge temperature was $76 \pm 1^{\circ}\text{F}$.
4. The mixed sponge was placed in a lightly greased fermentation pan, placed in the fermentation cabinet and allowed to ferment for 4 hrs at 85°F and 85% relative humidity.
5. The dough ingredients were then placed in a mixing bowl and mixed 30 sec at Speed 1.
6. The fermented sponge was added to the dough ingredients in the mixer bowl and mixed for 1 min at Speed 1, and then at Speed 2 until the dough had reached optimum development. The temperature at this stage was 82°F .
7. The dough was then placed in the fermentation cabinet for a 45 min resting period. Each piece of dough was then scaled to 539 g/piece.
8. Each dough was hand-punched, sheeted and rolled triple-fold; relaxed for 10 min for an intermediate proof period; molded, panned and proofed to height (1.5 cm above pan) at 105°F and relative humidity of 92%.
9. Breads were baked at 425°F for 22 min.
10. After cooling for 1 hr, breads were sacked in plastic bags.

PROCEDURE FOR PREPARATION OF BREAD SAMPLES FOR FOLATE ASSAY

(Association of Vitamin Chemists, Inc. 1966)

1. Frozen bread was homogenized in a Waring blender.
2. Two 1 g samples of each bread type for each baking were placed in 24x150 mm test tubes.
3. Five ml of M/5 phosphate buffer (containing 27.23 mg KH_2PO_4 and 5.6 mg NaOH/ml) were added to each tube.
4. The mixture was heated for 5 min in a water bath at 100°C.
5. After cooling, 20 mg chicken pancreas (in 5 ml M/5 buffer) were added.
6. The mixture was incubated, under toluene, at 37°C for 24 hr.
7. The toluene was removed by heating in a boiling water bath for 5 min.
8. The solution was quantitatively transferred to a 100 ml volumetric flask and diluted so that 1 ml of the diluted solution contained approximately 0.2 μg of folacin.

PROCEDURE FOR PREPARATION OF BREAD SAMPLE FOR VITAMIN B₆ ASSAY

(Association of Vitamin Chemists, Inc. 1966)

1. Frozen bread was homogenized in a Waring blender.
2. Two 1 g samples each baking of fortified bread and two 2 g samples of each baking of enriched bread were weighed into a 500 ml Erlenmeyer flask.
3. One ml of 10 N HCl and 179 ml of water were added (0.055 N solution). Each beaker was covered with foil.
4. The samples were autoclaved at 15 lb pressure for 4 hr.
5. The cooled samples were adjusted to a pH of 4.5 with NaOH.
6. The entire sample was transferred to a 200 ml volumetric flask, filtered and diluted so that 1 ml of the sample contained approximately 1 ng of vitamin B₆/ml.

MEDICAL HISTORY

This information is strictly confidential and will be used only by the researchers and their medical consultant.

Name: _____ Date: _____ Age: _____

Campus Address: _____ Phone: _____

Home Address: _____ Phone: _____

Birthdate: _____ Place: _____

Name of Nearest Relative: _____ Relation: _____

Address: _____ Phone: _____

Height: _____ Weight: _____

Do you smoke? yes _____ no _____ If so, how often: _____

List any serious illnesses or health complications:

Have any health factors caused a change in lifestyle? If so, how?

Do you have any complications (Pain, distress, etc.) with exercise?

Have you ever had an EKG done? yes _____ no _____

Do you take any regular medications? If so, for what purpose(s)?

List medications.

Have any relatives had any type of heart disease? If so, list relation and complication and date.

Are you currently taking any oral contraceptives? If so, list brand or type and length of period you have been taking the pill.

Check if you have any of the following:

- | | |
|--|---|
| <input type="checkbox"/> diabetes mellitus | <input type="checkbox"/> chronic constipation or irregularity |
| <input type="checkbox"/> thyroid disorder | <input type="checkbox"/> gall bladder disease |
| <input type="checkbox"/> any type of infection | <input type="checkbox"/> gastric or duodenal ulcer |
| <input type="checkbox"/> any type of tumor | <input type="checkbox"/> abnormal bleeding |
| <input type="checkbox"/> fainting spells | <input type="checkbox"/> chills or fever |
| <input type="checkbox"/> epilepsy | <input type="checkbox"/> recent weight change |
| <input type="checkbox"/> frequent colds | <input type="checkbox"/> poor appetite |
| <input type="checkbox"/> frequent sore throat | <input type="checkbox"/> excessive weakness or tiredness |
| <input type="checkbox"/> persistent cough | <input type="checkbox"/> dizziness |
| <input type="checkbox"/> heart murmur | <input type="checkbox"/> shortness of breath |
| <input type="checkbox"/> rapid or irregular heart beat | <input type="checkbox"/> blood in the stools |
| <input type="checkbox"/> chest pains | <input type="checkbox"/> tarry stools |
| <input type="checkbox"/> high blood pressure | <input type="checkbox"/> blood in urine |
| <input type="checkbox"/> kidney disorder | <input type="checkbox"/> pus in urine |
| <input type="checkbox"/> irregular menstrual cycle | <input type="checkbox"/> leg cramps |
| | <input type="checkbox"/> swelling in any of the extremities |

Other: _____

Do you consider yourself as having poor health because of any chronic physical or mental condition? If so, discuss.

Family Medical History

Have any relatives ever been treated for:

- ☐ diabetes
- ☐ high blood pressure
- ☐ epilepsy
- ☐ abnormal lipid metabolism
- ☐ cancer

Do you consider yourself a: ☐ sedentary individual
☐ slightly active individual
☐ moderately active individual
☐ very active individual

DIETARY HISTORY

NAME: _____

1. List any vitamin-mineral or protein supplements taken on a regular basis.

Name brand and nutrient composition Amount (list per day or week)

2. How many times a week do you usually eat the following foods?

<u>Frequency</u>	<u>Food</u>
_____	eggs
_____	chicken
_____	pork
_____	beef
_____	lamb
_____	variety meats (luncheon meats, bologna, etc.)
_____	liver or other organ meats
_____	shrimp or lobster
_____	butter or margarine (type of oil, if possible)
_____	_____
_____	cheeses (list type)
_____	_____
_____	_____
_____	mayonnaise
_____	salad dressing (ex: Miracle Whip, Spin-Blend, etc.)
_____	other mixed salad dressings
_____	sour cream
_____	potato chips
_____	nuts
_____	avocado
_____	cream or coffee whitener (designate which item)
_____	_____
_____	milk (designate whole, 2%, skim) in cups _____
_____	other dairy products (designate)
_____	_____
_____	_____

_____ highly fortified foods (ex: Metrecal or other liquid
diet foods, Breakfast Squares, Total or Product
19, etc.)

_____ citrus fruit or juices (list type)

_____ pineapple

_____ strawberries

_____ tomato

_____ potato

_____ broccoli

_____ collard, mustard, turnip greens

_____ green peppers

_____ cabbage

_____ spinach

_____ summer squash

_____ cakes and pastries

_____ coffee

_____ tea

AGREEMENT AND RELEASE

1. I, _____ volunteer to participate in the Dietary Research Study to be conducted January 12-March 13, 1981 in the Department of Foods and Nutrition, Kansas State University by Beth Fryer, Project Director and Margaret Entz, Graduate Research Assistant.
2. I am willing to have blood drawn from me by venipuncture at the beginning, midway and end of the study for analyses of vitamins and mineral elements.
3. I understand that during the 9 week study, I will consume all the foods served to me and will not consume any foods or drinks outside the Metabolic Suite unless authorized by the researchers. Caloric intake will be adjusted so that I will maintain my weight ± 3 pounds.
4. I will collect urine and fecal samples as requested for a week at a time at the beginning, midway and end of the study.
5. I realize reports will be made of the study and I consent to publication of such without disclosure of my name.
6. I have been completely informed as to and understand the nature and purpose of the research. The researchers have offered to answer any further inquiries I may have. I understand that I will be able to leave the study at any time of my own accord.
7. As compensation for my voluntary services as a participant in this study, I will receive my meals and \$3.00 per day (payable at the end of the study).
8. As an agency of the State of Kansas, Kansas State University does not provide financial compensation (and/or long term care) to human subjects for injuries resulting from participation in this research.

Date _____

Signed _____

6-DAY MENU CYCLE

DAY 1	DAY 2	DAY 3
<u>Breakfast</u>	<u>Breakfast</u>	<u>Breakfast</u>
Orange	Grapefruit half	Orange
131 g	170 g	131 g
Cinnamon toast	Egg, poached	Egg, boiled
18 g	50 g	50 g
Cinnamon/sugar	Bacon	Bread
10 g	7.5 g	50 g
Margarine	Toast	Margarine
50 g	50 g	10 g
Bread	Margarine	Jelly
64 g	10 g	18 g
Egg, scrambled	Skim milk	Skim milk
245 g	245 g	245 g
<u>Lunch</u>	<u>Lunch</u>	<u>Lunch</u>
B-L-T sandwich	Reuben sandwich	Beef and vegetable stew
Bread	Bread	Stew beef
50 g	50 g	71 g
Bacon	Corned beef	Carrots
15 g	24 g	18 g
Lettuce	Swiss cheese	Celery
25 g	28 g	10 g
Tomato	Sauerkraut	Onion
68 g	25 g	11 g
Mayonnaise	Margarine	Potatoes
14 g	10 g	39 g
Skim milk	Apple	Tomatoes
245 g	138 g	60 g
Apple		Bread
138 g		50 g
<u>Dinner</u>	<u>Dinner</u>	<u>Dinner</u>
Roast beef	BBQ beefburgers	Margarine
85 g	70 g	10 g
Bread	Ground beef, raw	Skim milk
50 g	11 g	245 g
Margarine	Bread crumbs	Grapes
10 g	30 g	100 g
Coleslaw	Sauce	
84 g	47 g	
Apricot halves	Celery sticks	Ground beef, cooked
65 g	18 g	40 g
Apricots	Carrot sticks	Sauce
Juice	Bread	Swiss cheese
64 g	38 g	25 g
	Margarine	Crust, bread dough
	Oatmeal cookie	55 g
	Peaches	Salad
	Peaches	Lettuce
	Juice	55 g
	Skim milk	Tomato
	245 g	70 g
<u>Snack</u>	<u>Snack</u>	<u>Snack</u>
Turkey sandwich	Ham sandwich	Tuna sandwich
Bread	Bread	Bread
50 g	50 g	50 g
Turkey roll	Ham	Tuna
57 g	28 g	43 g
Mayonnaise	Mayonnaise	Mayonnaise
14 g	14 g	14 g
		Pickle relish
		5 g

DAY 4		DAY 5		DAY 6	
<u>Breakfast</u>		<u>Breakfast</u>		<u>Breakfast</u>	
Tomato juice	182 g	Orange juice	124 g	V-8 juice	182 g
Egg, scrambled	64 g	Egg, poached	50 g	Egg, boiled	50 g
Bread	50 g	Bacon	8 g	Bread	50 g
Margarine	10 g	Bread	50 g	Margarine	10 g
Jelly	18 g	Margarine	10 g	Jelly	10 g
Skim milk	245 g	Skim milk	245 g	Skim milk	245 g
<u>Lunch</u>		<u>Lunch</u>		<u>Lunch</u>	
Fruit salad		Vegetable soup		Spaghetti casserole	
Cottage cheese	113 g	Carrots	18 g	Spaghetti, cooked	105 g
Apple	69 g	Celery	24 g	Ground beef, cooked	50 g
Orange	66 g	Onion	20 g	Sauce	60 g
Peach	122 g	Potatoes	52 g	Salad	
Pineapple	58 g	Tomatoes	75 g	Lettuce	55 g
Banana, peeled	40 g	Toasted cheese bread		French dressing	13 g
Bread	50 g	Bread	50 g	Bread	50 g
Margarine	10 g	Mozzarella cheese	28 g	Margarine	10 g
		Margarine	10 g	Grapefruit	170 g
<u>Dinner</u>		Apple	138 g	<u>Dinner</u>	
Hamburger pattie	57 g			Stir-fried chicken and vegetables	
French fries	50 g	<u>Dinner</u>		Chicken breast	56 g
Salad		Porcupine meatballs		Broccoli	40 g
Lettuce	98 g	Ground beef, raw	70 g	Onions	11 g
Tomato	70 g	Tomato paste	26 g	Celery	15 g
French dressing	26 g	Rice	70 g	Carrots	18 g
Bread	50 g	Green beans	65 g	Oil	5 g
Margarine	10 g	Carrot sticks	36 g	Rice	40 g
Orange	131 g	Bread	50 g	Bread	50 g
		Margarine	10 g	Margarine	10 g
		Ice cream	50 g	Skim milk	245 g
<u>Snack</u>				Banana, with peel	175 g
Bologna sandwich		<u>Snack</u>			
Bread	50 g	Roast beef sandwich		Peanut butter sandwich	
Bologna	28 g	Bread	50 g	Bread	50 g
Mayonnaise	14 g	Roast beef	84 g	Peanut butter	16 g
Lettuce	12 g	Mayonnaise	14 g	Jelly	18 g

TABLE XIV
NUTRIENT CONTENT OF 6-DAY BASAL DIET^a

Nutrient	Day						Mean ^b
	1	2	3	4	5	6	
Energy (kcal)	1267	1237	1205	1311	1292	1251	1261 ± 38
Protein (g)	75.7	61.8	68.2	62.8	73.4	64.6	67.8 ± 5.7
Vitamin A (IU)	5590	6235	5568	5502	10457	8333	6948 ± 2029
Thiamin (mg)	0.36	0.41	0.36	0.52	0.39	0.37	0.40 ± 0.06
Riboflavin (mg)	2.46	1.90	2.52	2.32	2.02	2.04	2.21 ± 0.26
Niacin (mg)	13.0	8.6	12.2	11.5	12.5	15.9	12.3 ± 2.4
Folacin (μg)	380	292	229	540	235	526	367 ± 40
Vitamin B ₆	1.4	0.8	1.3	1.5	1.6	1.9	1.4 ± 0.4
Calcium (mg)	636	691	694	498	582	538	606 ± 80
Iron (mg)	13.0	10.2	10.4	13.0	10.0	9.5	11.0 ± 1.6
Magnesium (mg)	157	133	153	164	147	204	160 ± 24
Zinc (mg)	14.0	11.5	13.2	13.8	12.6	10.0	12.5 ± 1.5

^aEnergy, protein, vitamin A and niacin values were based on data in USDA Home and Garden Bulletin No. 72 (1978). Thiamin, riboflavin, folacin, vitamin B₆, calcium, magnesium, iron and zinc values were obtained by direct analysis.

^bMean ± SD.

PREPARATION OF SERUM SPECIMENS FOR FOLATE ASSAY

Difco Laboratories

1. Serum samples containing ascorbic acid (1 mg/ml serum) were thawed.
2. The 1 ml sample was added to 9 ml rehydrated Bacto-Folic Buffer A (code-3246-21).
3. The serum-buffer solution was incubated at 37°C for 90 min and then autoclaved for 2.5 min at 15 lbs pressure.
4. The cooled mixture was filtered to remove the coagulated protein and the clear solution was diluted to use in the folic acid assay.

FOLACIN AND VITAMIN B₆ STATUS OF YOUNG WOMEN
INGESTING NAS/NRC FORTIFIED BREAD

by

MARGARET M. ENTZ

B.A., Bethel College, 1975

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Foods and Nutrition

KANSAS STATE UNIVERSITY
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An expanded fortification proposal for cereal-grain products by the National Research Council (NRC) of the National Academy of Sciences (NAS) prompted the investigation of the relationship between the ingestion of bread fortified with folacin and vitamin B₆ and the concentration of these vitamins in the serum and urine, respectively. Twelve normal women, aged 20-27 years, served as subjects in the 8-week controlled feeding study.

The subjects consumed a basal diet, which was composed of common foods and based on a 6-day menu cycle, and 200 g of bread daily. The experimental subjects ate bread fortified according to the 1974 NAS/NRC proposal. Their average daily dietary intakes of vitamin B₆ and folacin were 5.0 mg and 528 µg, respectively. The control subjects, who ate bread enriched according to present standards, ingested an average of 2.0 mg of vitamin B₆ and 288 µg of folacin daily.

Blood and urine samples were collected during the week preceding the study, at the midpoint (week 4) and at the end (week 8) of the study. Serum folates were measured microbiologically using Lactobacillus casei as the test organism. The urinary levels of free vitamin B₆ were obtained by a microbiological assay method using Saccharomyces uvarum as the assay organism.

Serum folate concentrations were not significantly different due to diet (experimental vs control); but were different ($P < 0.05$) due to time (week 4 vs week 8). Serum folate levels for the experimental and control groups declined significantly from week 4 to week 8. Urinary vitamin B₆ concentrations were significantly higher ($P < 0.01$) for persons consuming fortified bread than for those consuming the enriched bread, which indicated that the urinary excretion of vitamin B₆ was reflective of a dietary intake in a range higher than tissue saturation level. Based on the results of this study, there seems to be no evidence that bread fortified with folacin and vitamin B₆ was beneficial in the diets of healthy young women.