FOLATE STATUS RESPONSE OF
PREGNANT WOMEN AND NONPREGNANT CONTROLS
TO CONTROLLED FOLATE INTAKES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL
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This dissertation is dedicated to my mom and dad who from the beginning taught me that along with hard work comes opportunity. I thank them for their unconditional love and support.
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<tr>
<td>apABG</td>
<td>acetamidobenzoylglutamate</td>
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<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
<td></td>
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<tr>
<td>AICAR</td>
<td>5-formiminoimidazole 4-carboxamide ribonucleotide</td>
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<tr>
<td>ATP</td>
<td>adenosine 5’triphosphate</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Centers for Disease Control</td>
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<td>d</td>
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<td>DCI</td>
<td>deuterium chloride</td>
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<td>diethylaminoethyl</td>
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<td>dihydrofolate</td>
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<td>deoxyuridylic acid</td>
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<td>dUST</td>
<td>deoxyuridine suppression test</td>
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<td>EDTA</td>
<td>ethylenediamine tereacetic acid</td>
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<td>FBP</td>
<td>folate binding protein</td>
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<td>GCMS</td>
<td>gas chromatography mass spectrometry</td>
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<tr>
<td>GAR</td>
<td>glycinamide ribotide</td>
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<td>glu</td>
<td>glutamate</td>
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<td>h</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HCl</td>
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<td>mg</td>
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<tr>
<td>MeCN</td>
<td>acetonitride</td>
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<td>MRC</td>
<td>Medical Research Council</td>
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<td>MTHFR</td>
<td>methylenetetrahydrofolate reductase</td>
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<td>NaCl</td>
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<tr>
<td>NaOD</td>
<td>sodium deuteroxide</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>ng</td>
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<td>Second National Health and Nutrition Examination Survey</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>pABA</td>
<td>p-aminobenzoic acid</td>
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<td>p-aminobenzoylglutamic acid</td>
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<td>Pd</td>
<td>palladium</td>
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<td>PHS</td>
<td>Public Health Service</td>
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<td>PLP</td>
<td>pyridoxal 5'-phosphate</td>
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<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
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<td>RNA</td>
<td>ribonucleic acids</td>
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<td>THF</td>
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<td>µg</td>
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<td>US</td>
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

FOLATE STATUS RESPONSE OF PREGNANT WOMEN AND NONPREGNANT CONTROLS TO CONTROLLED FOLATE INTAKES

By

Marie Anne Caudill

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Chairperson: Dr. Lynn B. Bailey
Major Department: Food Science and Human Nutrition

Folate is a B vitamin that plays a major coenzymatic role in one-carbon metabolism and is a key participant in the metabolism of certain amino acids and biosynthesis of pyrimidines and purines. Folate requirements are higher during pregnancy, although the increment above that required by nonpregnant women has not been ascertained. The 1989 RDA for both pregnant and nonpregnant women was reduced by approximately one-half. The rationale for the reduction in pregnant women from 800 to 400 μg/d was based primarily on studies in which folate intake was not controlled.

The purpose of this 84 d study was to investigate folate status response of pregnant subjects (n=12) during their second trimester and nonpregnant controls (n=12) to defined folate intakes comparable to the current (400 μg/d) and former (800 μg/d) RDA for pregnant women. Subjects consumed either 450 or 850 μg/d
provided as a combination of dietary folate and synthetic folic acid. The outcome
variables used to assess folate status response included serum, red cell and urinary
folate and the folate catabolites pABG and apABG. Comparisons between and
within supplementation groups were made at steady state or the time at which the
subjects had acclimatized to their assigned folate intake.

No differences (P>0.05) were detected in serum and red cell folate
concentrations or in urinary 5-methyl-THF excretion between pregnant and
nonpregnant women consuming either 450 or 850 µg/d. Serum folate and 5-
 methyl-THF excretion were higher (P≤0.05) in subjects consuming 850 compared
to 450 µg/d. Urinary folic acid (14.5 µg/d) was detected only in nonpregnant
controls consuming 850 µg/d folate. No differences (P>0.05) were detected in
pABG or apABG concentrations between pregnant and nonpregnant women
consuming either 450 or 850 µg/d. Pregnant women consuming 450 compared to
850 µg/d excreted less (P≤0.05) of both catabolites by the end of the study.

In summary, no differences were detected in the outcome variables
measured between nonpregnant and pregnant women within the same
supplementation group. Further, normal folate status was maintained by all
groups. These data suggest that 450 µg/d of folate derived from both dietary and
synthetic sources is adequate for pregnant women during their second trimester.
This level of intake equates to approximately 600 µg/d dietary equivalents
assuming 50% and 75% availability of food folate and synthetic folic acid
consumed with meals, respectively.
CHAPTER 1
INTRODUCTION

Folate is a generic descriptor for compounds that have nutritional properties and chemical structures similar to those of folic acid (pteroylglutamic acid). Thus, it includes folic acid, the oxidized, monoglutamate form of the vitamin provided in supplements and fortified foods, and the reduced, naturally occurring, polyglutamyl folates, which are the predominant dietary forms. De novo biosynthesis of folates occurs only in bacteria and plants so humans must obtain this vitamin from their diet.

Folate in its fully reduced state and polyglutamyl form participates in a variety of coenzyme reactions involving one-carbon transfers required for amino acid metabolism and nucleic acid synthesis, as well as the biosynthesis of specific lipids, hormones and neurotransmitters. Deficiency of the vitamin leads to impaired cell division and to abnormalities in numerous synthetic pathways dependent on one-carbon transfer reactions. A wide array of clinical problems may result from a deficiency including megaloblastic anemia, growth failure, malabsorption syndromes and increased risk for certain types of cancer and atherosclerosis (Bailey 1990a, Giovannucci et al. 1993, Selhub et al. 1995).

Pregnancy is associated with increased requirements for folate, although controversy exists regarding the amount of folate required to meet the demands of
the pregnant woman and her developing fetus (NRC 1989, Subcommittee on Dietary Intake and Nutrient Supplement During Pregnancy 1990, Herbert 1987a, Bailey 1995). Adequate folate intake is essential during pregnancy since this time represents a state of increased cellular division due to the rapid growth of the fetus, the placenta and the maternal organs such as the uterus (Cunningham et al. 1993). In addition, there is an increase in erythropoiesis, a folate requiring process, and plasma resulting in the expansion of maternal blood volume (Cunningham et al. 1993). The pregnancy induced hypervolemia which starts during the first trimester serves to meet the demands of the enlarged organs (Cunningham et al. 1993). Due to the severe demands that are placed on the supply of folate during pregnancy for the synthesis of DNA, pregnant women are at increased risk of developing a folate deficiency (Bailey et al. 1980, O'Connor 1994). Sauberlich (1990) summarized numerous studies both within the United States and world wide that illustrated the widespread prevalence of poor folate nutriture during pregnancy. Bailey et al. (1980) reported that among a group of low income pregnant women, folate deficiency was significantly more prevalent than iron deficiency at the initiation of prenatal care. These data clearly illustrate that many women are unable to meet the increased demand for folate during pregnancy through diet alone which has resulted in the acceptance of routine prenatal folate supplementation (Sauberlich 1995).

Unfortunately, due to lack of data regarding the size of the folate body pool and equilibrium of the vitamin during pregnancy, the National Academy of
Sciences Committee on Dietary Intake and Nutrient Supplements During Pregnancy (1990) could not establish a specific supplementation recommendation for folic acid. Despite the widespread acceptance of folic acid supplementation for the vast majority of pregnant women, the Committee recommended that folic acid supplementation be reserved for specific "high risk" population groups such as pregnant adolescents but not for the pregnant population as a whole. However, this recommendation preceded conclusive research regarding NTDs and therefore was not addressed by this committee.

The 1989 RDA for folate was lowered from 800 µg/d (NRC 1980) to 400 µg/d (NRC 1989) for pregnant women. The reduction in the RDA for pregnant women was largely based on the findings of two studies (NRC 1989). Chanarin et al. (1968b) reported that 100 µg/d of synthetic folic acid, in addition to dietary folate, was able to maintain red cell folate concentrations in pregnant women throughout gestation. Bates et al. (1982) subsequently reported an estimated dietary folate intake of 190 µg/d for this population group. This estimate (190 µg/d) in conjunction with Chanarin's report that 100 µg/d plus diet maintained red cell folate concentrations was used by the 10th edition RDA committee to support the 400 µg/d RDA (NRC 1989). Colman et al. (1975) found that maize fortified with 300 µg/d folic acid consumed with a constant diet maintained normal serum and red cell folate concentrations during the last thirty days of pregnancy in a rural, poorly nourished, African population. Interpretation of these studies is complex, and illustrates the importance of investigating folate requirements in
healthy pregnant women under controlled metabolic conditions during which time the response of pregnant women to known levels of folate intake can be assessed. Currently, relatively little is known about the requirement for folate during pregnancy, the specific tissue needs for folate, or how to assess folate status during gestation (i.e. the range of acceptable values) (O’Connor 1994).

In recent years, studies have been conducted to assess the appropriateness of the decrease in the RDA for nonpregnant women of child-bearing age and adult men. O’Keefe et al. (1995) reported that the 1989 RDA for nonpregnant women of 180 µg/d was insufficient to maintain initial folate status in healthy, unsupplemented nonpregnant women and that 400 µg/d more adequately met the criteria of an RDA. Similarly, Jacob et al. (1994) reported that 200 µg of folate per day was insufficient to achieve normal plasma homocysteine concentrations after a moderate depletion period in a depletion-repletion protocol involving ten healthy male adults. These data suggested that both males and nonpregnant females require more than the current RDA to maintain normal folate nutriture.

McPartlin et al. (1993) used an alternative approach in estimating folate requirements in pregnant women at one time point during each trimester. Their approach was based on the quantitation of the urinary folate catabolites, p-aminobenzoylglutamate (pABG) and the more predominant form, acetamidobenzoylglutamate (apABG). These catabolites were hypothesized to represent folate turn over by McPartlin and associates (1993) and therefore a more direct way to estimate requirements. McPartlin et al. (1993) reported that based
on the urinary excretion of pABG and apABG, the current RDA for pregnant women was inadequate particularly in the second trimester. Limitations of this study included the fact that folate intake was not controlled and folate status was not assessed.

We conducted a controlled metabolic trial to investigate the response of pregnant women in their second trimester to folate intakes comparable to the current RDA (400 µg/d) and the former RDA (800 µg/d). Two nonpregnant groups consisting of women similar in age, lifestyle and demographics were included as a control. The decision to select the second trimester was based on the findings of McPartlin et al. (1993) which indicated that the second trimester represented the highest rate of folate catabolism and thus the greatest requirement for folate. The overall goal of the study was to provide metabolic data which could be used to assess the adequacy of the current RDA for pregnant women. One objective of this study was to determine if differences existed in folate status and catabolite excretion between pregnant subjects and nonpregnant controls consuming equivalent folate intakes. A second objective was to determine if differences existed in folate status and catabolite excretion between pregnant subjects consuming folate intakes approximating the current RDA (400 µg/d) compared to the former RDA (800 µg/d).
Hypotheses

The major hypotheses and specific aims of this research were as follows.

**Hypothesis 1.** The response of pregnant women and nonpregnant controls to two defined levels of folate intake (450 or 850 μg) would indicate a greater folate requirement for pregnant women as determined by changes in folate status indices and catabolite excretion.

**Hypothesis 2.** Folate response indices would reflect a decline in folate nutriture approaching negative folate balance among pregnant women consuming 450 μg/d of folate and maintenance of folate status in pregnant women consuming 850 μg/d.

**Hypothesis 3.** Folate response indices would reflect maintenance of folate status in nonpregnant women consuming 450 μg/d of folate and an excess exceeding the body's ability to metabolize or store folate in nonpregnant women consuming 850 μg/d of folate.

Specific Aims

**Aim 1.** To determine weekly serum and red cell folate concentrations via microbiological assay during the study period and monthly concentrations during the follow-up period. Differences between and within supplementation groups would help assess the adequacy of folate intake.

**Aim 2.** To determine weekly urinary excretion of intact urinary folate via microbiological assay and to determine the specific forms of excreted urinary intact folate by high performance liquid chromatography. Data would be
obtained related to the amount of excreted, unmetabolized urinary intact folate (folic acid) and the amount of excreted, metabolized urinary intact folate (5-methyl-tetrahydrofolate) and comparisons would be made between and within supplementation groups.

**Aim 3.** To quantitate the urinary excretion of the two major folate catabolites, pABG and apABG. Information on folate turnover and possibly requirements during pregnancy would be obtained based on the premise that catabolized folate reflects utilization and that which needs to be replaced daily.

Folate was provided as a combination of dietary and synthetic sources. During the first half of the study, a small percentage of the total daily intake of folate came from folate labeled with deuterium followed by provision of 100% unlabeled folate. Labeling of synthetic folic acid with deuterium, a staple isotope, can be used safely in humans (Gregory et al. 1990). Information on folate kinetics, including turnover rates and pool sizes, can be obtained by comparing the ratio of labeled folate to unlabeled folate in biological samples collected from humans consuming deuterated folic acid. A small subsample of subjects consisting of one subject from each group (n=4) was also given $^{13}$C-pABG midway through the 12 wk period so that the bioavailability of exogenous pABG could be evaluated and reported separately by a co-worker.

Several parameters were used to assess folate nutriture including serum folate, red cell folate, urinary intact folate and the urinary breakdown products, pABG and apABG. Kinetic data will also be obtained based on the isotopic
enrichment of biological samples including urine, red cell and plasma and reported separately. Plasma homocysteine, a functional index of folate status, was measured by a co-worker.
CHAPTER 2
LITERATURE REVIEW

History

In 1931, Dr. Lucy Wills described a macrocytic anemia in pregnant Hindu women that responded to treatment with yeast extract. Later, Wills and Bilimoria (1932) described a similar macrocytic anemia in monkeys that responded to a factor present in crude liver extracts. The active principle in liver and yeast extracts described by Wills had many properties in common with bacterial “growth factors” present in liver (Snell and Peterson 1940) and spinach leaves (Mitchell et al. 1941). The term folic acid, derived from the Latin word “folium” meaning “leaf,” was coined by Mitchell et al. (1941) after isolating this substance from spinach leaves. Folic acid was synthesized by Angier et al. (1945), and it was shown to be the common factor in the preceding observations.

Folate Biochemistry

The folic acid molecule consists of three parts: the pteridine moiety (with an amino group in position 2 and hydroxyl group in position 4); the para-aminobenzoic acid; and the L-glutamic acid (Figure 1). The pteridine component is linked by a methylene bridge to para-aminobenzoic acid which is joined by peptide linkage to glutamic acid. The first two fractions compose pteroic acid which leads to the classification of folic acid as pteroyl-glutamic acid. Folic acid
(C_{19}H_{19}N_{7}O_{6}) is named in the chemical literature as a derivative of L-glutamic acid: N-[4-[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-L-glutamic acid. Crystalline folic acid is yellow with a molecular weight of 441.41 g/mol. Folic acid, itself, is not biochemically active but becomes so after it has undergone reduction. Naturally occurring folates include a very large number of compounds because (a) the pirazine ring (atoms 4a,5,6,7,8 and 8a) of the pteridine moiety can occur in three states of reduction with (b) six different one-carbon substituents present at N-5 and/or N-10 and (c) the polyglutamyl chain may have as many as 12 glutamyl residues (Krumdieck 1990).

In nature, folate exists mainly in the conjugated forms. Pentaglutamates and hexaglutamates predominate as the intracellular folates in animal tissue, while
extracellular folates (i.e. in plasma, spinal fluid, bile and urine) are monoglutamate derivatives (Krumdieck 1990). Most natural folates are unstable and are degraded by heat, oxidation and ultraviolet light. Some tetrahydrofolate (THF) derivatives including 5-formyl-THF and 5-methyl-THF are relatively heat stable. Acid will destroy THF and 5,10-methylene-THF. The addition of ascorbic acid and other reducing agents has helped tremendously in preventing the oxidation and subsequent degradation of folates in biological samples.

Folates have limited solubility in water and in most organic solvents such as methanol. Folates are minimally soluble in the mildly acidic range (pH 2 to 4), with solubility increasing in proportion to the pH above this range (Gregory 1989). Folates are also highly soluble at very low pH.

**Food Distribution and Folate Bioavailability**

Folate is found in a wide variety of foods originating from both animal and plant sources. Liver, mushrooms, orange juice and green leafy vegetables are particularly good sources (Combs 1992). White bread, rolls and crackers, although not rich sources of folate, represent major contributors of folate in the U.S. diet according to the Second National Health and Nutrition Examination Survey (NHANES II) data, 1976-1980 (Subar et al. 1989). These sources are likely to remain major contributors secondary to the FDA mandating the fortification of bread products with folic acid (FDA 1996). Dietary folate exists primarily in the reduced form as polyglutamyl derivatives of THF with 5-methyl-THF and 10-formyl-THF predominating. Very little free folate (folyl monoglutamate) is found in foods. Food storage and preparation can greatly
affect the stability of folate. Exposure of the folate molecule to heat, ultraviolet light, air and metal ions will result in oxidation and subsequent cleavage rendering it inactive (Herbert 1987a, Gregory 1989), although a small portion of the oxidized folates may be converted back to biologically active forms (Gregory 1989, Shane 1995). Further, because folate is a water soluble vitamin, it can be leached out of foods during periods of boiling.

Before folates can be absorbed from the gut, glutamyl residues in excess of one must be removed because only monoglutamyl forms can enter the enterocyte. This cleavage is accomplished by an exocarboxypeptidase folyl \( \gamma \) glutamyl hydrolase, more commonly referred to as folate conjugase (EC 3.4.12.10) (Halsted 1979) which functions at a pH optimum of 6.5 (Gregory 1995). Medications or conditions that increase this pH will decrease the activity of this enzyme possibly leading to folate malabsorption. Natural conjugase inhibitors found in certain foods including yeast, beans and lentils may also reduce folate availability (Bailey 1988, Butterworth et al. 1974). However, Wei et al. (1996) reported high relative bioavailability of both monoglutamyl and hexaglutamyl folic acid when added to lima beans and tomatoes. Chronic exposure to alcohol and possibly zinc deficiency may also reduce folate bioavailability (Bailey 1988, Naughton et al. 1988, Tamura et al. 1978).

The monoglutamate form of folate is actively transported across the proximal small intestine by a saturable, pH-dependent process (Gregory 1995, Halsted 1990, Rose 1978). Two folate-binding proteins (FBPs) exist in the
jejunal brush border and are thought to be involved in folate binding and transport (Shoda et al. 1993). Folic acid can also be absorbed by a nonsaturable mechanism involving passive diffusion when high concentrations are consumed (Gregory 1995).

The term "folate bioavailability" refers to the efficiency of utilization of this vitamin, including physiological and biochemical processes involved in intestinal absorption, transport, metabolism and excretion (Gregory 1995). The bioavailability of dietary folate is estimated to be approximately 50% (Sauberlich et al. 1987). In contrast, synthetic folic acid consumed under fasting conditions is approximately 100% available (Gregory 1995). Foods fortified with synthetic folic acid were initially considered to be only 50% available (Colman et al. 1975). More recently, however, studies have found that foods fortified with synthetic folic acid are highly available (Cuskelly et al. 1996, Pfeiffer et al. 1997). Pfeiffer et al. (1997) determined the bioavailability of $^{13}$C$_5$ folic acid in various fortified cereal-grain products and in water as a control, relative to the intravenously injected $^2$H$_2$folic acid. Measurement of isotope excretion ratios of urinary folates (% of $^{13}$C$_5$ folate dose / % of $^2$H$_2$folate dose) indicated no differences among treatment groups or the control suggesting that folic acid fortified cereal-grain foods were highly available. Pfeiffer and associates (1997) also indicated that consumption of $^{13}$C$_5$folic acid after a light breakfast meal did not significantly effect its bioavailability. The influence of food on the bioavailability of simultaneously ingested folic acid supplements has not been firmly established.
However, based on recently reported data related to fortified foods, one could hypothesize that it is more available than endogenous folate present in food and less available than folic acid consumed under fasting conditions.

Once folate enters the enterocyte, it usually undergoes reduction to THF and either methylation or formylation (Perry 1973, Selhub et al. 1983). However, folate metabolism within the enterocyte is not required for entry into portal blood or for transport (Shane 1995). When high doses of various folates are given, little metabolism occurs, and the majority of folate appears in portal circulation unchanged (Herbert and Das 1994). The degree of metabolism in the intestinal mucosa is a function of the amount of folate administered (Gregory 1995). Because the reduction process is readily saturable (Gregory 1995), supplemental folic acid may be more efficiently reduced and methylated if divided into several smaller doses (Lucock et al. 1989b). Gregory (1995) suggested that this may increase the efficiency of its metabolic utilization and improve its bioavailability.

In 1966, Giles hypothesized that the high incidence of folate deficiency during pregnancy was a consequence of reduced folate absorption. Iyengar and Buba (1975) subsequently conducted a study to compare folic acid absorption in pregnant women to that of nonpregnant women. Two milligrams of folic acid along with 10 μCi of tritiated folic acid were administered to 9 pregnant women between 10 to 26 wk gestation undergoing therapeutic termination of pregnancy and 6 nonpregnant women undergoing sterilization. Absorption varied between 65 and 95 percent, with a mean absorption of 80 percent in both pregnant and
nonpregnant subjects. The results indicated that there was no alteration in absorption of a large dose of folic acid during the first two trimesters of pregnancy. A limitation to this study was lack of comparison between pregnant and nonpregnant women in their ability to absorb polyglutamyl folates. Studies (Bailey et al. 1988, Gregory et al. 1991) conducted in nonpregnant subjects to determine the bioavailability of mono- and polyglutamyl folate suggested partial availability of polyglutamyl folates compared to monoglutamyl forms. However, Wei et al. (1996) reported high availability of both polyglutamyl and monoglutamyl forms when added to lima beans and tomatoes.

**Transport and Tissue Distribution**

Monoglutamates, mainly 5-methyl-THF, and to a lesser extent folic acid, dihydrofolate (DHF) and formylated forms are present in portal circulation. Approximately two-thirds of the folate in plasma is bound to protein (Groff et al. 1995). The majority of these proteins bind folate with relatively low affinity and are non-saturable (Ratnam and Freisheim, 1990). Examples include albumin and α-2 macroglobin (Groff et al. 1995). In contrast, folate binding proteins present in low amounts in plasma and blood cells bind folate with high affinity (Wagner 1995). Free folate accounts for the remainder of folate found in the plasma. Folate can then be taken up by the liver or transported to extrahepatic tissues. Within the liver, folate is metabolized to polyglutamate derivatives by folyl polyglutamate synthetase and retained or released into blood or bile. The folate derivative, 5-methyl-THF, is a poor substrate for folyl polyglutamate synthetase
and in order to be conjugated must first have its methyl group removed once it enters the cell. This can be accomplished by methionine synthase, a vitamin $B_{12}$-dependent enzyme. Polyglutamates cannot cross biological membranes and deconjugation to the monoglutamate form (removal of glutamyl residues in excess of one) must occur prior to releasing folate into circulation (Combs 1992).

Tetrahydrofolate, 5-methyl-THF and 10-formyl-THF are the predominate folate derivatives found in the liver and are typically bound to glutamates varying in length from 4 to 7 (Groff et al. 1995). Most of the 5-methyl and 10-formyl derivatives are secreted into bile which are then reabsorbed via enterohepatic circulation or excreted via the feces. Approximately half of the total folate that reaches peripheral tissues occurs by this recirculation process (Steinberg 1984). Folate secreted directly into circulation is transported to other tissues bound to either low or high affinity folate binders as previously described.

Recent studies from numerous laboratories suggest the existence of several distinct mechanisms for transport of folate across membranes (Henderson 1990). Folate transport into tissue cells can occur by a carrier-mediated process that may or may not require ATP (Groff et al. 1995) or by folate binding proteins that are associated with the plasma membrane of cells (Da Costa and Rothenberg 1996). These membrane-associated folate binding proteins act as folate receptors and thereby facilitate cellular uptake of folate (Da Costa and Rothenberg 1996). These receptors are attached to the plasma membrane via a glycosylphosphatidylinositol anchor and can also be released from the membrane
as a soluble protein upon hydrolysis by a glycosyl phosphatidylinositol-specific phospholipase C (Lee et al. 1992). Although many cells do not express this membrane-associated FBP, high levels are found in placental trophoblastic cells, ovaries, fallopian tubes and mammary glands (Weitman et al. 1992). It was hypothesized that the membrane-associated folate receptor's of placental trophoblastic cells provides the following two advantages: 1) ensures adequate folate supply even under conditions of deficiency secondary to the receptors high affinity for folates; and 2) prevents potentially harmful folate analogues from entering the placental trophoblastic cells due to the high substrate specificity of the receptor (Selhub 1994). The folate receptors found in placental cells thereby appear to ensure adequate folate supply to the fetus even when maternal stores are depleted (Selhub 1994). Recently, an ex vivo placental cotyledon perfusion model was used by Henderson et al. (1995) to test the hypothesis that under physiologic conditions, placental folate receptors played a major functional role in transplacental folate transport. Their results indicated that maternal-to-fetal folate transfer is a two-step process. The first step concentrates 5-methyl-THF (or folic acid) by binding it to placental folate receptors on the maternally facing chorionic surface. Gradual release of 5-methyl-THF from this pool adds to incoming circulating folates to generate an intervillous blood concentration (placental compartment) three times that of maternal blood. The second step involves transfer of folates to fetal circulation along a downhill concentration gradient. Thus, folate bound to placental folate receptors are predestined for transplacental
folate transport, since incoming (dietary) folates displace placental folate receptor bound folates that then passively diffuse to the fetus (Antony 1996). This study provided a mechanism for the observation that the developing fetus drains maternal folate supplies as evidenced by the significantly higher plasma and red cell folate concentrations of newborns compared to maternal concentrations (Ek and Magnus 1981).

Folate receptors are also involved in folate transport across proximal renal tubular cells (Selhub 1994). Data suggest that after glomerular filtration, the luminal folate binds folate receptors in the renal tubular cells and is internalized rapidly via folate receptor mediated endocytosis. Dissociation of folate from the receptor occurs in the low pH of endocytotic vesicles allowing the return of folate to the blood, where it can be taken up by other cells, and the recycling of apo-folate receptor back to the luminal brush-border membrane.

Once within cells, 5-methyl-THF (predominate folate derivative in plasma or urine) is demethylated and converted back to a polyglutamyl form by folylpoly-γ-glutamate synthetase in order to become a functional coenzyme, accepting and transferring one-carbon fragments (Wagner 1995). The addition of glutamyl residues also represents a way of keeping the folate in the cell. The mitochondrial folate pool is distinct from the cytosolic pool in that 5-methyl-THF is located in the cytosol, while 10-formyl-THF is in the mitochondria (Shane 1995). Tissues are limited in their ability to store folate beyond their normal cellular requirements. The total body content of humans is estimated to be
approximately 10 mg, of which one-half resides in the liver (Herbert 1987, Combs 1992).

**Folate Functions**

Folate coenzymes are involved in numerous reactions that involve the transfer of one-carbon units as depicted by Figure 2. These one-carbon units exist at various levels of oxidation ranging from methane, the most reduced, to carbon dioxide, the most oxidized (Wagner 1995) and are carried by folate when the coenzyme is in the fully reduced THF form. As depicted by the diagram, folate is reduced in two steps to THF by dihydrofolate reductase which utilizes NADPH + H⁺. The majority of folate derivatives are required for amino acid metabolism and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis which is exemplified by the following reactions.

Tetrahydrofolate can accept a one-carbon unit from serine forming glycine and 5,10-methylene-THF (A). This reaction is reversible via the donation of a hydroxymethyl group from 5,10-methylene-THF to glycine producing serine and THF. In mammalian systems, the β-carbon of serine provides the majority of carbon units with THF serving as the carrier of these activated carbon atoms (Shane and Stokstad 1985). For example, the folate derivative, 5,10-methylene-THF, produced from the one-carbon donation by serine to THF, can either be reduced in a nonreversible reaction by NADPH and FADH₂ to 5-methyl-THF (B), oxidized to 5,10-methenyl-THF in a reversible reaction (F) or function as a one-carbon donor in a reaction required for DNA synthesis (G).
Enzymes involved in interconversions of coenzymes forms of THF

(A) Serine hydroxymethyltransferase (coenzyme-PLP)
(B) Methylene-THF reductase
(C) Methionine synthetase (coenzyme-B₁₂)
(D) Formiminotransferase
(E) Cyclodeaminase
(F) Methenyl-THF reductase
(G) Thymidylate synthetase
(H) Formate-activating enzyme
(I) Phosphoribosylglycinamidase transformylase
(J) Aminocarboxamide ribotide transformylase
(K) Cyclohydrolase

Figure 2. Functions and interconversions of coenzyme forms of THF. Modified from Groff JL, Gropper SS and Hunt, SM. Advanced Nutrition and Human Metabolism, New York: West Publishing Co. 1995; p 266.

The folate derivative, 5-methyl-THF, can donate its methyl group to homocysteine to form methionine and regenerate THF (C). This reaction (C), catalyzed by methionine synthase, requires vitamin B₁₂ in addition to folate.
(Figure 3). Vitamin B₁₂ is bound tightly to methionine synthase and serves to acquire the methyl group from 5-methyl-THF and transfer it to homocysteine thereby producing methionine and regenerating THF. Vitamin B₁₂ deficiency induces a secondary folate deficiency which is best explained by the "methyl trap" hypothesis (Shane and Stokstad 1985). When the activity of methionine synthase is reduced due to lack of vitamin B₁₂, 5-methyl-THF accumulates and prevents the regeneration of THF ultimately inhibiting the generation of other folate one-carbon forms. The megaloblastic anemia that may result can be explained by a lack of folate coenzymes for DNA precursor synthesis in blood cells (Shane 1995). Hence, megaloblastic anemia may develop not only as the result of a primary folate deficiency but also from a primary vitamin B₁₂ deficiency which causes a secondary folate deficiency.

Histidine metabolism also relies on the availability of folate (D). Urocanic acid is generated by the deamination of histidine which can undergo further metabolism to yield formiminoglutamate (FIGLU). At this point, THF accepts the formimino from FIGLU producing glutamic acid and 5-formimino-THF, the latter can be converted to 5,10-methenyl-THF (E) and then reduced to 5,10-methylene-THF (F). Hence, 5,10-methylene-THF, an important coenzyme in DNA synthesis, can be produced by two separate reactions (A) and (F). Thymidylate synthase requires 5,10-methylene-THF and deoxyuridylate (dUMP) to generate the pyrimidine, thymidylic acid and DHF (G). Tetrahydrofolate is regenerated from DHF by dihydrofolate reductase. Because both dihydrofolate
reductase and thymidylate synthase are very active during cell division, they have been the target of chemotherapeutic drugs. For example, methotrexate binds the active site of dihydrofolate reductase which prevents the regeneration of THF thereby reducing the availability of 5,10-methylene-THF needed for cell division.

Figure 3. The conversion of homocysteine to methionine and the regeneration of THF. Reproduced from Groff JL, Gropper SS and Hunt, SM. Advanced Nutrition and Human Metabolism, New York: West Publishing Co. 1995; p 266.

Formate is required for the conversion of THF to 10-formyl-THF (H) which is the folate coenzyme needed for purine ring formation and consequently both RNA and DNA synthesis. Carbon 8 of the purine atom is generated by the formylation of glycinamide ribotide (GAR), a reaction involving 10-formyl-THF, to form formylglycinamidine ribotide (FGAR) and THF (I). Purine ring atom C2
is acquired by formylation of 5-aminoimidazole 4-carboxamide ribonucleotide (AICAR) involving 10-formyl-THF to yield 5-formiminoimidazole 4-carboxamide ribotide (FAICAR) and THF (J). The folate derivative, 10-formyl-THF, can also be converted to 5,10-methenyl-THF (K) and then reduced to 5,10-methylene-THF as dictated by the needs of the cell.

**Turnover and Excretion of Folate**

Although studies conducted in both rat and humans confirm that folylpolyglutamates turn over in mammalian cells and tissues, the mechanism of turn over has not been well characterized (Shane, 1995). Since only a small proportion of supplemental or dietary folate is recovered in the urine when consumed in physiological amounts (Jukes et al. 1947), it has been speculated that the tissues alter these compounds in metabolism. In the late 1950s, Dinning and co-workers reported that upon incubating folic acid with liver slices, a "diazotizable amine" was formed. He predicted that the cleavage of folate to a "diazotizable amine" was one pathway for the metabolic disposal of folic acid.

His experiments with rats supported his prediction when a "diazotizable amine" was found to be a major urinary excretory product following administration of either folic acid or 5-formyl-THF. Approximately half of the excreted metabolite was in free form, while the other half was N-acetylated. The investigators reported that cleavage to a diazotizable amine was a pathway of major importance for the metabolic disposal of folate based upon the magnitude of the amine
excretion. Two decades later, administration of $^{3}$H and/or $^{14}$C labeled folic acid to rats revealed that folate catabolism takes place by cleavage of the C9-N10 bond, and not by excretion of inactive forms of the vitamin (Barford et al. 1978, Connor et al. 1979, Murphy et al. 1976, 1978 and 1979). The principle catabolites of a tracer dose of $^{3}$Hpteroylglutamate (labeled on the benzene ring) or $^{14}$C pteroylglutamate (labeled on the pterin moiety) were apABG or derivatives of the pterin component, respectively (Barford et al. 1978, Connor et al. 1979, Murphy et al. 1976, 1978 and 1979). This mode of catabolism was subsequently confirmed in humans (Krumdieck et al.1978, Pheasant et al. 1979, Saleh et al. 1980). Krumdieck et al. (1978) administered $[2-^{14}$C] folic acid to a human volunteer and determined both urinary and fecal radioactivity, as well as urinary excretion of folate over a 129 day period of observation. Urinary pterin and isoxanthopterin were found to be radioactive which supported the mode of catabolism described in rats in which folate is cleaved in half producing pterin and pABG moieties. A substantial amount of $^{14}$Cfolate and derivatives were also detected in the feces indicating that this is an important avenue of elimination of folate and derivatives (i.e. catabolites) (Krumdieck, 1978). Pheasant et al. (1979) gave an oral dose of $2-[^{14}$C], $3',5',9-[^{3}$H]folic acid (60 µg or 5 mg folic acid) to five subjects and obtained urine collections over the following 24 h period. Urinary scission products including apABG were identified as the major urinary species following the low dose. Saleh et al. (1980) administered an oral dose of a mixture of $2-[^{14}$C]- and $[3',5',7,9-^{3}$H]-Pte Glu to 14 subjects and
identified the metabolites present in the urine collected 0-24 h after administration of the radioactive tracers. Their data clearly illustrated that orally administered folic acid is incorporated into the reduced folate pool in man and undergoes scission at the C9-N10 bond to yield metabolites qualitatively similar to those produced in rats. Both free pABG and acetylated pABG were detected in urine in these studies.

It was hypothesized by Murphy et al. (1976) that both the oxidative cleavage and N-acetylation occur intracellularly and that free pABG may be the result of labile folates emerging from the cell and degrading in circulation or during passage into the bladder. Murphy et al. (1976) speculated that within the cell, folate is cleaved between the pteridine and the pABG moieties resulting in a mixture of pteridines and p-aminobenzoylpolyglutamate. This latter compound is recognized as an undesirable catabolite by the cell and is subsequently taken up by the lysosomes and converted to pABG. Acetylation of pABG follows and this compound is excreted into the urine. It was also hypothesized by Murphy et al. (1976) that folate deficiency so often associated with pregnancy may result from an increased catabolism of folate due to the presence of the fetus and not just from the increased folate requirement of rapidly growing tissue (Murphy et al. 1976 and 1978) or the increased excretion of the intact folate molecule (Fleming 1972, Landon and Hytten, 1971).

In 1993, McNulty et al. conducted a study in rats to examine the possible role of increased folate catabolism as a contributory factor to the folate deficiency
of pregnancy in rats. They utilized a newly developed high performance liquid chromatography (HPLC) method allowing for the quantitative determination of endogenous folate catabolites in rat urine. Three groups of female rats including one nonpregnant and two pregnant groups were investigated. One of the pregnant groups was allowed to feed freely whereas the other was pair-fed to the nonpregnant control group. Sources of exogenous (dietary) folate were kept to a minimum in order to limit excess serum folate concentrations, which could potentially contribute to the folate catabolites formed exogenously (i.e. folate break down in urine after excretion). The authors reported that pregnancy induces an elevation in the rate of folate catabolism in rats, even in the presence of low dietary folate. Pregnancy in rats caused a significant increase in urinary levels of the folate catabolite apABG compared with nonpregnant animals. Between the two pregnant groups, urinary apABG concentrations were consistently higher in the ad lib fed group which suggested that a certain amount of the observed elevation in folate break down was due to a greater food intake. However, because apABG concentration peaked prior to a reduction in weight at parturition for both pregnant groups (i.e. day 18 versus day 21, respectively), the observed increase in apABG was determined to be a real effect of pregnancy. The peak apABG concentration at day 18 was thought to reflect a change in placental growth from hyperplasia to hypertrophy. DNA synthesis, a folate requiring process, takes place during cell division (hyperplasia) and not during the increase in cell size. The authors suggested that increased folate catabolism during
pregnancy was a consequence of increased generation of DHF, a labile folate coenzyme, resulting from participation in DNA synthesis (i.e. deoxyuridylic acid accepts one-carbon unit from 5,10-methylene producing thymidylic acid and DHF) (McNulty et al. 1993). Later, this hypothesis was expanded to include the increased generation of THF which is also a labile folate coenzyme resulting from the increased participation of 10-formyl-THF in de novo purine synthesis (McNulty et al. 1995). In contrast to apABG, urinary pABG concentrations were not detectable in this investigation and appeared to be dependent on dietary folate intake which was minimal. Again, it was reiterated that unacetylated pABG in urine may arise from the cleavage of intact folates during collection or analysis or may be the result of either incomplete acetylation of the catabolite in vivo or deacetylation of apABG generated in vivo (McNulty et al. 1993). The authors noted that pABG does not contribute a substantial amount to normal folate catabolite excretion, nor does it vary during pregnancy. No significant differences in urinary folate concentrations were found between the pregnant and nonpregnant rats indicating that increased losses of intact folates through the urine would not be contributing factors to folate deficiency. Overall, it was concluded that pregnancy induces an elevation in the rate of folate catabolism and that this may impose a drain on available folates, thus offering an explanation for folate deficiency associated with pregnancy.

Following the rat study of McNulty et al. (1993), a similar approach involving measurement of urinary catabolite excretion was used by McPartlin et
al. (1993) in human pregnant women. Six normal pregnant women provided 24 h urine samples at one time point during each trimester and postpartum. Six nonpregnant women of the same age were used as controls. The women entered a metabolic ward for 42 h where they were provided with a defined diet containing no exogenous source of pABG and a known folate concentration. The first 18 h was considered a wash out period for the folate breakdown products arising from normal diet (prior to entering metabolic ward) and urine was not collected. Throughout the final 24 h, urine was collected and subsequently analyzed for pABG and apABG by HPLC. Mean total 24 h excretion of apABG and pABG in micrograms were converted to folate equivalents on the basis that the molecular weight of either catabolite is approximately one-half intact folate. Urinary apABG, the predominant catabolite, expressed as folate equivalents was significantly greater in the second trimester compared to the first or third trimester, postpartum or in the non-pregnant women. The change in the nature of growth from hyperplasia to hypertrophy was provided as an explanation for these findings. McPartlin et al. (1993) concluded that 24 h urinary concentrations of folate catabolites reflected daily metabolic turnover of folate and should thus indicate dietary requirement.

**Folate Requirements and Recommended Dietary Allowances (RDAs)**

Since 1941, RDAs have been prepared by the Food and Nutrition Board and are intended to reflect the best scientific evaluation on nutrient allowances for the maintenance of good health and to serve as the basis for evaluating the
adequacy of diets of groups of people (NRC 1989). Recommended dietary allowances are defined as the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons (NRC 1989). Once the average minimal requirement for a nutrient has been estimated, adjustments are made to account for bioavailability, individual variation and the need for adequate reserves. Hence, a safety factor is incorporated into the RDAs which, in theory, ensures that the requirements of 98% of the population will be covered. For many people, the RDA may exceed their actual requirement by approximately 50%.

RDAs are based upon a variety of study designs. Depletion-repletion studies determine nutrient requirements by measuring the amount of the vitamin needed to bring a deficient individual to a normal status. Nutrient balance studies measure nutrient status in relation to intake, and requirements are based upon the level of the vitamin needed to maintain normal status. Biochemical indices are often utilized in these study types. Requirements have also been estimated by quantitating how much of a specific nutrient is consumed by free living healthy populations who are maintaining normal nutrient status. The information derived from such studies dictate how the RDAs are revised approximately every five years.

The folate RDA was decreased by 50% or more for all the age-sex groups in the 10th edition (NRC 1989). One rationale for reducing the RDA in
nonpregnant women was the observation that folate intake estimated by population surveys including the Second National Health and Nutrition Examination Survey (NHANES II) (LSRO 1984) was approximately 50% lower than the RDA reported in the 9th edition of the RDAs (NRC 1980) and evidence of widespread folate inadequacy was lacking (Senti and Pilch 1984). There are limitations inherent in basing RDAs on estimated dietary folate intakes by normal populations. First, underreporting is a recognized limitation and would result in underestimation of requirements especially since actual intake may be twice as high as reported intake. Second, existing food composition tables underestimate the amount of folate contained in the food item. Recently modified folate extraction procedures from food including double extractions, pH optimizations and trienzyme treatments (Gregory et al. 1990, Martin et al. 1990, Tamura et al. 1997) improved folate recovery from the food matrix and yielded higher folate values when assayed microbiologically. For example, an extraction buffer with a pH of 7.85 was found to yield higher folate values than more acidic buffers because it prevents the partial interconversion and degradation of folates (Gregory et al. 1990, Wilson and Horne 1984). Trienzyme treatment facilitates retrieval of folates trapped in the food matrix and increases measurable folate levels by 20% in whole wheat flour compared with conjugase digestion alone (Martin et al. 1990).

The work of Sauberlich et al. (1987) was also used by the committee to support a reduction in the folate RDA, although it appears that these data were
misinterpreted by the committee. Ten adult nonpregnant women followed a folate depletion regimen for 28 d followed by a repletion period. Plasma folate levels decreased throughout the depletion period and continued to fall until 200 μg/d dietary folate was provided. The RDA committee concluded from these data that the 200 μg/d level satisfied the criteria for establishing an RDA. However, 200 μg/d represented the minimal dietary requirement for nonpregnant women and had not been adjusted to account for individual variation and allowance for storage. Sauberlich et al. (1987) suggested an intake of 300 μg/d was necessary in order to provide an allowance for storage. In addition, the study was not carried out long enough to determine the amount of folate required to achieve acceptable serum or red cell folate concentrations.

In 1992, Bailey suggested that the new RDAs for folate may not provide an adequate safety allowance for population groups at risk and should be reevaluated as new information evolves. Recently, two studies have been conducted which suggest that the current RDA for nonpregnant women is inadequate. O’Keefe et al. (1995) investigated the response of nonpregnant women of child-bearing age to three different levels of folate intake. Total intakes of 200, 300 or 400 μg folate per day were provided as a combination of dietary sources (30 μg) and either 170, 270 or 370 μg/d synthetic folic acid. The consumption of 200 and 300 μg/d resulted in significantly lower mean serum folate concentrations than the 400 μg/d. The authors reported that women consuming folate intakes of 200 and 300 μg/d were unable to maintain serum
folate concentrations in the acceptable range (>3 ng/mL) throughout the duration of the 10 week study. In addition, plasma homocysteine concentrations changed inversely to serum and erythrocyte folate levels with evidence of elevated levels (>16 μmol/L) in the subjects consuming the 200 μg/d level of intake. Based upon these data, the investigators concluded that 400 μg/d appears to more closely fulfill the criteria of a dietary allowance.

Based on the urinary excretion of folate catabolites, apABG and pABG, McPartlin et al. (1993) also demonstrated that 180 μg/d for nonpregnant women was inadequate. Assay of 24 h urine collections for pABG and apABG revealed that upon conversion of the catabolites to folate equivalents and addition of urinary intact folate, the requirement to replace the catabolites and urinary excretion of intact folate was 99 ± 22 μg/d. Following adjustments for bioavailability and the addition of two standard deviations, the RDA was computed as 280 μg for nonpregnant women. Since McPartlin and associates (1993) did not consider the other avenues of folate loss such as fecal excretion, folate requirements were underestimated resulting in an underestimation of the folate RDA. It is important to remember that urinary excretion of folate catabolites is just one component of folate requirements.

Perhaps the most compelling reason to revisit the current RDA for folate in nonpregnant women is the recent Public Health Service (PHS) recommendation that all women of child bearing age consume 400 μg/d of folate in order to reduce their risk of having a pregnancy affected with spina bifida or other neural tube
defects (NTDs) (CDC 1992). This recommendation was based upon numerous studies which convincingly illustrated the efficacy of 400 μg or more of folate in reducing the risk of NTDs (Czeizel and Dudas 1992, MRC 1991, Werler et al. 1993). The protective role of folate in reducing both the occurrence and the recurrence of NTDs has prompted the Department of Health and Human Services and the Food and Drug Administration (FDA) to require the fortification of all enriched grain products with folic acid as of January 1, 1998 (FDA 1996). It is estimated that the consumption of enriched grain products will increase folate intake by an average of 100 μg/d. Considering that the average diet provides an estimated 200 μg/day, improvement of food choices or supplementation may still be warranted.

The current RDA of 200 μg/d for adult males also appears to be inadequate based on the results of a recently conducted metabolic “depletion-repletion” study by Jacobs et al. (1994). Plasma homocysteine concentrations rose during folate depletion and correlated inversely with plasma folate concentrations. During folate repletion, the current RDA for adult men, 200 μg /d, was ineffective in normalizing high homocysteine concentrations resulting from folate depletion. The investigators concluded that the current RDA for adult men may not provide the expected margin of protection normally incorporated upon derivatization.

Since the publication of the 1989 10th edition of the RDA for folate, information has been compiled from effectively designed and controlled studies
that strongly suggests a need for revision for both the adult male and nonpregnant female RDA. When the extra demands of pregnancy for folate are considered, the adequacy of the current RDA of 400 µg/d is questionable. At this time, metabolically controlled studies have not been conducted that investigate the response of pregnant women to defined levels of folate intake. The basis for the change in the RDA for folate from 800 µg to 400 µg/d during pregnancy stems from investigations that provided either (a) known amounts of supplemental folic acid to an uncontrolled diet or (b) provided folic acid to malnourished pregnant women. Chanarin et al. (1968b) investigated folate requirements in 206 women from approximately wk 15 to 38. The women were randomly assigned to receive either an iron supplement or an iron supplement with 100 µg of folic acid. Red cell and serum folates were determined by microbiological assay using *Lactobacillus casei* (*L. casei*) as the test organism. At baseline, no differences in serum or red cell folate concentrations existed between the two groups. However, as the study progressed, red cell folate concentrations declined over time in those not receiving 100 µg/d supplemental folic acid compared to maintenance of red cell folate concentrations in those women receiving supplemental folic acid.

Dietary folate intake was estimated to be 676 µg/d based on the microbiological quantitation of food folate from one hundred eleven separate 24 h food collections which were duplicates of the actual meals consumed over 7 consecutive days (range 198 µg to 1,615 µg) (Chanarin et al. 1968a). The investigators concluded that folate requirements in pregnancy were met by a total folate intake of 776
which included the supplement of 100 µg of folic acid. This study supports the 1980 RDA (9th edition) for folic acid of 800 µg/d and was cited as a reference by this RDA committee (NRC 1980). Bates et al. (1982) subsequently estimated dietary intakes of 190 µg/d by this population group (United Kingdom). This estimation in conjunction with Chanarin’s report that 100 µg/d supplemental folic acid plus dietary folate maintained red cell folate concentrations was cited by the 10th RDA committee in support of the 400 µg/d RDA.

Another study cited by the RDA committee for supporting the 400 µg/d level for pregnant women was the investigation conducted by Colman et al. (1975) in which African women during their last 30 days of pregnancy consumed maize meal fortified with folic acid for daily intakes of either 300 or 500 µg folic acid. Changes in folate nutritional status (serum and red cell folate) were compared to the changes observed in pregnant women who were receiving folic acid as: (1) 300 µg in tablet form; (2) maize meal without folic acid; or (3) maize meal containing 1000 µg. The last two comparison groups were from a previous study (Colman et al. 1974) which indicated that folic acid-fortified maize meal prevents the progression of folate depletion during pregnancy. In the current investigation, daily intakes of maize meal containing 300 µg added folic acid were effective in preventing the progression of folate depletion in late pregnancy. The RDA committee assumed that the effective dose was 168 µg of synthetic folic acid since it is generally agreed that food folate is approximately 50% available. Based partly on the results of Colman’s study, the Committee
contended that 400 μg/d during pregnancy would be sufficient to build or maintain maternal folate stores and to keep pace with the increased folate needed to support rapidly growing tissue. This study, however, was not designed to assess folate requirements in pregnant women. It was conducted solely to determine the smallest amount of folic acid that, when added to maize meal, prevented folate deficiency in pregnancy. The authors did not state how much endogenous dietary folate was consumed on a daily basis (i.e. folate content of unfortified maize meal or other foods). This amount would have to be known in order to use this study as a basis for establishing an RDA for pregnant women. For example, if these women consumed 100 μg/d dietary folate from unfortified maize meal then their actual intake increases from 300 to 400 μg/d. Individual variation and allowance for storage would also have to be considered. Another reason that this study is not suitable for basing an RDA for pregnant women is that the population group studied was nutritionally compromised and not representative of a normal healthy population. Although, maize meal fortified with 300 μg folic acid plus low intakes of endogenous folate may be effective in preventing the progression of folate depletion in late pregnancy in this population group, it is unknown whether it would promote adequate folate nutriture in well-nourished pregnant populations. The adequacy of maternal folate stores throughout gestation is an important consideration due to increased transfer of folate from maternal to fetal stores during the last weeks of pregnancy (Ek 1980, Ek and Magnus 1981). Women with inadequate folate stores are more likely to
become deficient which may not become evident until the post-partum period. Martinez (1980) found that the best predictor of a woman’s red blood cell folate concentration was the age of her youngest child.

Since the publication of the 10th edition of the RDAs for pregnant women, the study conducted by McPartlin et al. (1993) not only refuted the current RDA for nonpregnant women but also for pregnant women. They hypothesized that the increased requirement for folate during pregnancy may relate to its accelerated breakdown to apABG and its deacetylated derivative pABG due to folate’s participation in DNA biosynthesis. The results of their study supported their hypothesis that folate catabolism is accelerated in pregnancy particularly during the second trimester. The investigators were also able to clearly demonstrate that the increased excretion of catabolites noted during the second trimester was independent of the increase in glomerular filtration rate which increased around the 8th wk and remained elevated throughout the entire pregnancy.

Recommended dietary allowances were computed by McPartlin et al. (1993) by converting both catabolites to folate equivalents, adding the small quantity of urinary intact folate and adjusting the total for bioavailability and population variance. The estimated RDAs based on the urinary excretion of folate catabolites and intact folates corresponded to 280 μg/d (nonpregnant and 1st trimester), 660 μg/d (2nd trimester) and 470 μg/d (3rd trimester). Again the RDAs have been underestimated as urinary excretion of folate catabolites is only one component of folate requirements. Limitations to this study included the fact that
dietary intake was not controlled between hospital admissions and the 18 h “wash out” period may not have been sufficient for eliminating exogenous pABG arising from previous uncontrolled dietary folate intake. It is possible that the breakdown products are retained by the body for a longer duration than that allotted by the investigators, and if so, the catabolites found in the urine may not represent an endogenous source. The absorption and fate of folate catabolites derived from food remains to be determined. Furthermore, McPartlin and his coworkers assessed urinary excretion of catabolites at one time point only for each trimester, and data on other indices of folate status were not reported. Another possible limitation of this study is the inclusion of unacetylated pABG along with the major catabolite, apABG. However, until the origin of pABG is known, it seems prudent to include this catabolite in the calculations. McPartlin and coworkers analyzed each catabolite separately, so as information becomes available, appropriate adjustments can be made to their interpretation of the data if necessary.

The importance of adequate folate nutriture and appropriate folate intake during pregnancy is widely recognized, yet the data we have for basing recommendations regarding folate requirement during pregnancy, is plagued with limitations. A controlled metabolic study which investigates the response of pregnant women to defined levels of folate intake utilizing a variety of folate indices to assess folate status response is long overdue.
Folate Deficiency and Pregnancy

Since folate governs the synthesis of the precursors of DNA and RNA, the formation and development of every human cell is dependent upon an adequate supply of this vitamin. Pregnancy represents a state of rapid cell division involving both maternal and fetal tissue. In a relatively short period, a two celled embryo develops into a two billion celled fetus (Hibbard 1964). This occurs simultaneously with the rapid proliferation of various maternal tissues including the uterus and the hematopoietic system (Hibbard 1964). The increased demand that pregnancy imposes on folate nutriture is reflected in the doubling of the RDA from nonpregnant conditions. Cooper et al. (1970) concluded that 500-600 µg/d of dietary folate was required to prevent all evidence of folate deficiency in pregnancy and many women can not meet the increased requirement for folate through diet alone. Poor nutrition has been recognized as a major factor in the development of folic acid deficiency since the 1960s (Stone et al. 1967). The decline in folate status (both serum and red cell folate) during pregnancy has been well-documented in both developed (Bailey et al. 1980, Cooper et al. 1970, Ek et al. 1981, Lowenstein et al. 1966, Qvist et al. 1986) and underdeveloped countries (Colman et al. 1975). Bailey et al. (1980) conducted a study to investigate folate status at the initiation of prenatal care in 269 pregnant low-income subjects. They found that serum folate was low (3 to 6 ng/mL) and deficient (< 3 ng/mL) in 48% and 15% of the subjects, respectively and 29% of the subjects had subnormal (<140 ng/mL) red cell folate concentrations. Qvist et al. (1986) reported
decreasing plasma folate levels with advancing pregnancy so that, at term, almost 50% of the women had subnormal plasma folate concentrations which persisted over the following 2 months. The investigators also found that 10% of the women had subnormal red cell folate concentrations at term which increased to 33% during the post-partum period. The authors contended that this observation could be explained by the delayed reflection of folate stores by the red cell folate values in periods of increased demand (Qvist et al. 1986). Ek and Magnus (1981) observed similar folate status changes throughout gestation in 43 women. They found a positive correlation between plasma folate and red cell folate concentrations at different stages of pregnancy supporting the delayed reflection of folate stores by red cell folate concentrations (Ek and Magnus 1981).

The problems associated with folic acid deficiency have been recognized since the late 1950s. The most overt sign of folate deficiency is megaloblastic anemia which is most commonly observed in the late stages of pregnancy. Earlier indications (preanemia) include serum folate concentrations below 3 ng/mL and red cell folate concentrations below 140 ng/mL. Folate deficiency has been associated with low birth weight, cessation of pregnancy, delayed maturation of the nervous system, growth retardation, megaloblastic anemia and congenital anomalies (Hibbard 1964, Shojania 1964, Strelling et al. 1966, Baumslag et al. 1970, Iyengar and Rajalakshmi 1975, Smithells et al. 1976). Recently, O’Scholl et al. (1996) conducted a prospective study examining the influence of folate intake on two pregnancy outcomes associated with poverty and disadvantage:
preterm delivery and infant low birth weight (LBW). The investigators reported that women with a low mean daily folate intake (≤ 240 μg/d) had an approximately twofold greater risk of preterm delivery and incidence of infant low birth weight after controlling for maternal characteristics, energy intake and other related nutrients. In addition, lower concentrations of serum folate at wk 28 were also associated with a greater risk of preterm delivery and low birth weight. Although overt megaloblastic anemia is infrequent in the United States, it seems desirable to consume enough folate to maintain maternal stores to keep pace with the increased demand for folate during pregnancy.

Folate Supplementation During Pregnancy

Several studies have been conducted to determine the amount of supplemental folic acid required to maintain normal folate status during pregnancy (Cooper et al. 1970, Dawson 1966, Hansen and Rybo, 1960, Lowenstein et al. 1966, Willoughby and Jewel 1966). Unfortunately, dietary folate intake was not controlled and interpretation of these data is difficult. Willoughby (1967) and Willoughby and Jewel (1968) reported that 300-350 μg/d synthetic folic acid in addition to diet was able to maintain normal folate status in pregnant women throughout gestation, whereas supplemental amounts of either 100 or 200 μg/d were inadequate. Dawson (1966) also reported that 150 μg/d folic acid plus dietary folate was insufficient to maintain normal serum folate concentrations. In contrast, Chanarin et al. (1968b) reported that 100 μg/d supplemental folic acid, in addition to diet, was adequate. Lowenstein et al.
(1966) found that consumption of 500 μg/d synthetic folic acid plus diet resulted in higher serum folate concentrations in pregnant women compared to nonpregnant controls (11.0, 7.0 ng/mL, respectively) and therefore may be above the minimal daily requirement. Cooper et al. (1970) concluded that the decline in folate status during pregnancy could be prevented with 200-300 μg folic acid/day in addition to diet. Overall, these data support a supplementation level of approximately 300 μg/d.

Programs are in place to aid pregnant women in obtaining a sufficient amount of folate. Unfortunately, not enough is known about folate metabolism and requirements during pregnancy to provide women with the optimal amount, and problems arising with oversupplementation of folate have been raised. The biggest reluctance to give prophylactic folic acid during pregnancy is due to the possible risk of masking a developing B₁₂ deficiency. However, since the incidence of pernicious anemia is low among women of child-bearing age, the masking of a B₁₂ deficiency leading to neurological complications would be extremely rare (Tamura 1995). Another concern is the possibility of interfering with zinc absorption. In 1984, Mukherjee et al. conducted a study involving 450 pregnant women and reported that high maternal plasma folate concentrations during pregnancy were associated with an increased rate of pregnancy complications and fetal distress. They reasoned that folic acid supplementation adversely affected the intestinal absorption of zinc which resulted in the complications. In contrast, Tamura et al. (1992) found high serum folate
concentrations were associated with favorable pregnancy outcome and reported higher birth weight and Apgar scores of newborns, reduced prevalence of fetal-growth retardation, and lower incidence of maternal infection around delivery. Recently, Kauwell et al. (1995) conducted a study to determine the effect of supplemental folate on zinc nutriture and found that folic acid supplementation did not adversely affect zinc status.

Overall, the detrimental effects of folic acid supplementation on pregnancy outcome are speculative considering supplementation has been in use for the past 40 y with no identifiable adverse effects. However, because the effects of oversupplementation with folic acid on the developing fetus are unknown (Scott et al. 1991) and the fetus has demonstrated its ability to sequester folate from maternal circulation, high supplemental doses of folic acid (> 1mg) should be avoided under normal circumstances.

Neural Tube Defects

Neural tube defects (NTDs) are a group of serious malformations which affect the developing nervous system and include anencephaly, spina bifida and encephalocele. Approximately 2,500 babies with these abnormalities are born each year in the US which represents approximately 1 to 2 NTD affected infants per 1000 births; many more are aborted either spontaneously and electively (Crandall et al. 1995). Neural tube defects arise within one month of conception and over 90% are born to women without a prior family history of this disorder (Crandall et al. 1995). Although the cause of NTDs is unknown, it is now well
established that maternal folic acid supplementation during the periconceptual period significantly reduces the risk of giving birth to a baby with a NTD. Several studies have confirmed that for women without a prior history of NTDs, a 60 to 100% decrease in risk was obtained if folic acid was taken prior to conception and throughout the first trimester (Milunsky et al. 1989, MRC 1991, Mulinare et al. 1988). Studies which have investigated the effect of folic acid supplementation on the recurrence of NTDs have also reported similar reductions after folic acid supplementation (MRC 1991, Laurence et al. 1981, Smithells et al. 1983, Vergel et al. 1990). The effective dose of folic acid ranged between 0.4 mg to 5 mg/d. The neural tube closure occurs within 28 d after conception, a time when many women do not even know they are pregnant. Hence, NTD prevention is best achieved by adequate folic acid intake throughout the reproductive years (Crandall et al. 1995). In 1992, the Public Health Service recommended that all women of childbearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of having a pregnancy affected with an NTD (CDC 1992). Daly et al. (1995) subsequently demonstrated that red cell folate concentrations of 400 ng/mL or higher were associated with a low risk of folate-responsive NTDs. Brown et al. (1997) found that red cell folate concentrations $\geq 400$ ng/mL could be achieved by folate intakes of at least 450 $\mu$g/d (supplement users) or 500 $\mu$g/d (food and folic acid fortified cereals, only). A limitation to the study of Brown et al. was that dietary estimates of folate consumption were obtained during the
study period and not 2-3 months earlier during folate incorporation into reticulocytes.

The average consumption of dietary folate by women in the United States is approximately 200 µg/d (National Academy of Sciences 1990). It is possible for women to increase their consumption of dietary folates by increasing their consumption of fruits, vegetables and whole grains. Unfortunately, for various reasons, adherence to dietary guidelines is not widespread. Fortification of staple foods stuffs with folic acid is an effective and inexpensive approach to assist all women in increasing their daily intake of folic acid. Recently, the FDA amended the standards of identity for enriched cereal-grain products and has given manufacturers two years to comply with its decision requiring the fortification of enriched grain products with folic acid (FDA 1996). The level of folic acid fortification permitted (140 µg per 100 g of cereal grain product) is low enough to keep daily folate intake below 1 mg yet high enough to enhance folate intake by an average of 100 µg/day.

As stated before, the cause of NTDs is unknown at this time. Davis et al.(1995) found that the absorption of folic acid routinely consumed in supplements and fortified food products was not impaired in women with a history of a pregnancy with a NTD. Recent evidence suggests impairment of homocysteine remethylation resulting in a possible methionine shortage at a crucial stage of development (Mills et al. 1995). The investigators suspect a defect in methionine synthase, the enzyme responsible for converting
homocysteine to methionine. Methionine synthase requires both the presence of folate (5-methyl-THF) and vitamin B_{12}. It is a key enzyme in many methylation reactions, including the production of myelin, and it also catalyzes the synthesis of THF, a compound required for DNA synthesis, following its conversion to 5,10-methylene-THF. Further, homocysteine, itself, is reportedly a putative embryotoxin (Lucock et al. 1995). An earlier study conducted by Kirke et al. (1993) found that both plasma folate and plasma B_{12} were independent risk factors for NTDs, and they suggested that vitamin B_{12} should be considered, as well as folate, in any supplementation program aimed at preventing NTDs. In lieu of the recent discovery of elevated homocysteine concentrations and the possibility of impaired homocysteine metabolism, supplementation with B_{12}, in addition to folic acid, may result in a further reduction of NTDs. However, the findings of van der Put et al. (1995) of a mutation in the gene coding for methylenetetrahydrofolate reductase (MTHFR) as a risk factor for spina bifida would support folic acid supplementation alone. Reduced MTHFR activity would lead to lower 5-methyl-THF concentrations which would result in elevated homocysteine concentrations. However, this is a common gene mutation in the general population and is not a definitive marker for those at risk of delivering a baby with a NTD.

An association between NTDs and obesity, independent of the effects of folic acid intake, has also been reported (Werler et al. 1996). These investigators collected data from 1988 to 1994 in a case-control surveillance program of birth
defects and found a positive association between prepregnant weight and NTD risk which was not alleviated by adequate folate intake among the heavier women. The authors noted that the mechanism by which increased weight may affect NTD risk is not clear but speculated that their diets may be deficient in nutrients (other than folate) that may be necessary for closing of the neural tube or that obesity affects some metabolic factors which may have a direct effect on neural tube development (i.e. insulin).

Assessment of Folate Status

Assessment of folate nutriture during pregnancy is complicated by the physiological changes that occur over this nine month period. A healthy woman bearing a normal-sized fetus will increase her plasma volume from 25% to 80% with an average value of 40% (Bartels et al. 1989). Plasma volume begins to rise during the latter part of the first trimester, reaches a maximum at approximately 25 wk gestation and remains relatively constant until the 34th wk of gestation (Bartels et al. 1989). As a result of this hemodilution, the concentration of several nutrients, including folate, decline. Unfortunately, little is known as to what constitutes normal plasma folate concentrations in pregnancy. As O'Connor (1994) emphasized in a recent review, it is difficult to discern the meaning of a low serum folate concentration during pregnancy secondary to the physiological changes that occur during this time and a reduction in serum folate may be normal. Hall et al. (1976) found that the fall in serum folate concentration was due principally to plasma volume expansion and should therefore be regarded as
physiological. However, women consuming supplemental folic acid (\(~300\) \(\mu\)g) plus low dietary folate are able to maintain serum folate concentrations within the normal range for nonpregnant women (Cooper et al. 1970, Willoughby 1967, Willoughby and Jewel 1968). Currently we can only evaluate the nutriture of pregnant women against nonpregnant standards and based on the critical role folate plays in producing a healthy baby and the link between low serum folate concentrations and folate deficiency, it seems desirable to maintain serum folate concentrations in the range deemed appropriate for nonpregnant women.

Altered renal function is another physiological change which makes assessing folate status during pregnancy a challenge. In the non-pregnant state, the renal tubules are very effective in reabsorbing water-soluble vitamins like folate. During pregnancy, however, Landon and Hytten (1972) reported that urinary folate excretion increases approximately four fold.

Several folate status indices are available to clinicians and researchers to obtain information on folate nutriture in both pregnant women and the general population. Importantly, all have certain limitations which must be considered. Determinations of serum and erythrocyte folate concentrations are commonly performed and represent the most practical approach for population studies. The main folate derivative in serum is the reduced, monoglutamate form, 5-methyl-THF (Gibson 1990). Serum does not contain polyglutamates. Serum folate concentration is reflective of acute folate status that changes in response to current dietary intake. For example, serum folate values increase rapidly after the
ingestion of folate (dietary or supplemental) and decrease rapidly on a folate deficient diet. Hence, serum folate concentration is not a valid index of long term folate status or tissue stores unless it is used in conjunction with controlled dietary intake over time. Furthermore, normal serum folate concentrations have been documented in individuals with known folate deficiency and low levels have been found in individuals with normal status (Lindenbaum and Allen 1995). Several non-nutritional factors can also affect serum folate concentrations. An acute drop in serum folate results after alcohol ingestion because reabsorption of folate by the kidney is impaired (Hillman et al. 1977). Smoking has also been shown to lower serum folate concentration possibly by enhancing erythropoiesis, which in turn, increases folate requirements (Gibson 1990). An elevated serum folate concentration may result from hemolysis of red blood cells occurring during phlebotomy. Stagnant-loop syndrome, acute renal failure and liver damage may also result in elevated serum folate levels (Gibson 1990). Serum folate values below 3.0 ng/mL are considered to reflect deficiency; values between 3.0 - 6.0 ng/mL reflect borderline deficiency; while values above 6.0 ng/mL are considered to be within an acceptable range (6.0- 20 ng/mL).

In contrast to serum folate concentration, folate within the erythrocyte is not influenced by hemodilution (Bailey 1990) and therefore, provides a better measure of folate nutriture during pregnancy. However, it is important to remember that the red cell folate concentrations reflect folate available for incorporation in the red cell at the time of erythropoiesis and, therefore, represents
folate status 2 to 3 months prior to the time of blood sampling. Red cell folate concentrations are less sensitive to short-term fluctuations in folate status than serum folate levels and are therefore, a good indicator of long term folate status. Because changes in erythrocyte folate concentrations take several months to reveal any significant changes in folate nutriture, they cannot be used to assess recent changes in folate nutriture. Red cell folate concentrations are a more reliable index of folate status than serum folate concentrations and are the preferred biochemical measurement for population studies. A major limitation, however, of erythrocyte folate levels is they cannot be used to distinguish between megaloblastic anemia due to folate deficiency and that due to a B<sub>12</sub> deficiency or a combination deficiency. However, low folate levels found both in the red cell and serum or plasma is highly suggestive of a folate deficiency (Sauberlich 1995). Red cell folate concentrations below 140 ng/mL are indicative of folate deficiency. Values between 140 - 160 ng/mL are borderline while values above 160 ng/mL are within acceptable range.

Serum and erythrocyte folate concentrations can be assayed using either microbiological or radioisotope dilution techniques. The microbiological assay is considered to be the gold standard and is widely accepted for assaying folate concentrations in biological samples including blood and urine, as well as folate concentrations in tissues and food. The test organism most commonly used is *L. casei* because it responds to the greatest number of different folate derivatives, including those with up to three L-glutamic acid residues (Herbert, 1987b). Other
microorganisms that have been used include *Streptococcus faecium* which does not respond to 5-methyl-THF and *Pedicococcus cerevisiae* which does not respond to either folic acid or 5-methyl-THF (Tamura 1990). A major limitation to the microbiological assay is that it cannot be used for serum or erythrocyte samples containing antibiotics or methotrexate because these substances inhibit bacterial growth. Since the polyglutamate form of folate is present in erythrocytes, an incubation period is required to ensure complete hydrolysis to the mono, di or tri glutamate form by the conjugase enzyme. Ascorbic acid is added to biological samples to act as a reducing agent and prevent the oxidation of the folate. Pfeiffer and Gregory (1996) recently determined that the pH of the biological sample is important during the incubation period since the human serum pteroylpolyglutamate hydrolase (conjugase) enzyme functions optimally at pH 4.5 (Krungkrai 1987). For this reason ascorbic acid should be used as the preserving agent in the red blood cell hemolysate as it yields a final pH of approximately 4.0 rather than the sodium ascorbate which yields a higher pH (Pfeiffer and Gregory 1996).

The radioisotope dilution technique is not as widely accepted as the microbiological technique but is used frequently in the clinical setting because it is fast and is not affected by antibiotics, tranquilizers, and or antimitotic agents which can interfere with the growth of *L. casei* (Gibson 1990). This technique uses a folate binding protein derived from milk and \(^{125}\text{I}\)-labeled folic acid or methyl-tetrahydrofolate. The unlabeled folate present in the biological sample
competes with the labeled folate for available binding sites on the folate binding protein. Charcoal is used to adsorb both the unbound labeled and unlabeled folate which is removed later by centrifugation. A scintillation counter is used to measure the amount of bound labeled folate existing in the supernatant. The decrease in the radioactivity is proportional to the folate concentration in the sample. High performance liquid chromatography techniques have also been developed for the assessment of folate in biological samples (Gregory et al. 1984, Selhub 1990).

Formiminoglutamate (FIGLU) excretion is a functional index of folate nutriture. Normally histidine is converted to glutamic acid via the action of formimino transferase and its coenzyme tetrahydrofolate. Because folate is required in this reaction, a folate deficiency will result in the accumulation and excretion of FIGLU in the urine particularly after a histidine load. Although the FIGLU test is highly sensitive, it is not specific for folate. Deficiency of vitamin B_{12} or the enzyme formimino transferase will also produce high urinary excretion of FIGLU after a histidine load. The use of the FIGLU excretion test in pregnant women is controversial. Rothman (1970) reviewed several studies showing increased FIGLU excretion in early pregnancy with a progressive decrease in the level toward term and a second increase post partum. Hence the use of the FIGLU excretion as a functional index of folate status during pregnancy is not widespread.
Polymorphonuclear leukocyte lobe count is also utilized as a functional index. Neutrophils normally have three to four segments. In megaloblastic anemia hypersegmentation occurs increasing the number of lobes and is a characteristic early feature of folate and vitamin B_{12} deficiency (Colman 1981). Neutrophil segmentation also occurs under other conditions and is thus not very specific. However, for persons who may be both iron and folate deficient, evaluation of neutrophil hypersegmentation is particularly helpful in diagnosing the folate deficiency that is being masked by the co-existing iron deficiency (Das et al. 1978).

Deoxyuridine suppression test (dUST) is yet another way to assess functional folate status. This is an in vitro test performed on short term cultures of proliferating cells. The test is based on the need for folate in the methylation of deoxyuridine to thymidine. Bone marrow cells, lymphocytes or whole blood are preincubated with unlabeled deoxyuridine. Tritiated thymidine is then added to the cells and if folate deficiency exists, [^{3}H]thymidine will not get incorporated into DNA. Sauberlich et al. (1987) found that this test did not correlate well with either plasma or red cell folate concentrations of non-pregnant women. Furthermore, the use of this test has never been validated using pregnant or lactating women (O'Connor 1994).

Determining plasma homocysteine concentration is gaining momentum as a functional index of folate status. Plasma homocysteine concentration is inversely correlated with serum folate and red cell folate concentrations and has
proven to be a sensitive, although not specific, index of folate status because plasma homocysteine concentrations are also elevated with a vitamin $B_6$ or $B_{12}$ deficiency.

**Stable-Isotope Methods**

Isotopes are forms of an element that have the same number of protons (atomic number), but which differ in the number of neutrons and thus atomic weight. Because an atom's chemical characteristics are defined by its atomic number, isotopes behave similarly. Stable isotopes do not undergo nuclear decay and are therefore, nonradioactive. Both stable and radioisotopes act as tracers in that they follow or outline the pathway or fate of a tracee. In order to be an effective tracer, the isotopically labeled compound must act the same as the nonlabeled species in the system of interest. Gregory et al. (1990) conducted a study to determine whether stable-isotope-labeled folates could be employed for studies of folate absorption and metabolism in human subjects. After a saturation regimen, eleven adult males consumed 677 nmol (300 μg) each of 3',5'-labeled bideuterofolic acid and glutamate labeled tetradeutero folic acid. The molar ratio of labeled folates in the urine were compared to the molar ratio in the ingested dose and found to be similar (not significantly different). In addition, the percentage of labeled folate in the urine over a forty-eighty hour period approached the percentage of the ingested labeled folate. These data revealed that the two labeled forms of folate behaved similarly in vivo when compared to each other and when compared to unlabeled folate.
The interest in utilizing stable isotopes as tracers resurfaced in the 1970s primarily due to the interfacing of gas chromatography with mass spectrometry. The power of gas chromatography as a separation technique made it possible to analyze biological samples in complicated matrixes. In order to analyze a sample by gas chromatography mass spectrometry (GCMS), the sample must be volatile and heat stable. For this reason organic compounds are often derivatized. Prior to derivatization, folate is cleaved and the pABG fraction which contains the label is isolated. Trifluoroacetic anhydride and trifluoroethanol are added forming a lactam of (N-trifluoroacetyl-α-trifluoroethyl)-p-aminobenzoylglutamate as the major product (Toth and Gregory 1988). This compound is well suited for GCMS analysis because it is volatile and stable. The isotope ratios (i.e., D2 to D0) or the enrichment of the sample (refers to the abundance of the label in excess of natural abundance) can then be determined in the biological sample.

There are several advantages to using stable isotopes with safety being the most important one. The only possible toxicity that may result from substitution of a stable isotope for its lighter and more abundant isotope is an “isotope effect” which is a slowing of biochemical reactions because of the greater mass of the stable isotope (Van Langenhove 1986). Browne (1986) contended that concern about toxicity from stable-isotope labels should not limit the use of stable-isotope-labeled drugs, because there is now abundant evidence that the amount of stable-isotope label (including deuterium) that can be accumulated with repeated administration of even large amounts of drug does not pose any risk to humans.
Importantly, due to the increased sensitivity and specificity that GCMS analysis provides, only minimal amounts of tracers are required and they can be chronically administered to the same subject. In addition, a molecule can be labeled with more than one different isotope and each one can be detected by GCMS. This concept enables us to study the bioavailability of pABG labeled with $^{13}$C at the same time we are investigating $^{2}$H$_2$ folic acid turnover. However, the availability of commercially labeled stable isotopes is limited, so synthesis is a major consideration (Gregory and Toth 1990).
CHAPTER 3  
STUDY DESIGN AND METHODS

Study Design

A two-by-two factorial study design was used in which pregnant women (n=12) and nonpregnant controls (n=12) were randomly assigned to consume folate intakes of either 450 μg/d or 850 μg/d for 84 d. These intakes approximated the current and former RDA for pregnant women. Four experimental groups (n=6) were thus established: pregnant subjects fed either 450 or 850 μg/d and nonpregnant subjects fed the same two levels. Subjects consumed folate as a combination of food folate (120 ± 15 μg/d) and synthetic folic acid (either 330 or 730 μg/d) taken with meals. Approximately fifteen percent of the total folate intake was provided as deuterated folic acid (D2) during the first one-half of the study then switched to 100% unlabeled folic acid (D0) at the beginning of wk 7 allowing for future investigation of folate kinetics. In addition, 4 subjects (one from each group) consumed [13C]pABG at the beginning of wk 7 to provide information as to the fate of exogenous pABG. Blood and urine were collected at baseline and thereafter on a weekly basis for 12 wk (wk 14-25 gestation for pregnant subjects) except during wk 7 and 8 when multiple collections were obtained. Folate status was assessed by measurement of serum
folate, red cell folate, urinary 5-methyl-THF, urinary folic acid and urinary pABG and apABG. After completion of the study, a subsample of our subjects consumed either 200 μg/d (4 pregnant; 3 nonpregnant) or 600 μg/d (4 pregnant; 4 nonpregnant) synthetic folic acid plus uncontrolled dietary folate and folate and over the following 3 months returned to the clinical research center for monthly blood draws. See Figure 4 for an overview of the protocol.

Figure 4. Overview of Protocol. 1Controlled dietary folate (120 μg/d); 2Uncontrolled dietary intake; 3Two levels of supplemental folic acid (330 and 730 μg/d) during the 12 wk metabolic study and two levels (200 and 600 μg/d) during the follow-up study; 4Administration of 13C-pABG to 4 subjects; 5One blood draw/wk except following the switch to unlabeled folic acid then six collections over 2 wk and except during follow-up period then 1 draw/mo; and 6One 24 h urine collection/wk except following switch to unlabeled folic acid then seven collections over 2 wk. NP = Non Pregnant; P = Pregnant
Subjects

Healthy pregnant female subjects (18-35 y, 14 wk gestation) and nonpregnant controls (18-35 y) with normal blood chemistry profiles, normal blood folate concentrations and normal health status as determined by medical histories were eligible for participation. Exclusion criteria included chronic drug (including oral contraceptives and folate antagonists), alcohol or tobacco use. The majority of pregnant subjects (n=10) were consuming prenatal vitamins containing folic acid (0.4 -1 mg) prior to starting the study. Gestational age in pregnant subjects was determined by sonogram in conjunction with the date of last menstrual period. Approval of the study protocol by the Institutional Review Board of the University of Florida and signed informed consents by participants were obtained. Compliance to the study protocol was ensured by close daily contact in a positive environment monitored by the research team who observed consumption of folic acid with meals. Nonpregnant women maintained their body weight within 5% of base line throughout the study period and pregnant women gained approximately 0.45 kg per week.

Diet

A 5-d menu cycle consisting of five dinners and three breakfasts and lunches was designed as detailed in Table 1. Conventional foods were selected to provide meals that were varied and palatable. Folate content was minimized by thrice boiling chicken, ground beef, green beans and white potatoes and by using
Table 1. Five-day cycle menus.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waffles</td>
<td>Shredded Wheat</td>
<td>Biscuits</td>
<td>Waffles</td>
<td>Shredded Wheat</td>
</tr>
<tr>
<td>Margarine</td>
<td>Milk</td>
<td>Margarine</td>
<td>Margarine</td>
<td>Milk</td>
</tr>
<tr>
<td>Syrup</td>
<td>Raisins</td>
<td>Jelly</td>
<td>Syrup</td>
<td>Raisins</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>Brown sugar</td>
<td>Canned pears</td>
<td>Cranberry juice</td>
<td>Brown sugar</td>
</tr>
<tr>
<td></td>
<td>Apple Sauce</td>
<td></td>
<td></td>
<td>Apple Sauce</td>
</tr>
<tr>
<td></td>
<td>Cranberry juice</td>
<td></td>
<td></td>
<td>Cranberry juice</td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandwich,</td>
<td>Sandwich,</td>
<td>Crackers</td>
<td>Sandwich,</td>
<td>Sandwich,</td>
</tr>
<tr>
<td>Pita</td>
<td>Pita</td>
<td>Tuna</td>
<td>Pita</td>
<td>Biscuit</td>
</tr>
<tr>
<td>Turkey breast</td>
<td>Ham</td>
<td>Mayonnaise</td>
<td>Turkey breast</td>
<td>Ham</td>
</tr>
<tr>
<td>Cheese</td>
<td>Cheese</td>
<td>Raisins</td>
<td>Cheese</td>
<td>Cheese</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>Mustard</td>
<td>Chocolate chip</td>
<td>Mayonnaise</td>
<td>Mustard</td>
</tr>
<tr>
<td>Canned pears</td>
<td>Canned peaches</td>
<td>Cookies</td>
<td>Canned pears</td>
<td>Canned peaches</td>
</tr>
<tr>
<td>Granola bar</td>
<td>Apple newtons</td>
<td>Cola</td>
<td>Granola Bar</td>
<td>Apple newtons</td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casserole,</td>
<td>Turkey</td>
<td>Casserole,</td>
<td>Macaroni and</td>
<td>Chicken breast</td>
</tr>
<tr>
<td>Chicken</td>
<td>Rice</td>
<td>Ground beef</td>
<td>Cheese</td>
<td>Barbeque sauce</td>
</tr>
<tr>
<td>Rice</td>
<td>Gravy</td>
<td>Onion</td>
<td>Turkey Ham</td>
<td>Potato</td>
</tr>
<tr>
<td>Cheese soup</td>
<td>Green beans</td>
<td>Cream of mushroom soup</td>
<td>Green beans</td>
<td>Margarine</td>
</tr>
<tr>
<td>Margarine</td>
<td>Margarine</td>
<td>Margarine</td>
<td>Margarine</td>
<td>Green Beans</td>
</tr>
<tr>
<td>Green beans</td>
<td>Icecream</td>
<td>Green beans</td>
<td>Ginger Snaps</td>
<td>Canned pears</td>
</tr>
<tr>
<td>Newtons</td>
<td>Chocolate syrup</td>
<td>Frozen juice flavored bar</td>
<td></td>
<td>Devil’s food</td>
</tr>
<tr>
<td>Apple juice</td>
<td>Apple juice</td>
<td></td>
<td></td>
<td>Cookies</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Apple juice</td>
</tr>
<tr>
<td><strong>Snacks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oreo cookies</td>
<td>Oatmeal cookies</td>
<td>Graham crackers</td>
<td>Granola bar</td>
<td>Rice cakes</td>
</tr>
<tr>
<td>Milk</td>
<td>Milk</td>
<td>Milk</td>
<td>Milk</td>
<td>Milk</td>
</tr>
<tr>
<td>Yogurt</td>
<td>Yogurt</td>
<td>Yogurt</td>
<td>Yogurt</td>
<td>Yogurt</td>
</tr>
<tr>
<td>Popcorn</td>
<td>Popcorn</td>
<td>Popcorn</td>
<td>Popcorn</td>
<td>Popcorn</td>
</tr>
</tbody>
</table>
canned fruits and vegetables and refined starches. The menus were analyzed for their folate content in our laboratory and provided a mean of 120 ± 15 μg/d.

Energy and all other nutrients were analyzed by the Minnesota Nutrient Data System version 2.7 (Nutrition Coordinating Center 1994). Table 2 includes the nutrient composition of the three menus. The menu cycle provided ≈ 2500 calories/d of which ≈ 60% came from carbohydrate, 25% from fat and 15% from protein. A custom formulated supplement (Tischon Corp., Westbury, NJ) was used to provide the RDA for all essential nutrients not met by the diet except folate. Loss of water soluble vitamins/electrolytes in the boiled food items was accounted for in the supplement. Caloric energy was modified to maintain weight or ensure weight gain in nonpregnant and pregnant subjects, respectively.

Examples of non-nutritive food sources included margarine, candy, Jello®, Cool-Whip® and sweetened or unsweetened beverages.

Table 2. Nutrient composition of 5-d cycle menus¹,²

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal</td>
<td>2540</td>
<td>2791</td>
<td>2472</td>
<td>2603</td>
<td>2490</td>
</tr>
<tr>
<td>Protein, g</td>
<td>83</td>
<td>85</td>
<td>85</td>
<td>78</td>
<td>88</td>
</tr>
<tr>
<td>Fat, g</td>
<td>60</td>
<td>65</td>
<td>81</td>
<td>74</td>
<td>50</td>
</tr>
<tr>
<td>Folate, μg</td>
<td>114.6</td>
<td>134.7</td>
<td>99.2</td>
<td>118.2</td>
<td>135.1</td>
</tr>
</tbody>
</table>

¹Supplementation for pregnant subjects was provided by Tischon Corporation (Westbury, New York) - 0.4 mg thiamin mononitrate, 18 mg iron fumarate, 4.5 mg zinc sulfate, 230 RE retinyl palmitate, 3 TE d-α tocopheryl acetate, 120 IU Vitamin D₃, 7 mg niacinamide, 1.5 mg pyridoxine HCl, 16 μg phylloquinone.

²Supplementation for nonpregnant controls was provided by Tischon Corporation (Westbury, New York) - 3 mg iron fumarate, 1.5 mg zinc sulfate, 230 RE retinyl palmitate, 120 IU Vitamin D₃, 5 mg niacinamide, 0.9 mg pyridoxine HCl, 14 μg phylloquinone.
Folate Extraction from Menus

The three day cycle breakfast and lunch menus and the five day cycle dinner menus were analyzed for folate content three separate times during the study period using the method described by Gregory et al. (1990) and Martin et al. (1990). Each menu was prepared as though it was being consumed by the subjects. The food items were weighed, blenderized and aliquoted into 10 mL tubes. The blenderized samples were protected from light and stored at -20° C until analyzed for folate content. Six grams of blenderized menu were extracted in 20 mL boiling buffer containing 50 mM Hepes/50 mM Ches, pH 7.85 with 2% ascorbate and 0.2 M 2-mercaptoethanol. The buffer containing the sample was placed in a boiling water bath for 10 minutes and then cooled on ice for 10 minutes. Following homogenization using a Polytron (Type P100/35, Brinkman Instruments, Westbury, NY) at a setting of 5 for 1 minute, rat plasma conjugase (500 µl) was added to the homogenate to deconjugate polyglutamyl folates. Rat plasma conjugase was obtained from Pel-Freeze (Rogers, AR) and dialyzed for 24 h through 12,000-13,000 mw tubing in cold 50 mM Hepes/50 mM Ches, pH 7.85 buffer containing 2% (w/v) sodium ascorbate, 10 mM 2-mercaptoethanol, and 2 teaspoons of acid washed charcoal. The conjugase was stored at -85 °C in 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Alpha-amylase (20 mg/mL) (Sigma Chemical Co., St. Louis, MO, No. A-0273) was also added at this time to aid in the release of folate from the carbohydrate matrix. The samples incubated for 4 h at 37 °C, after which protease (2 mg/mL) (Sigma
Chemical Co., St. Louis, MO, No. #P-5147) was added to release any folate in the protein matrix of the food. This was followed by an additional 1 h incubation period at 37 °C. The samples were next placed in a boiling water bath for 5 minutes to inactivate the enzymes and cooled on ice for 10 minutes. Samples were centrifuged at 15,000 rpm, 5 °C, for 25 minutes using a Sorvall RC-5 Superspeed refrigerated centrifuge (DuPont Instruments, Newton, CT). The supernatant was filtered through Whatman #1 paper and kept on ice in a graduated cylinder. The pellet was resuspended in 5 mL Hepes-Ches buffer, homogenized and centrifuged again at 15,000 rpm, 4° C, for 25 minutes. The supernatant was then filtered through Whatman #1 paper and added to the previous filtrate. The graduated cylinders were filled to 30 mL with Hepes-Ches buffer and 1 mL portions were dispensed into five 1.5 mL vials for each menu. Nitrogen was bubbled through each vial for 10 seconds prior to storage at -20° C. Extractions were performed in duplicate for each menu for the three different times it was sampled. The folate extracted from the menus was quantitated in triplicate by microbiological assay using L. casei (Tamura 1990). A blank was utilized throughout the extraction procedure so that folate derived from the addition of the enzymes could be subtracted.

Synthesis, Purification and Identification of Deuterated Folic Acid

Deuterated folic acid was synthesized according to the method described by Gregory (1990). The first step involved bromination of folic acid with
bromine at the 3' and 5' positions of the benzoic acid ring as described by Cosulich et al. (1951). Six grams of unlabeled folic acid (#F-7876) were dissolved in 75.6 mL of 6 N hydrochloric acid (HCl), diluted with an equal volume of distilled water and placed in an ice bath on a stirrer. A large excess of bromine vapor was used (126 mmol) to brominate the folic acid molecule at the specified positions via nitrogen gas. The reaction was conducted until all the bromine was consumed (approximately 20 minutes). Following dilution of dibromo folic acid with 75 mL distilled H2O, the sample was filtered through a sintered glass funnel under vacuum and refrigeration, washed several times and dried in a dessicator for several days.

The second step involved synthesis of deuterated folic acid from dibromo folic acid. A 2.0 g portion of dry crude brominated folic acid was suspended in 60 mL deuterium oxide (D2O) and dissolved by the addition of 1.0 mL of 40% (w/w) sodium deuteroxide (NaOD). The use of D2O and NaOD allowed for the removal of the exchangeable protons from the 3',5'-dibromofolate. After 5 minutes, 1.6 mL of 35% (w/w) deuterium chloride (DCl) was added to precipitate the 3',5'-dibromofolic acid. Following centrifugation for 15 minutes at 5,000g (4° C), the pellet was suspended in 30 mL D2O, dissolved with 0.5 mL NaOD (40%) and transferred to a hydrogenation bottle. Deuterium oxide and NaOD were added to achieve a 200 mL and 2.5 mL total of D2O and NaOD, respectively. The catalyst, 0.25 g of 10% palladium (Pd) on carbon (Kodak; Rochester, NY), was then added and nitrogen gas was bubbled into the solution to exclude dissolved
oxygen. Deuterium gas was utilized to replace the bromine at the 3' and 5' positions of the benzene ring with deuterium. This catalytic dehalogenation was performed at room temperature for approximately 1 h. At the end of the reaction period, the catalyst was removed by filtering the mixture through Whatman #1 paper under vacuum and washing several times. Crude [$^2$H$_2$]folic acid was recovered from the filtrate by acidification to pH 2 with HCl followed by centrifugation. The crude [$^2$H$_2$]folic acid was dried under a dessicator for several days and weighed before starting the final step of purification. The weighed crude [$^2$H$_2$]folic acid (1.3 g) was then divided in half and ~636 mg was purified by ion-exchange chromatography on a column containing DEAE-Sephadex A-25 (Sigma Chemical Co., St Louis, MO).

Diethylaminoethyl (DEAE) sephadex A-25 (75 g) was hydrated and suspended in 0.025 M sodium phosphate buffer containing 0.1 M NaCl, pH 7 (Buffer A) and poured into the column (50 cm length, 2.5 cm ID). Approximately 1 L of Buffer A ran through the column for equilibration. Half of the crude [$^2$H$_2$]folic acid sample (636 mg) was dissolved in 2-3 mL of 0.1 M NaOH and approximately 10 mL of Buffer A. The sample required sonication to ensure it was fully in solution. Litmus paper was used to check the pH which was subsequently adjusted to pH 8. The dissolved sample was applied onto the column and a fraction collector was assembled to collect 300 drops per tube or about 15 mL. Once the sample was loaded, a 2-L gradient containing Buffer A and 0.025 M sodium phosphate buffer with 0.6 M NaCl (Buffer B) was used to
elute $^2\text{H}_2$folic acid. Fractions containing pure $^2\text{H}_2$folic acid were identified via HPLC using a 0.033 M phosphate buffer containing 15% acetonitrile (MeCN) as a mobile phase. The detector was set at 280 nm and a folic acid standard was injected first to determine the retention time of folic acid. The $^2\text{H}_2$folic acid fractions eluting at the retention time of folic acid were pooled and precipitated by acidification with HCl. Following centrifugation, the purified $^2\text{H}_2$folic acid was desiccated over P$_2$O$_5$ and weighed. The purity of the crude $^2\text{H}_2$folic acid (630 mg) was assessed spectrophotometrically and via HPLC and proton NMR. NMR analysis indicated complete labeling of the 3',5' positions of the benzoic acid ring as no signal was detected for 3',5'-protons.

**Supplemental Folic Acid**

Commercially available folic acid (Sigma Chemical, St. Louis, MO) was used to prepare the folic acid supplements. Subjects received either 330 or 730 μg/d as synthetic folic acid. Deuterated [3',5'-$^2\text{H}_2$] folic acid was synthesized by the method of Gregory (1990) as previously described. In order to increase absorption of the synthetic folic acid, the day’s requirement was divided into two portions so that half was consumed in the morning and the other half in the evening. Stock solutions were prepared containing the required amounts of both the deuterated and the unlabeled folic acid for the first 6 weeks of the study. At a later date, stock solutions containing only unlabeled folic acid were prepared for
the second half of the study period. Food grade phosphoric acid and sodium phosphate (dibasic) were used to make the buffer in which \( ^{2}\text{H}_2 \) folic acid and unlabeled folic acid were dissolved. For the 450 µg level, 60 µg of deuterated folic acid (~15% of total folate) was required per day. Since the subjects were consuming supplemental folic acid twice per day, only 30 µg was required per supplement tube. The deuterated folic acid stock solution was prepared for the 450 µg/d groups by weighing out 45 mg deuterated folate, dissolving in 0.1 N NaOH, and filling to 150 mL for a final concentration of 30 µg/100 µL. The exact concentration of all stock solutions was determined spectrophotometrically at 282 nm using a molar absorptivity coefficient at pH 7.0 of 27,600 L · (mol · cm)\(^{-1}\) (Blakley 1969). The volume needed per tube was adjusted according to the actual concentration. The subjects on the 450 µg/d level also received 270 µg/d of unlabeled folic acid or 135 µg twice/d. Folic acid (202.5 mg) was weighed and dissolved with 0.1 N NaOH and filled to 150 mL with buffer for a final concentration of 135 µg/100 µL. For the 850 µg/d group, 120 µg of the deuterated folic acid (~15% of total folate) was required per day or 60 µg per tube. To make the deuterated stock solution for this level, 90 mg of deuterated folic acid was weighed out and dissolved with 0.1 N NaOH and filled to 150 mL with buffer for a final concentration of 60 µg/100 µL. The 850 µg groups required 610 µg of unlabeled folic acid or 305 µg per tube. Folic acid (457.5 mg) was weighed out and dissolved with 0.1 N NaOH and filled to 150 mL with buffer for
a final concentration of 305 μg/100 μL. The stock solution for the second half of the study was prepared in a similar fashion except the folate contribution from the deuterated folic acid was replaced with unlabeled folic acid. All stock solutions were stored in a -70°C freezer and the concentration was verified spectrophotometrically before making up a new batch of supplements to ensure that no degradation of folate had occurred during storage. For human consumption, stock solutions in appropriate volumes (~100 μL/tube) were dispensed into 50 mL conical tubes to which approximately 40 mL of apple juice was added. The tubes were then capped tightly, vortexed and stored at -20°C until used (within 3 months). Supplemental folic acid content was confirmed periodically by HPLC and found to be fully stable.

Purification and Administration of [13C]pABG

Labeled pABG was provided to a subsample of the subjects (n=4) in order to obtain information on the absorption and excretion of exogenous pABG which is found in food. [13C]Folic acid, kindly provided by the lab of Dr. Gregory, was cleaved at the C9 and N10 bond via zinc dust suspended in water (0.75g/3mL H2O) and 5 N HCl. After removal of the zinc dust by centrifugation, the supernatant containing both the pteridine and pABG moieties was removed and adjusted to pH 7. At this point, the supernatant became cloudy due to formation of a precipitate and required a second centrifugation. The final supernatant containing both fragments of the molecule was then applied to a size exclusion
gel (P2). The two fragments were separated on the basis of size upon the addition of water to the gel after the sample was loaded. Small 1-2 mL fractions were collected and analyzed by HPLC to identify the fractions containing the pABG fragment. A pABG standard (Sigma #A-0879) was used to identify the retention time of pABG and the appropriate fractions were pooled. The concentration of the pooled pABG was then determined by HPLC using a pABG standard curve. One hundred microgram portions of the pABG water mixture was dispensed into 50 mL conical tubes and filled to 40 mL with apple juice. Four subjects (one from each group) received the one time administration of 100 μg [13C]pABG at the half way point of the study (beginning of week 7). This was followed by five consecutive 24 h urine collections. The enrichment of the urine with [13C]-labeled pABG will be determined by GCMS and evaluated in terms of bioavailability. Another aspect of this study involves the quantitation of pABG in the diet. This method has been worked out by the laboratory of Dr. Gregory and may be an ancillary project.

**Blood Collection and Processing**

Weekly fasting blood samples were drawn by a certified phlebotomist throughout the twelve wk study. Multiple blood collections (i.e. six in a 2 wk period) were obtained at the switch-over period starting at the beginning of the 7 wk. Blood processing occurred within one h of the blood draw. A total of 17 blood collections per subject were obtained during the 12 wk study period. The initial blood draw obtained on day one was representative of baseline
concentrations of serum folate, red cell folate and plasma homocysteine, while the last blood draw at the end of wk 12 represented final concentrations. Precautions were taken to protect all blood samples from light during collection, processing and storage. As mentioned previously, upon completion of the twelve wk study, a subgroup of the pregnant (n=8) and nonpregnant women (n=7) returned once a month for phlebotomy during a three month follow-up period. In addition, maternal and cord blood and a blood chemistry profile (SMAC 25) were obtained at the time of delivery for a subgroup of the pregnant women (n=8).

Serum was collected in 13 mL silica-coated tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) and held at room temperature for 30 to 60 minutes to allow time for clotting. The clotted portion of each sample was removed by centrifugation at 2,000 rpm for 30 minutes (International Equipment Company Model HN-S II Centrifuge, Needham Heights, MA). The serum was then dispensed in duplicate into 1.5 mL vials containing approximately 1.5 mg of ascorbate (to ensure folate stability during storage) and subsequently frozen at -20°C until analysis of serum folate by microbiological assay.

Plasma and whole blood were collected in 10 mL ethylenediamine tetraacetic acid (EDTA) tubes (Vacutainer®, Becton, Dickinson, Rutherford, NJ). For a small subset of subjects (n=4) an extra 13 mL EDTA tube (Vacutainer®, Becton, Dickinson, Rutherford, NJ) was used to collect plasma to be analyzed at a later date by GCMS to obtain information on folate kinetics.
Plasma to be assayed for homocysteine was placed immediately on ice and centrifuged at 2,000 rpm (IEC Model HN-SII Centrifuge, Needham, MA) for 30 minutes in the cold room. After centrifugation, the plasma was pipetted in duplicate into 5 mL scintillation vials and placed in the freezer at -20° C for subsequent analysis by a coworker. Plasma to be assayed for deuterated folic acid was kept at room temperature and centrifuged at 2,000 rpm for 30 minutes (International Equipment Company Model HN-S II Centrifuge, Needham Heights, MA). The plasma was then transferred to a 10 mL conical tube. The plasma obtained after centrifuging the whole blood for the packed red blood cells was added to the appropriate tube to increase the total plasma obtained (about 8 mL).

Whole blood was mixed at room temperature and 75 μL was dispensed in duplicate into 1.5 mL vials. A 1:20 dilution of whole blood was then performed with a 0.1% ascorbate solution made with ascorbate (Na salt) and milli-Q water (Millipore, Bedford, MA). The whole blood folate diluted with ascorbate and milli-Q water was incubated at room temperature for at least 30 minutes in order to allow for deconjugation of the polyglutamates to mono-, di- or tri- glutamates. From the same sample, duplicate samples of whole blood were drawn into capillary tubes and centrifuged for 7 minutes at 2,000 rpm. Hematocrit levels were determined using a microhematocrit tube reading device.

The packed red cells from the homocysteine tube and the blood remaining in the whole blood tube were combined in a 10 mL conical tube and centrifuged
at 2,000 rpm for 20 minutes to obtain approximately 5 mL of packed red cells. An equal volume (1:1) of ascorbate dissolved in milli-Q water (0.2% solution) was added to the red cells following the removal of plasma. After mixing, the red cells were stored at -20 °C until subsequent analysis of the deuterated folate by a coworker.

**Urine Collection and Processing**

Brown, opaque, 2.5 L bottles containing 3 g of ascorbic acid (Na salt) to protect the urinary folates from degradation were used to collect the 24 h urine samples. Urine was collected weekly and for five consecutive days during the switch-over period. A total of eighteen, 24 h urine collections were obtained per subject. The subjects were instructed to start the collection after the first void and to end the collection after their first void the next morning. The urine was kept cold at all times during the collection process and returned to the investigator after the 24 h period. The volume of each 24 h urine collection was recorded, and urine was transferred into 200 mL containers and 1.5 mL vials and stored prior to analysis at -20° C.

**Analyses Performed by Microbiological Assay**

**Serum Folate** Total serum folate concentrations were assessed microbiologically using the test organism *L. casei*. A slightly modified version of the method described by Tamura (1990) utilizing the 96-well microtiter plate and serial dilutions was used for analyzing the samples. The *L. casei* (microbe #9640) was
grown in ATCC 7830 media (Difco Laboratories, Detroit, MI). Serum contains only the monoglutamate form (mainly 5-methyl-THF) to which the *L. casei* microbe responds. The samples were added to assay medium containing minimal folic acid and were subsequently inoculated with the microorganism. The samples incubated over night, and the growth response of the organism was estimated the next day by measuring the increase in turbidity of the medium which is proportional to the folate concentration in the serum or red blood cell hemolysate. Calibration curves for the assay were constructed using 10 ng/mL folic acid standard.

Falcon (Becton Dickinson, Rutherford, NJ), 96-well, flat bottomed, sterile, low evaporation tissue culture plates with lids were used for this assay. For each assay, 20 μl of control, standard and samples were added in duplicate to selected wells of the plate followed by the addition of 130 μl buffer to these select wells. One hundred and fifty microliters of buffer was then dispensed into all wells. A total of five serial dilutions of the samples, standard and pooled control was made using an 8-channel pipette (Costar8-Pette #04670, Costar Corp., Cambridge, MA). All wells were inoculated with 150 μl of the medium containing the test organism (5 μL/mL media), covered and placed in the incubator at 37° C for 18 hours.

After incubation, the growth of the microorganism was measured by reading the turbidity of each well at 650 nm using a microtiter plate reader (Molecular Devices UV Max, Menlo Park, CA) interfaced with a computer. Data
were calculated using Soft Max data analysis software (Molecular Devices). The turbidity reading of each sample was translated by the computer into a folate concentration (ng/mL) based on the known amount of folate in the standard. A standard curve was generated from the standards using a four parameter equation. The linear portion of the standard curve was used to determine the folate concentration (ng/mL) of the samples. Procedures for preparing the medium, the reagents and the standard are detailed in the Appendix.

Within assay and day-to-day variation was assessed by measuring previously determined folate concentrations of pooled plasma each time the assay was performed. The inter- and intra- coefficient of variation for this assay were ~8% and 7%, respectively.

Red cell folate Red cell folate concentrations were also analyzed microbiologically using the microorganism, L. casei. Prior to analysis, the pH of each sample was reduced to approximately 4.0 with 50 µl 0.1 M HCl and incubated for sixty minutes in order to allow the conjugase to cleave the polyglutamate form of the reduced folate to a mono-, di- or tri-glutamate. Due to the addition of the acid, the dilution factor was adjusted from 20 to 21. The acidification and incubation steps were necessary at this point due to the use of sodium ascorbate instead of free ascorbic acid. All samples were analyzed in triplicate and the intra- and inter- coefficient of variation were less than 10%.

Red blood cell folate concentrations were determined by multiplying the whole blood folate (ng/mL) obtained directly from the assay by the dilution factor (21)
followed by subtraction of serum folate and hematocrit and division of this value by the hematocrit as illustrated in the following equation.

\[
\frac{[\text{Whole blood folate}] - [\text{serum folate} \times (1 - \frac{\text{hematocrit}}{100})]}{\text{hematocrit}}
\]

**Urinary folate**  Concentrations of intact urinary folate were determined microbiologically using *L. casei* as the test organism (Tamura 1990). Samples were analyzed in triplicate and the intra- and inter- coefficient of variation were < 10%. Appropriate dilutions were performed on urine samples that were running higher than what could be determined using the standard curve (> 20 ng/mL).

**Urine and Serum Creatinine Determinations (Glomerular Filtration Rate)**

Urine and serum creatinine determinations were measured using a Sigma Diagnostics kit (Sigma Chemical Co., St. Louis, MO. No. 555-A). Three milliliter of water, 3 mL of creatinine standard and 3 mL of sample were added to cuvettes labeled as blank, standard and sample(s) (serum or urine), respectively. Urine samples were diluted 10:1 with Milli-Q water. Alkaline picrate solution was added to all cuvettes, mixed and allowed to stand at room temperature for 8-12 min. The absorbance of standard and sample were read against a blank at 500 nm. Acid Reagent (No. 555-2) (0.1 mL) was added to all cuvettes, mixed and allowed to stand at room temperature. Following 5 minutes, the absorbance of standard and sample versus a blank were read. Concentrations of unknowns were
calculated from the absorbance of a 3 mg/dL creatinine standard ranging in concentration from 0 to 10 mg/dL.

Calculations: Creatinine (mg/dL)
= 3 x [(Initial - Final Absorption Test) / (Initial - Final Absorption Std)]

Creatinine Clearance (ml/min) = urine (mg/dL)\times urine (mL/min) / serum (mg/dL)

HPLC Quantitation of Urinary Intact Folates, pABG and apABG and Preparation for GCMS

Overview

Weekly urine samples (30 - 200 mL) for each subject (n=312) were passed through affinity chromatography columns in duplicate containing immobilized folate binding protein. Urinary folates bind tightly to the column material at neutral pH. The non-folate sample effluent from the affinity columns was collected and frozen at -20 °C for subsequent pABG and apABG analysis. Following the removal of contaminants with a high salt neutralizer solution, folate was eluted in 5 mL 0.1 N HCl and collected separately. Of the 5 mL folate effluent, 1 mL was saved for quantitation of urinary 5-methyl-THF and folic acid at weeks 0, 4, 8 and 12. The remaining 4 mL was prepared for GCMS analysis in order to determine isotopic enrichment of urine with deuterium.

Urinary pABG and apABG were measured in duplicate on a biweekly basis in pregnant women (weeks 0, 2, 4, 6, 8, 10 and 12) and on a monthly basis in nonpregnant controls (weeks 0, 4, 8 and 12) by the method of McPartlin et al. (1992) with minor modifications. The eluant containing both pABG and apABG
(from above) was loaded onto ion exchange chromatography columns to separate pABG and apABG. Tritiated pABG and \[^{3}\text{H}]\text{apABG} served as internal standards and were added to urine samples prior to application on ion exchange chromatography columns. After separation, the catabolites remained isolated throughout the remainder of the analysis. Acetamidobenzoylglutamate was deacetylated to form pABG and reapplied to the ion exchange columns. The pABG fractions were diazotized and loaded onto sep-pak cartridges for further purification and concentration. Azo-pABG was eluted in methanol and pABG was regenerated. High performance liquid chromatography was used to quantitate both endogenous pABG and pABG derived from apABG. High performance liquid chromatography was also used to isolate and collect the pABG fractions which were then prepared for GCMS so information can be obtained on folate kinetics. See Figure 5 for overview of catabolite purification and quantitation process.

**Procedures**

**A. Purification of the Folate Binding Protein from Dried Whey**  
An affinity chromatography sorbent coupled to purified bovine milk folate-binding protein (FBP) was prepared by a minor modification of the method of Selhub et al. (1980) as described by Gregory and Toth (1988). Folate-sepharose column material (Sigma Chemical Co., St. Louis, MO, No. F-7889) was gently poured into a glass column for the purpose of binding the FBP in dried whey (Meloskim, Kerry Ingredients). The column was washed with 500 mL 0.2 M acetic acid and 1 L of
water followed by equilibration with 1 L of 1.0 M KH₂PO₄. Dried whey (825 g) was suspended in 6 L of water (best to do in batches). The pH of the mixture was adjusted to 7.0 with 5 M NaOH and subsequently centrifuged for 10 minutes at 8,000 rpm. The supernatant was filtered through glass wool to remove excess lipid and then loaded onto the folate sepharose column at room temperature. After loading, the column was washed with 1 M NaCl (1 L) containing 0.05 M Tris HCl, pH 7.4 for the purpose of removing compounds other than the FBP. Following a wash with H₂O (1L), adsorbed FBP was eluted in 0.2 M acetic acid (approximately 500 mL) and collected in 5 to 7 mL portions by a fraction collector. A spectrophotometer (λ = 280 nm) was used to determine which fractions contained the protein. The protein containing fractions were pooled for a total of approximately 300 mL. Dialysis tubing was used to remove the acetic acid. Following placement of the sample in the dialysis tubing, it was placed in 11 L of refrigerated milli Q-water and allowed to sit on a stirrer overnight. The next day, the 11 L refrigerated water was exchanged for fresh refrigerated Milli Q-water and allowed to sit overnight on a stirrer for the second time. The dialyzed FBP solution was freeze dried and stored at -20 °C until coupled to Affigel 10.

B. Preparation of Folate Binding Protein Affinity Columns. A 25 mL bottle of Affi-gel® 10 Gel (Bio-Rad Laboratories, Richmond, CA; No. #153-6046) was gently shaken and transferred to a glass fritted funnel in order to drain the excess liquid without letting the bed go dry. The bed was washed with 75-100 mL ice
cold distilled water and the moist gel transferred to a small (125 mL) erlenmeyer flask. Purified folate binding protein (100 mg) from step A was suspended in 20 mL of 0.1 M KH2PO4, pH 7.0 at 4° C and mixed with gel in erlenmeyer flask. The mixture was agitated gently at 4° C for 4 hours in a shaking ice water bath. After agitation, the gel was filtered on a fritted glass filter and the effluent from each wash was collected separately. The gel was washed with 3 x 20 mL cold 0.05 potassium acetate, pH 6.6 buffer and 2 x 20 mL cold 0.05 potassium acetate containing 1.0 M NaCl, pH 6.6 buffer. The Bio-Rad method was used to analyze the collected fractions for protein that did not couple to the affinity columns. Bovine serum albumin (BSA) was utilized to make a five point standard curve ranging from 0 to 25 µg/mL. The protein concentration of the fractions was determined spectrophotometrically at 595 nm.

C. Binding Capacity Determination of Affigel-10 FBP Columns  Affigel-10 FBP material containing immobilized FBP (section B) was loaded into 12 glass columns (7 mm i.d. x 20 cm) with a long pasteur pipette. The columns were filled to a height of 6 cm which was approximately 2.5 mL of material and equilibrated with 10-20 mL 0.1 M KH2PO4, pH 7.0. Affinity chromatography columns containing immobilized FBP were previously found to retain folic acid, 10-formyl-folic acid, THF, 5-methyl-THF, 10-formyl-THF, and 5,10,methenyl-THF by Gregory and Toth (1988a). The amount of folate required to saturate the affinity columns was determined by applying ten 1 mL fractions of 10 µg folic acid/mL 0.1 M potassium phosphate buffer, pH 7.0 (determined
spectrophotometrically). After discarding the first 2 mL, the 10 mL eluates were collected separately and the concentrations determined spectrophotometrically at pH 7.0 using 0.1 M potassium phosphate buffer, pH 7.0 as a blank. The columns were subsequently washed with 5.0 mL of 0.025 M KH$_2$PO$_4$ containing 1.0 M NaCl (pH 7.0), followed by 5.0 mL of 0.025 M KH$_2$PO$_4$ (pH 7.0). Both effluents were discarded. The binding capacity of the columns was determined by elution of the bound folates with 0.1 N HCl. The first 2 mL effluent was discarded and the following 5 mL of 0.1 N HCl collected in a 10 mL volumetric flask which was filled to 10 mL with 0.1 N HCl. The concentration of the eluted folate was measured spectrophotometrically at 296 nm using a molar absorptivity coefficient at an acidic pH of 20,000 L · (mol · cm)$^{-1}$ (Blakley 1969). Hydrochloric acid (0.1 N) was used as the blank. The sum of the folate found in the load and the folate found in the acidic effluent should equal the amount of folate initially applied to the columns. The columns were then washed with 5 mL 0.1 N HCl and re-equilibrated with approximately 15 mL of 0.1 M KH$_2$PO$_4$, pH 7.0. The total binding capacity of the columns was approximately 25 µg (56 nmol) of folate.

D. Purification of Urine Samples using Affinity Chromatography The volumes of urine that were loaded on the affinity columns were calculated so that one third of the total binding capacity (C) was not exceeded. This determination was based on the urinary folate concentrations predetermined microbiologically. Following adjustment of urine to pH 7.0, up to 200 mL of urine (volume varied depending on urinary folate concentration) was filtered through Whatman #1 filter paper.
The filtered urine samples were loaded onto affinity columns pre-equilibrated with 0.1 M KH₂PO₄, pH 7.0. A peristaltic pump was used to maintain a flow rate of 0.3 mL/min. The non-folate effluent from the load was saved for analysis of pABG and apABG, as described later. The columns were washed with 5 mL of 0.025 M KH₂PO₄ containing 1.0 M NaCl, pH 7.0 and 5 mL of 0.025 M KH₂PO₄, pH 7.0. The folate was eluted with 0.1 N HCl. The first 2 mL contained no folate and was discarded. The following 5 mL were collected in a volumetric flask. Four milliliter were saved for cleavage reactions and subsequent GCMS analysis. No ascorbate was added to these samples. Ascorbate (50 µl of a 10% mixture) was added to the remaining 1 mL solution and stored at -20 °C for subsequent quantitation of urinary 5-methyl-THF and folic acid by HPLC (E). The columns were washed with an additional 5 mL of 0.1 N HCl to ensure all compounds had eluted followed by re-equilibration with 15-20 mL of 0.1 M KH₂PO₄, pH 7.0 buffer.

E. HPLC Quantitation of Urinary 5-Methyl-THF and Folic Acid

The concentrations of 5-methyl-THF and folic acid in the affinity column eluate were determined in duplicate for wk 0, 4, 8 and 12 by a reverse phase HPLC procedure described by Gregory et al. (1984) with minor modifications. A fluorescence detector (Spectrovision FD-300 Dual-Monochromator) connected to a U120 Universal Interface was used to monitor the native fluorescence of 5-methyl-THF. The detector was set at 295 nm excitation and 356 nm emission wavelengths. An ultraviolet (UV) absorption detector monitoring at 280 nm was used for detection
of folic acid. Both metabolites were quantitated under the following conditions: 50-μl injection volume, octadecylsilica column (Ultramex C18, 5 μm particle size, 4.6 mm i.d. x 250 mm; Phenomenex®, Torrance, CA USA), flow rate of 1.0 mL/min, isocratic mobile phase composed of 12% (v/v) acetonitrile and 0.033 M phosphoric acid, pH 2.3. Quantitation of folic acid and 5-methyl-THF was accomplished relative to standards ranging in concentration from 30 -1000 ng/mL. Standards were prepared from commercially available 5-methyl-THF and folic acid (Sigma Chemical, St. Louis, MO) using published molar absorptivities (Blakley 1969) to determine concentrations of stock solutions.

F. Chemical Cleavage of Intact Folates to pABG  The 4 mL affinity column eluate (D) containing 5-methyl-THF, folic acid and possibly THF was subjected to chemical cleavage of the C9-N10 bond forming pteridines and pABG (Toth and Gregory 1988). Para-aminobenzoylglutamate, containing the deuterium, was then isolated by HPLC and subsequently analyzed by GCMS. Cleavage of 5-methyl-THF was accomplished by adding 2 M potassium acetate (150 μl) and 1N NaOH (150 μl) to the 4 mL eluate yielding a pH of 5.7- 6.0. Five percent (v/v) hydrogen peroxide (220 μl) was added to oxidize 5-methyl-THF to 5-methyl-DHF. After exactly 30 seconds, 450 μl of 0.1% bovine hepatic catalase (Sigma Chemical Co., No. C-40) was added to decompose the remaining hydrogen peroxide and stop the reaction. To cleave 5-methyl-dihydrofolate, samples were acidified by the addition of 500 μl of 5 N HCl and incubated at room temperature for 60 minutes.
Folic acid was reductively cleaved in the acid mixture by the addition of 270 μl of a suspension of zinc dust (1 g zinc in 3 mL milli-Q water) with periodic agitation for 15 minutes. The zinc was subsequently removed by centrifugation in a clinical centrifuge at 2,000 rpm for 5 minutes. Supernatant was removed with a Pasteur pipette and stored at -20 °C until HPLC isolation of the pABG. These procedures yielded complete conversion of intact folates to pABG as monitored by HPLC.

G. HPLC Isolation of pABG and derivatization

HPLC isolation and derivatization of pABG was performed by a co-worker. The pABG fragment from step F was isolated and collected by HPLC using a Prodigy preparative C18 column (10 μm particle size; 250 x 10 mm; Phenomenex®, Torrance, CA USA). Formic acid (0.1 M) containing 6% (v/v) acetonitrile was used as the mobile phase at a flow rate of 4.0 mL/min. When collecting peaks for GCMS analyses, the pH of the mobile phase should not be adjusted. The retention time of pABG was predetermined with a pABG standard (Sigma #A-0879) dissolved in 0.1 N NaOH. Detection was achieved with an absorbance detector monitoring at 280 nm. The pABG peak (~ 4 mL) was manually collected and evaporated to dryness at 90 °C under a constant flow of nitrogen gas, capped tightly and kept refrigerated overnight. The dried pABG fractions were brought to room temperature, flushed with nitrogen gas for 30 seconds and derivatized by addition of 200 μl trifluoroacetic anhydride and 100 μl trifluoroethanol. Samples were then capped and placed in a heating block at 90° C for one h. After cooling to
room temperature, excess reagent was evaporated under nitrogen gas at room temperature and the residue dissolved in 50 \( \mu l \) dry ethyl acetate. Samples were transferred to autosampler vials with polyspring inserts and stored at -20 °C before GCMS analysis. The end product of the above reaction was a lactam of N1-trifluoroacetyl-p-aminobenzoyl-glutamate-\( \alpha \)-trifluoroethyl ester that retains all isotopic labeling on the pABG component of the folates (Gregory and Toth 1988a). This lactam is well suited for GCMS analysis for determination of isotopic ratios.

H. Synthesis of \(^3\)H)pABG Tritiated pABG was prepared from 250 microcuries \(^3\)H]folic acid (Amersham International, No. TRK 212) which is labeled at positions C7, C9 and 3' and 5' of the pABG moiety. To 200 \( \mu l \) of the \(^3\)H]folic acid, 100 \( \mu l \) 5 M HCl and 100 \( \mu l \) of Zn dust suspended in water (1.0 g per 3 mL milli-Q water) was added in order to cleave the intact molecule. The mixture was shaken intermittently for 15 minutes at room temperature followed by centrifugation for 10 minutes at 2,000 rpm to pellet the zinc. The supernatant was removed and stored in the refrigerator. Unlabeled folic acid (100 \( \mu g/mL \)) was diluted with a 1% sodium ascorbate solution to a final concentration of 4 \( \mu g/mL \) in order to resemble the conditions of the commercially available \(^3\)H]folic acid and cleaved as described above. Unlabeled pABG standards were prepared from commercially available pABG (Sigma Chemical Co., St. Louis, MO No. A-0879). Following adjustment of 100 \( \mu l \) pABG stock solution (100 \( \mu g/mL \)) to pH 13 by
diluting 1:10 with 0.1N NaOH (10μg/mL), unlabeled pABG concentration was verified spectrophotometrically at 272 nm using 15,500 as the extinction coefficient (Blakley 1969).

I. Purification of [3H]pABG by HPLC

Tritiated pABG was purified under the following conditions: 100 μl injection volume; octadecylsilica column (Microsorb C18, 3 μm particle size, 4.6 x 100 mm; Rainin; Woburn, MA), mobile phase consisting of 0.1 M formic acid, pH 4.0, 6 % acetonitrile. Before running the radioactive pABG-pteridine mixture, unlabeled pABG was injected to determine the retention time of pABG. Cleaved, unlabeled folate containing both the pteridine moiety and pABG was also run to assure the pteridine and pABG moieties were eluting separately. Once the retention time of 3.55 minutes for pABG was determined and lack of interfering compounds assured, the tritiated sample was injected four separate times and pABG was collected and counted via a scintillation counter. In order to assure the isolation and collection of [3H]pABG, unlabeled pABG was spiked with 20 μl of [3H]pABG, injected into the HPLC, collected in one milliliter fractions for 10 minutes, and counted. The isolation of [3H]pABG was verified since the majority of counts matched the retention time of unlabeled pABG. To further confirm [3H]pABG had been isolated, a small portion (20 μl) was applied to equilibrated ion exchange columns and after washing with 0.1 N HCl was eluted with 0.6 N HCl. Both effluents were collected and counted. Recovery of 95-100% of [3H]pABG in the 0.6 N
HC1 eluate provided additional evidence that $[^3\text{H}]p\text{ABG}$ had been isolated. One-half of the tritiated pABG was used for the synthesis of $[^3\text{H}]p\text{ABG}$ and the other half (~3 mL) was diluted to 500 mL with 0.1% sodium ascorbate solution and mixed well. Five hundred microliters of the diluted $[^3\text{H}]p\text{ABG}$ were transferred to 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and stored at -20 °C. Each tube (500 µl) contained ~ 58,000 dpm.

J. Synthesis of $[^3\text{H}]p\text{ABG}$  The $[^3\text{H}]p\text{ABG}$ starting material (2.5 mL) was concentrated by solid phase adsorption (C$_{18}$ Sep Pak, Waters). The $[^3\text{H}]p\text{ABG}$ fraction was converted to its diazotized derivative by the method of Bratton and Marshall (1939). Two hundred microliters of 5N HCl and 200 µl of 1% sodium nitrite (1g/100 mL) were added to 2.5 mL of the isolated tritiated pABG (J) and allowed to sit five minutes. Para-aminobenzoylglutamate, a primary amine, reacts in acid conditions with the nitrite to form a diazonium salt. Next, 200 µl of ammonium sulfamate (5%) was added to destroy excess HNO$_2$. After five minutes, 200 µl naphthylethylene diamine (1%) was added to couple with pABG. A minimum incubation period of 30 minutes was allowed for complete formation of the purple colored azo compound. The diazotized radiolabeled derivative was applied to and retained on a preactivated (3 mL methanol followed by 5 mL water) C$_{18}$ Sep Pak cartridge (Waters). The cartridge was washed with 10 mL HCl (0.05 M) and azo-pABG eluted with 3 mL methanol. Tritiated para-aminobenzoylglutamate was regenerated by the addition of 50 µl 5M HCl and 50
µl zinc dust suspended in water. The mixture was vortexed intermittently for 15 minutes followed by centrifugation at 2,000 rpm for 10 minutes. The acetamido derivative was prepared by transferring the supernatant to a conical glass tube and evaporating it to dryness under a stream of nitrogen gas in a reactitherm set at 40° C. Thirty-four microliters of 50% (v/v) acetic acid and 5 µl of acetic anhydride was added to the dried extract. The tube was plugged with cotton, vortexed gently and incubated at room temperature for 24 h. A nonradioactive apABG standard was prepared by the method of Baker et al. (1964). One gram of pABG was dissolved in 10 mL of 50% acetic acid and following addition of 1.5 mL acetic anhydride, the mixture sat overnight at ambient temperature. Whatman paper (#1) was used to filter the mixture followed by washing with milli-Q water. The residue was dessicated to remove water and stored at -20 °C.

K. Purification of [3H]apABG by HPLC The same conditions as described in (I) were used to isolate the radioactive apABG. Nonradioactive apABG standard synthesized previously (J) was dissolved in water and injected to determine the retention time of this fragment (~ 8 minutes). The tritiated sample was injected and the peak corresponding to the retention time of unlabeled apABG manually collected. To verify isolation of [3H]apABG, unlabeled apABG was spiked with 10 µl [3H]apABG, injected into the HPLC, manually collected in 1 mL fractions for the following 30 minutes and then counted. Since the majority of counts matched the retention time of unlabeled apABG, it was assumed that [3H]apABG
had been isolated. The pooled tritiated apABG was diluted with 0.1% sodium ascorbate solution up to a final volume of 160 mL and 1 mL portions containing 23,000 dpm were transferred into 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and stored at -20 °C.

L. Preparation of Ion-Exchange Columns and Fractionation of Urine  One column per 20 mL urine sample was prepared by filling a glass column (15 mm i.d. x 200 mm) with a slurry of Dowex 50W (Sigma Chemical Co. St. Louis, MO) cation exchange resin (7 g/10 mL H2O) to a height of approximately 5.0 cm. The column was activated with 50 mL HCl (6 M), washed with 50 mL H2O and equilibrated with 10 mL HCl (0.1M). The sample eluate from the affinity columns (step D) was divided into two 20 mL portions and acidified to a final concentration of 0.1 M with 5 M HCl. At this point, tritiated internal standards were added to samples analyzed over the first 3 wk (~ 110 samples). Following addition of tritiated internal standards, samples were applied to the ion exchange chromatography columns. Acetamidobenzoylglutamate eluted in the sample volume and the following 50 mL 0.1 mol/L HCl effluent, which were pooled and retained. A second 0.1 mol/L HCl wash was applied (higher concentrations resulted in pABG elution) and discarded. Para-aminobenzoylglutamate was eluted and collected in 100 mL of 0.6 mol/L HCl. The columns were reactivated, washed and re-equilibrated as described for reuse in step N.

M. Deacetylation of Urinary apABG and Rechromatography  The fraction containing apABG (M) was acidified to 0.2 mol/L HCl and heated at 100 °C for
60 minutes to deacetylate apABG and then cooled at ambient temperature. Following adjustment to 0.1 mol/L HCl by a 1:1 dilution with H2O, the samples were reapplied to equilibrated ion exchange columns and washed with 100 mL 0.1 M HCl. Para-aminobenzoylglutamate representing apABG was eluted and collected in 100 mL 0.6 mol/L HCl. The two pABG fractions were kept separate throughout the remainder of the purification and quantification process.

![Diagram](image-url)

Figure 5. Overview of catabolite isolation and purification process.

**N. Diazotization and Solid-Phase Extraction** The 100 mL fractions containing the native pABG (step L) and the deacetylated pABG (step M) were derivatized by the method of Bratton and Marshall (1939). One milliliter of 5.0 mol/L HCl and 1 mL sodium nitrite solution (1%) were added to each sample and allowed to react for five minutes at room temperature. One milliliter ammonium sulfamate...
solution (5%) was then added to destroy excess HNO₂. After five minutes, 1 mL N-(1-naphthyl)ethylenediamine solution was added to couple with pABG. The samples sat overnight at room temperature to allow complete formation of the purple-colored azo-N-(1-naphthyl)-ethylenediamine derivative of pABG (azo-pABG). Each sample (~104 mL) was applied to a preactivated C₁₈ Sep Pak cartridge (Waters) under negative pressure for further purification and concentration. Occasionally, samples turned a dark purple color and the volume loaded was reduced to prevent overloading the C₁₈ Sep Pak cartridge. Following a 10 mL 0.05 mol/L HCl wash, azo-pABG was eluted in 4 mL 100% methanol. The solution was evaporated to dryness at 40 °C under a stream of nitrogen.

Q. Regeneration of pABG  The residue (N) was reconstituted in 250 µl H₂O and pABG was regenerated upon the addition of 25 µl HCl (5M) and 25 µl Zn dust suspension (1 g /3 mL milli-Q water). After 15 minutes of intermittent mixing, the reaction mixture was transferred to a 1.5 mL microcentrifuge tube, and centrifuged at 2,000 rpm for ten minutes. The supernatant was removed, filtered to remove zinc particles, and stored at -20° C prior to HPLC analysis. Recovery of [³H]pABG and [³H]apABG was ~ 80 and 60%, respectively, and were the values used to correct final estimates of catabolites.

P. HPLC Quantitation of Urinary pABG  The amount of pABG (representing both the endogenous pABG and apABG) in a sample was determined by reverse-phase HPLC under the following conditions: 100-uL injection volume, octadecylsilica column (Ultramex C₁₈, 5 µm particle size; 3.5 mm i.d. x 250 mm;
Phenomenex, Torrance, CA USA), flow rate 1.0 mL/min, isocratic mobile phase composed of 0.1 M formic acid, pH 4.0 and acetonitrile (2%). An ultraviolet (UV) absorption detector (Dionex AD20) monitoring at 280 nm was used for detection of pABG. Quantification of pABG was accomplished relative to standards ranging in concentration from 100 - 1500 ng/mL prepared from commercial pABG (Sigma Chemical Co., St. Louis, MO) using published molar absorptivities (Blakley 1969) to determine the concentration of the pABG in stock solution. The computed value for each urine extract was adjusted by the final percentage recovery of the radioactive standards, either tritiated pABG or tritiated apABG that were added to the initial aliquot of urine. This value was then adjusted for total urinary volume to give a daily output value of catabolite.

Q. Recovery of $[^3\text{H}]\text{pABG}$ and $[^3\text{H}]\text{apABG}$ added to Urine Samples  Prior to catabolite quantitation, the method of McPartlin et al. (1992) was optimized through utilization of tritiated standards. Although McPartlin and associates (1992) also utilized tritiated internal standards, the author of this dissertation was able to identify steps in which significant amounts of either $[^3\text{H}]\text{pABG}$ or $[^3\text{H}]\text{apABG}$ were lost. Identifying these losses was accomplished by removing and counting 500 µL sample after each step. Appropriate adjustments were made such as substituting a 0.05 M HCl wash for the 25% methanol wash during the solid phase extraction enabling greater recovery of the tritiated standards at the end of the procedure. Following optimization of the method, recovery experiments were conducted on ten samples on ten different days. After
establishing that inter and intra coefficients of variation were < 10%, thereby verifying the reproducibility and accuracy of the method, tritiated standards were only added to the first 110 urine samples which were analyzed within the first four weeks of analysis. It was noted that after 4 wk, the standards started decomposing yielding lower recovery percentages due to both breakdown of pABG and loss of tritium and therefore were not used after this 4 wk period. No attempts were made to repurify the standards. Recovery of radiolabeled material prior to HPLC injection was expected to vary between 30 to 40% based on the findings of McPartlin et al. (1992). In the current study, recovery of $[^3$H]pABG and $[^3$H]apABG were ~ 80% and 60%, respectively, and were the values used to adjust our final catabolite concentrations.

R. Preparation of Catabolite Samples for GCMS Analysis The catabolites were isolated and manually collected from the HPLC and prepared for GCMS as described in sections F and G. However, the pH of the mobile phase add been adjusted to 4.0 and these collections may need to be repeated for GCMS analyses.

Statistical Analyses

Our study design was a two-by-two factorial with repeated measures. Two groups of women (pregnant and nonpregnant) were assigned to consume one of two supplement levels (450 or 850 μg/d) over a 12 wk period. One objective of this study was to determine if differences existed in folate status and catabolite excretion between pregnant women assigned to consume folate intakes of 450
μg/d compared to 850 μg/d. A second objective was to determine if differences existed between nonpregnant controls and pregnant subjects within the same supplementation group. Data were obtained at baseline (i.e. immediately prior to consuming defined diet) and then on a weekly basis. Prior to starting the study, the majority of pregnant women were consuming prenatal vitamins containing ~ 1 mg folic acid. Most folate status indices were affected by this prior folate supplementation and differences (P ≤ 0.05) were detected at baseline between pregnant and nonpregnant women. Thus, acclimatization to assigned folate intake was necessary to compare folate status indices between groups. After trying a variety of monotone models, the data were modeled using a negative exponential model: \( \mu + \beta_0 ((1 - \exp(-\beta_1 \times \text{week})) \) as it was the best fit for the data and allowed for estimation of steady state. Two key assumptions underlying this model included the following: (1) steady state was achievable within 12 wk; and 2) the data were approximately monotone for each group (always increasing, decreasing or maintaining). Steady state means for each group were assessed essentially by comparing the area under the curve at one time interval (i.e., week 0 to week 1) to the mean area under the curve of the following time intervals. When the ratio of these areas was ≥ 0.95 or ≤ 1.05 (the slope approximated zero), steady state was presumed (Gibaldi and Perrier 1982). Comparisons were made for differences in initial, final and steady state group means. All tests of initial, final and steady state means were performed at alpha = 0.05 using standard ANOVA techniques with appropriate contrasts (SAS/STAT Version 6, SAS
Institute, Cary, NC). A standard repeated measures analysis (MANOVA) was used to compare overall urinary apABG excretion among the groups under the assumption that prior folate supplementation did not affect urinary apABG excretion over time (i.e., the trend over time remained fairly constant for each group). Initial and final group means were compared using ANOVA with appropriate contrasts. Comparisons were made at alpha = 0.05 between groups for differences in initial, overall and final group means. Pearson correlations were used to describe associations between folate status indices, catabolite excretion and other pertinent parameters.

**Interpretation of the Data**

A similar approach to that taken in the controlled metabolic studies conducted by O'Keefe et al. (1995) and Jacob et al. (1994) was used to interpretate the folate status data. Interpretation of urinary folate catabolite excretion was based on the study conducted by McPartlin et al. (1993). The treatment response variables evaluated were as follows: (a) serum folate; (b) red cell folate; (c) urinary 5-methyl-THF; (d) urinary folic acid; and (e) urinary catabolites (apABG and pABG). Kinetic data and plasma homocysteine will be addressed in ancillary projects. The significance of changes in each of these response variables was determined statistically as described above. Each response variable was evaluated
independently and in conjunction with other variables. The data interpretation, as discussed below, was tailored to address the following hypotheses:

**Hypothesis 1.** The response of pregnant women and nonpregnant controls to two defined levels of folate intake (450 or 850 μg/d) will indicate a greater folate requirement for pregnant women as determined by changes in folate status indices and catabolite excretion.

**Hypothesis 2.** Folate response indices will reflect a decline in folate nutriture approaching negative folate balance among pregnant women consuming 450 μg/d of folate and maintenance of folate status in pregnant women consuming 850 μg/d.

**Hypothesis 3.** Folate response indices will reflect maintenance of folate status in nonpregnant women consuming 450 μg/d of folate and an excess exceeding the body's ability to metabolize or store folate in nonpregnant women consuming 850 μg/d of folate.

**Serum Folate** In a controlled metabolic study, serum folate concentration should provide a sensitive measure of the overall folate status of the individual. As the requirement for folate increases, serum folate was expected to decrease under conditions of constant folate intake. Rapidly declining serum folate concentrations may be suggestive of inadequate folate consumption, whereas maintenance of serum folate concentrations in the acceptable range may be reflective of sufficient folate consumption. In contrast, serum folate concentrations that have increased significantly and subsequently leveled off may
be reflective of folate consumption beyond the need for metabolism and storage. The results of the other variables aided in interpretation of the above mentioned scenarios. The effect of hemodilution on serum folate concentrations in pregnant women as a result of an increase in plasma volume was considered. Similar declines were expected in pregnant women consuming either 450 or 850 µg/d. Initial concentrations of serum folate were also considered, and comparisons were made between groups after acclimatization to the prescribed folate intake.

**Red Cell Folate** Red cell folate concentrations reflect liver stores and are considered to be a measure of long-term status (Herbert 1990). Folate is taken up and retained only by the reticulocyte and not by the mature cell as the latter possesses negligible folylpolyglutamate synthetase activity (Shane 1995). Since the life span of the red cell is ~ 120 days, red cell folate concentrations are reflective of folate status 2 to 3 months prior to analysis and significant changes in red cell folate concentrations may not occur within the time constraints of this study. However, changes in red cell concentrations (perhaps not significant) are expected to be observed if folate status changes and should parallel those observed in serum folate concentrations. Red cell folate concentrations that gradually decline over time may be suggestive of inadequate folate intake and declining folate stores. Maintenance of red cell folate concentrations in the normal range would be indicative of adequate folate consumption whereas an increase in red cell folate concentrations followed by a leveling off may reflect excess folate consumption. Red cell folate concentrations obtained during the
follow-up period were very informative as these values were reflective of folate status during the study period when subjects were consuming defined folate intakes. Other indices were used in conjunction with red cell folate concentrations to most appropriately interpretate the response.

**Urinary Folate** Urinary folate is predominately comprised of both unmetabolized folic acid and 5-methyl THF. Dramatic increases over time in the urinary excretion of unmetabolized folic acid may be reflective of folate intake above that necessary to maintain metabolism, whereas significant decreases over time may be indicative of inadequate folate consumption. Pregnant women were expected to excrete more intact folate during pregnancy secondary to the increase in glomerular filtration rate. Therefore, creatinine clearance was monitored throughout the study period in both the pregnant subjects and their nonpregnant controls so that urinary excretion of unmetabolized intact folate could be evaluated independent of the increase in glomerular filtration rate.

**Urinary Catabolites (apABG and pABG)** Excretion of the catabolites was expected to remain constant throughout the study period in nonpregnant women, regardless of folate intake. In the pregnant women, catabolite excretion was expected to vary secondary to changes in utilization rates during the second trimester also regardless of the level of folate intake. We expected to see similar variations in all pregnant women since they were gestationally, the same age. It was hypothesized that the pregnant women on either supplementation level would be excreting greater quantities of the breakdown products throughout the entire
study period (12 weeks) secondary to their greater requirement for folate compared to the nonpregnant controls. Importantly, data on glomerular filtration rate (serum creatinine and urinary creatinine) were utilized to determine if the potential increase was independent of glomerular filtration rate. The combined pABG and apABG fractions were used as representation of folate utilization and as a possible guideline for requirements when assessed with the other parameters discussed above.
Folate Content of Menus

The folate content of each menu is shown in Table 3. All menus were extracted in duplicate and measured in triplicate at three separate time points.

Table 3. Folate content of menus.

<table>
<thead>
<tr>
<th>Menu</th>
<th>1 (µg)</th>
<th>2 (µg)</th>
<th>3 (µg)</th>
<th>MEAN (SD) (µg)</th>
<th>INTRA/INTER CV %</th>
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<tr>
<td>B1</td>
<td>12.3</td>
<td>13.2</td>
<td>11.0</td>
<td>11.8 (0.8)</td>
<td>5.0 / 9.1</td>
</tr>
<tr>
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<td>11.8</td>
<td>11.4</td>
<td>11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>31.8</td>
<td>22.7</td>
<td>34.5</td>
<td>30.6 (4)</td>
<td>7.0 / 13.7</td>
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<td></td>
<td>30.8</td>
<td>29.5</td>
<td>34.3</td>
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</tr>
<tr>
<td>B3</td>
<td>12.7</td>
<td>16.6</td>
<td>15.8</td>
<td>14.6 (2.2)</td>
<td>8.5 / 14.4</td>
</tr>
<tr>
<td></td>
<td>13.5</td>
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<td>17.2</td>
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<td>34.9</td>
<td>37.8 (4.0)</td>
<td>6.9 / 8.5</td>
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<tr>
<td>D2 + PM SNACK</td>
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<td>48.9</td>
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<td>D3 + PM SNACK</td>
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<td>33.0</td>
<td>44.0</td>
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<td>5.3 / 16.1</td>
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<tr>
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<tr>
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<td>52.3</td>
<td>42.9</td>
<td>48.7 (4.1)</td>
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<td>53.8</td>
<td>47.7</td>
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</tr>
</tbody>
</table>
throughout the study. The diet provided a mean folate intake of 120 ± 15 µg/d. The intraassay and interassay coefficient of variation (CV) were 7.0 % and 12 %, respectively.

**Food Record Analyses**

During the 3 month follow-up study, the mean dietary folate intake for pregnant and nonpregnant subjects was 293 ± 39 and 379 ± 78 µg/d, respectively in the ≈450 µg/d group and 278 ± 18 and 197 ± 75 µg/d, respectively in the ≈850 µg/d group. In addition to dietary folate, subjects previously consuming 450 or 850 µg/d during the study consumed either 200 or 600 µg/d supplemental folic acid, respectively. Total folate intake (dietary folate + supplemental folic acid) during the follow-up period was estimated to be 493 ± 39 and 579 ± 78 µg/d for pregnant and nonpregnant women in the ≈450 µg/d group, respectively, and 878 ± 18 and 797 ± 75 µg/d for pregnant and nonpregnant women in the ≈850 µg/d group, respectively.

**Serum Folate**

Weekly serum folate concentrations were measured microbiologically in triplicate. Serum folate response for each experimental group throughout the 12 wk study is illustrated in Figure 6. At baseline, differences in serum folate concentrations (P ≤ 0.05) existed between pregnant and nonpregnant women assigned to consume 450 µg/d (22.32 ± 8.5, 11.63 ± 7.4 ng/mL, respectively; mean ± S.D.) or 850 µg/d (20.33 ± 9.9, 8.70 ± 4.6 ng/mL, respectively) (Figure 7). No differences (P > 0.05) existed among pregnant women assigned to
consume either 450 or 850 μg/d (22.32 ± 8.5, 20.33 ± 9.9 ng/mL, respectively) or among nonpregnant women assigned to consume these levels (11.63 ± 7.4, 8.70 ± 4.6 ng/mL, respectively) (Figure 7). At steady state, no differences (P > 0.05) were detected between pregnant and nonpregnant women within the 450 μg/d (11.78 ± 4.1, 11.61 ± 5.0 ng/mL, respectively) or within the 850 μg/d groups (19.68 ± 5.9, 19.16 ± 4.0 ng/mL, respectively) (Figure 8). Differences (P ≤ 0.05) were found between pregnant subjects (11.76 ± 4.1, 19.98 ± 5.9 ng/mL) and nonpregnant subjects (11.53 ± 5.0, 19.16 ± 4.0 ng/mL) consuming 450 μg/d compared to 850 μg/d, respectively.

Figure 6. Serum folate concentration (group means) throughout the 12 wk study. Steady state was achieved at wk 1 and 9 for the 450 and 850 μg/d nonpregnant groups, respectively, and at wk 8 and 1 for the 450 and 850 μg/d pregnant groups, respectively.
No differences were detected in final mean serum folate concentrations between pregnant and nonpregnant women consuming 450 µg/d (11.93 ± 4.7, 12.22 ± 5.7 ng/mL, respectively) or 850 µg/d (18.98 ± 6.8, 19.85 ± 4.2 ng/mL, respectively) (Figure 7). Differences (P<0.05) were found between pregnant subjects (11.93 ± 4.7, 18.98 ± 6.8 ng/mL) and nonpregnant subjects (12.21 ± 5.7, 19.85 ± 4.2 ng/mL) consuming 450 or 850 µg/d, respectively (Figure 7). All subjects maintained acceptable serum folate concentrations (> 6.0 ng/mL) (Sauberlich et al. 1974) throughout the study period. Acceptable serum folate concentrations were also maintained throughout the follow-up period in pregnant and nonpregnant women consuming ~450 µg/d (15.09 ± 6.3, 11.39 ± 4.0 ng/mL, respectively) and ~850 µg/d (21.00 ± 6.0, 18.49 ± 9.2 ng/mL, respectively).

![Figure 7](image)

*Figure 7.* Group comparisons of initial and final mean serum folate concentration (mean ± SD) analyzed separately. Bars designated by the same small letter were not significantly different (P>0.05).
Figure 8. Serum folate concentration (mean ± SD) at steady state. Bars designated by the same small number were not significantly different (P>0.05).

Red Cell Folate

Weekly red cell folate concentrations were measured microbiologically in triplicate. Red cell folate response throughout the 12 wk study is illustrated in Figure 9.

Figure 9. Red cell folate concentration (group means) throughout the 12 wk study. Steady state was achieved at wk 1 for the 450 μg/d nonpregnant group and at wk 1 and 7 for the 450 and 850 μg/d pregnant groups. Steady state was not achieved within the 12 wk period by the 850 μg/d nonpregnant group.
At baseline, differences (P<=0.05) were not observed in red cell folate concentrations between pregnant and nonpregnant subjects assigned to consume either 450 µg/d (610.43 ± 69.8, 491.72 ± 175.1 ng/mL, respectively) or 850 µg/d (518.05 ± 155.1, 338.80 ± 85.6 ng/mL, respectively) (Figure 10). No differences (P>0.05) were detected at baseline between pregnant women (610.43 ± 69.8, 518.05 ± 155.1 ng/mL) or nonpregnant controls (491.71 ± 175.1, 338.80 ± 85.5 ng/mL ) assigned to the consume 450 or 850 µg/d, respectively (Figure 10). At steady state, no differences (P>0.05) existed between pregnant and nonpregnant women consuming 450 µg/d (640.94 ± 111.1, 480.87 ± 170.8 ng/mL, respectively) or between pregnant women consuming 450 or 850 µg/d (640.94 ± 111.1, 764.21 ± 91.6 ng/mL, respectively) (Figure 11). Steady state was not achieved by the nonpregnant group consuming 850 µg/d within the 12 week study period and comparisons to this group could not be made. Differences (P<0.05) in final red cell folate concentrations were not detected between pregnant and nonpregnant women consuming 450 µg/d (537.37 ± 111.2, 459.73 ± 167.0 ng/mL, respectively) or 850 µg/d (700.50 ± 139.7, 543.6 ± 161.9 ng/mL, respectively) (Figure 10). No differences in final mean red cell folate concentrations were detected between pregnant women (537.37 ± 111.2, 700.50 ± 139.7 ng/mL) or nonpregnant controls (459.73 ± 167.0, 543.58 ± 161.9 ng/mL) consuming either 450 or 850 µg/d, respectively (Figure 10). Red cell folate correlated positively (r=0.45; P = 0.03 ) with serum folate. Acceptable red cell
folic acid concentrations (>160 ng/mL) (Sauberlich et al. 1974) were maintained throughout the study period. In the subjects who participated in the follow-up study, red cell folate was also maintained within normal concentrations by pregnant and nonpregnant women consuming ≈ 450 µg/d (576.21 ± 120.0, 436.98 ± 105.0 ng/mL, respectively) and ≈ 850 µg/d (753.00 ± 97.1, 550.01 ± 136.2 ng/mL, respectively).

Figure 10. Group comparisons of initial and final mean red cell folate concentration (mean ± SD) analyzed separately. Bars designated by the same small letter were not significantly different (P>0.05).
Figure 11. Red cell folate concentration (mean ± SD) at steady state. Final mean red cell folate concentration was used for the 850 μg/d nonpregnant group. Bars designated by the same small number were not significantly different (P>0.05).

Hemocrit.

Since folate intake did not affect hemocrit, data from pregnant and nonpregnant subjects were pooled, respectively. Acclimatization to the diet was not necessary so comparisons were made between initial and final mean hemocrits. Hemocrit changes over time are illustrated in Figure 12. At baseline, no differences (P>0.05) were detected between pregnant and nonpregnant women (37.08 ± 2.3, 38.83 ± 1.7 %, respectively) (Figure 13). Differences (P≤0.05) were detected in final mean hemocrit between pregnant and nonpregnant women (34.46 ± 2.3, 38.83 ± 1.6 %, respectively) (Figure 12). These differences would have been greater if additional iron had not been consumed by several pregnant subjects to prevent further decline in hemocrit.
Figure 12. Hematocrit (group means) throughout the 12 wk study.

Figure 13. Group comparisons of initial and final hematocrit (mean ± SD). Bars designated by the same small letter were not significantly different (P>0.05).
Urinary 5-Methyl-Tetrahydrofolate

Urinary 5-methyl-THF excretion was measured by HPLC in duplicate at baseline and thereafter on a monthly basis (i.e. wk 0, 4, 8 and 12) for all subjects. The urinary excretion of 5-methyl-THF for each experimental group is shown in Figure 14.

Figure 14. Urinary 5-methyl-THF excretion (group means) throughout the 12 wk study. Steady state was achieved at wk 1 and 6 for the 450 and 850 μg/d nonpregnant groups, respectively and at wk 6 and 2 for the 450 and 850 μg/d pregnant groups.
Differences (P≤0.05) in baseline urinary 5-methyl-THF excretion were observed between pregnant and nonpregnant women assigned to consume 450 µg/d (66.11 ± 106.1, 13.66 ± 17.8 µg/d, respectively) or 850 µg/d (158.30 ± 52.9, 19.00 ± 40.0 µg/d, respectively). Differences (P≤0.05) were not detected among pregnant (66.12 ± 106.1, 158.30 ± 52.9 µg/d) or nonpregnant women (13.66 ± 17.8, 18.95 ± 40.0 µg/d) assigned to consume 450 compared to 850 µg/d, respectively largely due to the enormous variability in the urinary excretion of this metabolite (Figure 15).

Figure 15. Group comparisons of initial and final mean 5-methyl-THF excretion (mean ± SD) analyzed separately. Bars designated by the same small letter were not significantly different (P>0.05).

At steady state, no differences were detected between pregnant and nonpregnant women consuming 450 µg/d (4.20 ± 1.4, 6.48 ± 4.7 µg/d, respectively) or 850
\( \mu g/d \) (87.38 ± 44.4, 64.59 ± 26.2 \( \mu g/d \), respectively). (Figure 15). Differences were detected between pregnant women (4.20 ± 1.4, 87.38 ± 44.4 \( \mu g/d \)) and nonpregnant controls (6.48 ± 4.7, 64.59 ± 26.2 \( \mu g/d \)) consuming 450 \( \mu g/d \) compared to 850 \( \mu g/d \), respectively. No differences (\( P \leq 0.05 \)) were detected in final mean 5-methyl-THF excretion between pregnant and nonpregnant women consuming 450 \( \mu g/d \) (3.75 ± 1.0, 4.23 ± 3.0 \( \mu g/d \), respectively) or 850 \( \mu g/d \) (79.02 ± 39.8, 63.85 ± 30.6 \( \mu g/d \), respectively) (Figure 15). Differences (\( P \leq 0.05 \)) were detected between pregnant women (3.75 ± 1.0, 79.02 ± 39.8 \( \mu g/d \)) and nonpregnant controls (4.23 ± 3.0, 63.85 ± 30.6 \( \mu g/d \)) consuming 450 \( \mu g/d \) compared to 850 \( \mu g/d \), respectively. Urinary 5-methyl-THF was positively correlated with serum (\( r = 0.74; \ P = 0.0001 \)) and red cell folate (\( r = 0.27; \ P = 0.21 \)).

![Figure 16](image-url)  
Figure 16. Urinary 5-methyl-THF excretion (mean ± SD) at steady state. Bars designated by the same small number were not significantly different (\( P > 0.05 \)).
Urinary Folic Acid

Urinary folic acid excretion was measured by HPLC in duplicate at baseline and thereafter on a monthly basis (i.e. wk 0, 4, 8 and 12) for all subjects. At baseline, only 2 of the 12 nonpregnant subjects were excreting folic acid in the amounts of 14.02 and 63.04 μg/d. Among the pregnant women, 2 subjects assigned to the 450 μg/d and 5 subjects assigned to the 850 μg/d were excreting folic acid (45.35 ± 41.2, 42.12 ± 41.6). At the end of the 84 d protocol, no detectable folic acid was being excreted by either of the 450 μg/d groups nor the 850 μg/d pregnant group. Final mean urinary folic acid excretion of the nonpregnant women consuming 850 μg/d was 14.57 ± 12.6 μg/d (19% of total urinary folate; 2% folate intake) (Figure 17).

![Figure 17](image)

Figure 17. Comparisons in final group urinary folic acid concentrations (mean ± SD). Bars designated by a star were significantly different.
Urinary Total Folate

Weekly urinary excretion of total intact folate was measured microbiologically. Urinary folate excretion was higher than that measured by HPLC (5-methyl-THF + folic acid). However, the change over time and differences detected between and within supplementation groups were similar (Table 4).

Table 4. Comparison of initial, steady state and final total urinary folate excretion (µg/d) measured by microbiological assay (MA) and HPLC.

<table>
<thead>
<tr>
<th></th>
<th>Baseline* (MA)</th>
<th>Baseline* (HPLC)</th>
<th>Steady State* (MA)</th>
<th>Steady State* (HPLC)</th>
<th>Final* (MA)</th>
<th>Final* (HPLC)</th>
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<tbody>
<tr>
<td>450 NP</td>
<td>18.2 (24.4)</td>
<td>16.0 (18.2)</td>
<td>14.8 (8.1)</td>
<td>6.5 (5.3)</td>
<td>18.0 (16.1)</td>
<td>4.2 (3.1)</td>
</tr>
<tr>
<td>450 P</td>
<td>111.5 (172.1)</td>
<td>81.1 (106.1)</td>
<td>14.1 (4.2)</td>
<td>4.2 (2.2)</td>
<td>9.7 (3.0)</td>
<td>3.8 (1.4)</td>
</tr>
<tr>
<td>850 NP</td>
<td>29.2 (53.1)</td>
<td>29.4 (39.8)</td>
<td>175.6 (50.7)</td>
<td>79.2 (25.7)</td>
<td>131.9 (55.3)</td>
<td>80.9 (31.4)</td>
</tr>
<tr>
<td>850 P</td>
<td>266.2 (214.2)</td>
<td>193.4 (60.3)</td>
<td>157.5 (72.7)</td>
<td>87.4 (44.1)</td>
<td>132.0 (62.4)</td>
<td>79 (39.6)</td>
</tr>
</tbody>
</table>

* Values are mean ± SD (n=6).

Glomerular Filtration Rates

Glomerular filtration rates were computed from serum and urine creatinine concentrations at baseline and thereafter on a monthly basis. The results are shown in the following table. Since folate intake should not affect glomerular filtration rates, pregnant subjects were compared to nonpregnant subjects. No
differences in GFR existed between pregnant subjects and nonpregnant controls nor did GFR increase in pregnant women with progression of pregnancy.

Table 5. Glomerular filtration rates (mL/min) at weeks 0, 4, 8 and 12.

<table>
<thead>
<tr>
<th></th>
<th>Wk 0*</th>
<th>Wk 4*</th>
<th>Wk 8*</th>
<th>Wk 12*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpregnant</td>
<td>84.5 ± 24</td>
<td>107.3 ± 19</td>
<td>110.8 ± 32</td>
<td>103.8 ± 36</td>
</tr>
<tr>
<td>Pregnant</td>
<td>93.6 ± 26</td>
<td>98.4 ± 31</td>
<td>98.2 ± 37</td>
<td>81.1 ± 26</td>
</tr>
</tbody>
</table>

* Values are mean ± SD (n=12).

Urinary apABG

Urinary apABG excretion was measured bimonthly (wk 0, 2, 4, 6, 8, 10,12) in pregnant subjects and monthly (wk 0, 4, 8, 12) in nonpregnant subjects. Urinary apABG excretion throughout the 12 wk period is illustrated in Figure 18.

Figure 18. Urinary apABG excretion (group means) throughout the 12 wk study.
No differences (P>0.05) existed in baseline group means between pregnant and nonpregnant subjects assigned to consume either 450 μg/d (36.18 ± 13.4, 29.40 ± 9.3 μg/d, respectively) or 850 μg/d (35.58 ± 11.0, 29.85 ± 8.9 μg/d, respectively) or between pregnant women (36.18 ± 13.4, 35.6 ± 11 μg/d) or nonpregnant women (29.40 ± 9.3, 29.85 ± 8.9 μg/d) assigned to consume either 450 or 850 μg/d folate, respectively (Figure 19).

Figure 19. Group comparisons of mean initial and final apABG excretion (mean ± SD) analyzed separately. Bars designated by the same small letter were not significantly different (P>0.05).

No differences (P>0.05) were detected in overall group means between pregnant and nonpregnant women consuming 450 μg/d (26.53 ± 8.3, 31.04 ± 7.2 μg/d, respectively) or 850 μg/d (30.27 ± 7.6, 36.86 ± 8.6 μg/d, respectively).

Differences (P<0.05) were not detected between pregnant subjects (26.53 ± 8.3, 30.27 ± 7.6 μg/d) or nonpregnant controls (31.04 ± 7.2, 36.86 ± 8.6 μg/d)
consuming either 450 or 850 µg/d, respectively (Figure 20). No differences (P>0.05) were detected in final means between pregnant and nonpregnant women consuming 450 µg/d (21.28 ± 7.3, 28.86 ± 9.5 µg/d, respectively) or 850 µg/d (35.71 ± 6.5, 32.56 ± 9.4 µg/d, respectively). Pregnant women consuming 450 µg/d compared to 850 µg/d excreted less (P<0.05) urinary apABG (21.3 ± 7.3, 35.7 ± 6.5 µg/d, respectively) at the end of the study (Figure 19).

![Figure 20. Comparison of overall mean urinary apABG excretion (mean ± SD). Bars designated by the same small number were not different (P>0.05).](image)

Little or no change was observed over time within the groups except for the pregnant women consuming 450 µg/d in which urinary apABG decreased by 40% (Figure 19). Urinary apABG was not significantly correlated with serum folate (r=0.13; P=0.54), red cell folate (r=0.017; P=0.94) or urinary 5-methyl-THF (r=0.03; P=0.89).
Urinary pABG

Urinary pABG excretion was measured bimonthly (wk 0, 2, 4, 6, 8, 10, 12) in pregnant subjects and monthly (wk 0, 4, 8, 12) in nonpregnant subjects. Urinary pABG excretion throughout the study period is illustrated in Figure 21. No differences (P > 0.05) in urinary pABG excretion at baseline were detected between pregnant women (9.07 ± 3.8, 16.75 ± 10.9 µg/d) or nonpregnant controls (6.42 ± 2.5, 5.11 ± 1.4 µg/d) assigned to consume either 450 or 850 µg/d, respectively (Figure 22). The pregnant women assigned to consume 850 µg/d were excreting more pABG (P ≤ 0.05) than either nonpregnant group at baseline.

![Figure 21](image)

Figure 21. Urinary pABG excretion (group means) throughout the 12 wk study. Steady state was achieved at wk 1 and 2 for the 450 and 850 µg/d nonpregnant groups, respectively, and at wk 6 and 2 for the 450 and 850 µg/d pregnant groups.

At steady state, no differences (P > 0.05) were detected in urinary pABG excretion between pregnant and nonpregnant women consuming 450 µg/d (4.46 ±
2.8 vs. 8.23 ± 2.1 μg/d, respectively) or 850 μg/d (11.36 ± 4.4 vs. 8.14 ± 2.3 μg/d, respectively) (Figure 23). Differences (P<0.05) were detected at steady state between pregnant women (4.46 ± 2.8, 11.36 ± 4.4 μg/d) consuming 450 μg/d compared to 850 μg/d, respectively. No differences (P>0.05) were detected at steady state between nonpregnant consuming 450 μg/d compared to 850 μg/d. No differences (P>0.05) were detected in final group means between pregnant and nonpregnant women consuming 450 μg/d (4.62 ± 2.3, 5.73 ± 2.1 μg/d, respectively) or 850 μg/d (8.53 ± 2.1, 8.57 ± 2.3 μg/d, respectively) (Figure 21). Differences (P<0.05) were detected in final group means between pregnant women (4.62 ± 2.3, 8.53 ± 2.1 μg/d) consuming 450 μg/d compared to 850 μg/d, respectively. Urinary pABG was positively correlated with serum folate (r = 0.42; P = 0.04) and urinary 5-methyl-THF (r = 0.52; P = 0.001).

![Figure 22](image-url)

**Figure 22.** Group comparisons of mean initial and final pABG excretion (mean ± SD) analyzed separately. Bars designated by the same small letter were not significantly different (P>0.05).
Figure 23. Urinary pABG excretion (mean ± SD) at steady state. Bars designated by the same small number were not significantly different (P>0.05).
CHAPTER 5
DISCUSSION AND CONCLUSION

This 84 d metabolic study was designed to investigate the folate status response of pregnant women consuming defined folate intakes comparable to the current (400 µg/d) and former (800 µg/d) RDA for folate in pregnant women. This is the first controlled metabolic study conducted to investigate folate status response of pregnant women to defined folate intakes. Pregnant women during their second trimester (wk 14-25) and nonpregnant controls consumed either 450 or 850 µg/d as a combination of synthetic folic acid and dietary folate. Several folate status parameters were affected by prior consumption of prenatal vitamins containing approximately 1 mg of folic acid by pregnant women and differences were observed at baseline between pregnant and nonpregnant women. Therefore, groups were compared at steady state or the time at which the groups had acclimatized to their defined folate intake.

Serum folate concentration is considered a sensitive index of recent folate status (Herbert 1987b) as it is highly influenced by recent dietary intake. However, under metabolic conditions, in which folate intake is constant, serum folate concentration should reflect the overall folate status of the individual. The rapid decline in serum folate concentrations in the pregnant women consuming 450
μg/d illustrated serum folate response to a lower folate intake. Once acclimatized to this lower intake, serum folate concentrations were maintained within normal limits in the pregnant group consuming 450 μg/d and were equivalent to the nonpregnant controls on the same supplementation level. Hemodilution did not appear to be a factor in the initial decline in serum folate concentration since the 850 μg/d pregnant group did not experience any decline throughout the 12 wk period and steady state serum folate concentrations were equivalent to the 850 μg/d controls. Hemodilution or expansion of blood volume is a known physiological consequence of pregnancy (Cunningham et al. 1993) and a significant decline in hematocrit from baseline was observed in the pregnant women participating in this study (Figures 12 and 13). It can be assumed, therefore, that the pregnant women had higher total amounts of serum folate at steady state than the nonpregnant controls.

Red cell folate concentration reflects liver folate concentration and is considered to be an indicator of long term folate status (Herbert 1987). Folate is accumulated only by developing reticulocytes (Shane 1995) and since the life span of red cells is about 120 days, red cell folate concentration more accurately reflects folate status 2-3 months prior to the time of analysis. However, since red cells are being synthesized daily over a 12 wk period, one should be able to detect some change in concentration. This is especially true during pregnancy when red cell production increases by approximately 33% (Blackburn and Loper, 1992) resulting in greater changes in red cell folate concentration if inadequate amounts
of folate are available at the time of incorporation. In our pregnant group consuming 450 μg/d, red cell folate was maintained throughout the study period and was equivalent to the 450 μg/d control group at steady state. Perhaps more importantly, normal red cell folate concentrations were also maintained throughout the three month follow-up period in pregnant subjects who had consumed 450 μg/d during the study. In addition, 450 μg/d (food folate + supplemental folic acid) was sufficient to maintain mean red cell folate concentrations above 400 ng/mL throughout the study and follow-up period thus supporting the findings of Brown et al. (1997) who reported that red cell folate concentrations of 400 ng/mL could be achieved by folate intakes of at least 450 μg/d. This is informative as red cell folate concentrations of ≥ 400 ng/mL are associated with a low risk of folate-responsive NTDs. Red cell folate concentrations were also maintained above 400 ng/mL in both pregnant and nonpregnant women consuming 850 μg/d throughout the study and follow-up period.

Although urinary excretion of folate is not considered to be a reliable index of folate status (Sauberlich et al. 1987), one might expect to observe differences between pregnant and nonpregnant women and between supplementation groups. These differences may reflect metabolic differences thereby enhancing the information gained from blood indices. Pregnant women have been found to excrete significantly more folate than nonpregnant women which is hypothesized by some investigators to partially account for the increase
in requirements during pregnancy (Fleming 1972, Landon and Hytten 1971). The 450 μg/d supplementation groups were excreting similar amounts of 5-methyl-THF (≈5 μg/d) at steady state and no detectable folic acid. The pregnant and nonpregnant women in the 850 μg/d group excreted similar amounts of 5-methyl-THF (≈ 80 vs. 60 μg/d, respectively) at steady state, approximately 15 fold higher than the 450 μg/d groups. Only the 850 μg/d nonpregnant group excreted detectable folic acid (19% of total urinary folate). Saleh et al. (1980) reported a lower folic acid to 5-methyl-THF ratio in patients with malignant disease (state of increased cellular proliferation) compared to controls. They suggested that malignant disease increased the demand for folate and led to more rapid metabolism of folic acid to the reduced folate pool as indicated by an increase in 5-methyl-THF relative to folic acid. Their explanation may apply to our finding that only nonpregnant women in the 850 μg/d group excreted unmetabolized folic acid since pregnancy also represents a period of increased cellular proliferation. The higher urinary excretion of 5-methyl-THF (metabolized form) in the 850 μg/d groups may reflect the saturable process of folate reabsorption from glomerular filtrate by proximal tubular cells (Williams and Huang 1982). Overall, these data on urinary folate excretion indicate that pregnant women are not excreting more folate than nonpregnant women and support the blood data in which no differences between pregnant and nonpregnant women within the same supplementation group were detected.
Glomerular filtration rate (GFR) was measured at baseline and thereafter on a monthly basis in order to assess differences in urinary excretion of folate and catabolites between pregnant and nonpregnant women independent of changes in GFR. Cunningham et al. (1993) reported that GFR increases as much as 50 percent by the beginning of the second trimester and has been found to persist to term. Our data indicated no difference in GFR between pregnant and nonpregnant women at baseline or at the end of the study for reasons unexplained. Glomerular filtration rate was thus not considered when assessing urinary excretion of folate derivatives or catabolites.

Folate catabolites, pABG and the more predominant form, apABG, were measured at numerous time points throughout the 12 wk study. Our results did not confirm the work of McPartlin et al. (1993) who reported a doubling of folate catabolism by pregnant women during the 2\textsuperscript{nd} trimester. In contrast, we found that the mean urinary excretion of both pABG and apABG was equivalent to that of our nonpregnant controls. No differences (P>0.05) in baseline, overall or final means were detected in urinary apABG excretion between pregnant and nonpregnant women within the same supplementation group which suggests that pregnancy did not affect urinary apABG excretion during this 84 d study. Final mean apABG excretion was lower (P \leq 0.05) in pregnant women consuming 450 \mu g/d compared to 850 \mu g/d indicating that over the long-term, urinary apABG was affected by folate intake. The 40\% decline in urinary apABG excretion from baseline measurements in pregnant women consuming 450 \mu g/d also illustrated
the effect of folate intake during pregnancy on apABG excretion since these women were previously consuming approximately twice the amount of folate. These observations may suggest that pregnant women become more efficient at conserving or recycling folate when folate intake is somewhat restricted.

Urinary pABG excretion was greater (P≤0.05) in pregnant women compared to nonpregnant controls at baseline which may have reflected partial degradation of urinary intact folate which was also higher at baseline in pregnant women due to prior supplementation with folic acid. McNulty et al. (1993) found that urinary excretion of pABG in rats appeared to be dependent on dietary folate level whereas as apABG excretion was not. At steady state, urinary pABG excretion in pregnant women was equivalent to nonpregnant controls within the same supplementation group indicating that pregnancy did not affect urinary pABG excretion. Pregnant women consuming 850 μg/d compared to 450 μg/d were excreting more (P≤0.05) urinary pABG. This may be explained by the positive correlation (r=0.53; P<0.01) between urinary pABG and 5-methyl-THF excretion which suggests that intact folate may have degraded in the urine. Differences (P≤0.05) were observed in urinary 5-methyl-THF excretion at steady state between pregnant subjects consuming 850 versus 450 μg/d. However, nonpregnant women consuming 850 μg/d versus 450 μg/d were also excreting more urinary 5-methyl-THF but not more pABG at steady state. As the study progressed, however, folate intake appeared to be affecting the urinary excretion of pABG in nonpregnant subjects although significant differences in final group
means were not obtained. The origin of urinary \( p\text{ABG} \) may represent any of the following: (1) endogenous \( p\text{ABG} \) that does not get acetylated prior to excretion; (2) folate breakdown occurring in circulation or in the bladder (Geohegan et al. 1995); (3) dietary \( p\text{ABG} \); (4) degradation of urinary intact folates; or (5) \( p\text{ABG} \) that gets deacetylated (McNulty et al. 1993).

Although our findings of equivalent \( p\text{ABG} \) excretion between nonpregnant and pregnant women differed from McPartlin et al. (1993) who reported a doubling of \( p\text{ABG} \) excretion by pregnant women during the second trimester, agreement existed in nonpregnant catabolite values between this study, McPartlin et al. (1993) and Kownacki-Brown et al. (1995). The mean urinary excretion of \( p\text{ABG} \) and \( p\text{ABG} \) in our study were \( \sim 34 \) and \( 7.2 \, \mu\text{g/d} \), respectively compared to \( \sim 32 \) and \( 13 \, \mu\text{g/d} \), respectively (McPartlin et al.1993), and \( \sim 66 \, \mu\text{g/d} \) (\( p\text{ABG} + p\text{ABG} \)) prior to supplementation (Kownacki-Brown et al. 1995). Our mean urinary \( p\text{ABG} \) excretion is lower than that of McPartlin et al. (1993) and may reflect removal of intact folates by affinity chromatography columns containing folate binding protein prior to catabolite isolation and quantitation. If affinity chromatography columns are not used, unremoved intact folates may break down upon acidification or during elution from the ion exchange columns. The lower urinary \( p\text{ABG} \) concentrations may also be the result of lower dietary \( p\text{ABG} \) intake since our subjects received the majority of folate from folic acid, the most stable form of the vitamin.
The concept of deriving requirements based solely on catabolite excretion may be inappropriate during pregnancy and even in nonpregnant humans since other avenues of folate loss or accretion are not considered. For example red cell production increases by 33% (Blackburn and Loper, 1992) resulting in greater folate uptake by reticulocytes. This extra demand for folate will not be reflected in urinary catabolite concentration. The doubling in uterus size may require more folate coenzymes of which the majority may be recycled upon utilization and not degraded and excreted as urinary catabolites. It is recognized that the developing fetus drains maternal folate supplies as evidenced by the significantly higher plasma and red cell folate concentrations in newborns compared to maternal concentrations (Ek and Magnus 1981, Giles 1966). Folate accumulated by the placenta and delivered to the developing fetus will not be estimated by quantitation of urinary folate catabolites. In addition, measurement of urinary catabolite excretion alone does not account for endogenous fecal folate loss which may be significant (Krumdieck et al. 1978). Aside from other avenues of folate loss, studies conducted in rats (Connor et al. 1979, Pheasant et al. 1981) and humans (Saleh et al. 1980) have identified p-acetamidobenzoate (pABA) as a folate catabolite whose quantitation would increase folate requirements when based on catabolite excretion. However, these studies compared rates of catabolism only and did not measure true endogenous urinary pABA total excretion, hence the contribution of pABA to folate requirements is unknown.
The differences between our findings and those of McPartlin et al. (1993) may be the result of differences in protocols since, in the present study, folate intake was highly controlled for a 12 wk period during which time 24 h urine collections were obtained. In contrast, folate intake was only controlled for a 42 h period in the study by McPartlin et al. (1993). It is possible that pregnant women consumed more folate containing food items prior to study participation and thereby consumed more pABG. In rats, intravenous administration of radiolabeled pABG resulted in substantial conversion to apABG (Murphy et al. 1976). In addition, Pheasant et al. (1981) reported that urinary pABG and its acetylated derivative were detected following an oral dose of radiolabeled pABG.

The fate of dietary pABG in humans is unknown although it has been reported (unpublished data) that pABG cannot enter cells and is rapidly excreted in urine (Johns et al. 1961) suggesting that it is excreted unacetylated. Another potential explanation for the discrepancy is the effect of weight gain on apABG excretion. In rats, urinary apABG excretion was consistently higher in the pregnant rats with free access to food compared to a pregnant pair fed group (pair fed to nonpregnant controls) suggesting that one component of the observed elevation in folate catabolism was due to greater food intake (McNulty et al. 1993). Furthermore, comparisons of urinary apABG excretion in nonpregnant female rats at weights comparable to those attained by the pregnant rats indicated a positive association between apABG excretion and weight gain (McNulty et al. 1993). In our protocol, weight gain was strictly controlled (5.0 kg during the 12 wk period).
which provides another possible explanation for differences between protocols since in the study of McPartlin et al. (1993) food intake was not controlled long-term. Although unrestricted calorie intake and perhaps greater weight gain may partially account for the higher apABG excretion observed by McPartlin et al. (1993), it does not fully explain the lack of difference in apABG excretion between our nonpregnant and pregnant subjects. Our definitions of second trimester also differed (personal communication) since urinary catabolite excretion was measured from wk 14 to 26 in this study and at one time point between wk 26 to 32 in the study of McPartlin et al. (1993).

The equivalent response of pregnant and nonpregnant to folate intakes of either 450 \( \mu g/d \) or 850 \( \mu g/d \) was not anticipated secondary to the pregnant woman’s increased demand for folate coenzymes required for DNA synthesis and greater folate uptake by developing red cells and fetus. In order to detect differences in folate status response between pregnant and nonpregnant women, it is likely that lower folate intakes are necessary. It is also possible that differences may have been detected with the progression of pregnancy. Our finding of equivalent response between pregnant and nonpregnant women does not suggest that pregnant women do not require more folate but does suggest that 450 \( \mu g/d \) was sufficient in maintaining folate status equivalent to that of nonpregnant controls.

Overall, these data suggest that 450 \( \mu g/d \) derived from both food (120 \( \mu g \)) and synthetic folic acid (330 \( \mu g \)) is adequate to maintain normal folate status in
well-nourished pregnant women during their second trimester. However, the question remains as to how to translate this finding into food folate and an RDA. Recent studies (Cuskelley et al. 1996, Pfeiffer et al. 1997) indicate that foods fortified with synthetic folic acid are highly available unlike naturally occurring food folates. Therefore, one may hypothesize that synthetic folic acid consumed with meals is more available than endogenous food folate (≈50%) (Sauberlich et al. 1987) but less available than the essentially complete absorption of synthetic folic acid (≈100%) consumed under fasting conditions (Gregory 1995). A reasonable, although somewhat conservative, approach is to assume that synthetic folic acid consumed with meals is approximately 75% available. Based on the above assumptions, our subjects in the 450 μg/d group consumed ≈ 307 μg/d available folate which translates into 615 μg/d dietary equivalents.

Our dietary equivalent estimate resembles the estimations of Chanarin et al. (1968a, 1968b), Cooper et al. (1970) and McPartlin et al. (1993). It also supports the recommendation of Willoughby et al. (1968) regarding the administration of 300-350 μg/d synthetic folic acid throughout gestation assuming an average folate intake of 150 μg/d (low dietary folate). It is questionable whether the majority of pregnant women can consume ≈ 600 μg/d of folate from diet alone (LSRO 1980, Brown et al. 1997, Huber et al. 1988) although folic acid enrichment of cereal-grain foods (140 μg/100g product) effective January 1, 1998 (FDA 1996) is estimated to increase average daily consumption by 80-100 μg (Brown et al., 1997, FDA 1996). Our findings indicate that prenatal vitamins
containing more than 500 µg/d are not necessary to maintain adequate folate status in well-nourished pregnant populations and support the findings of Lowenstein et al. (1966) which suggested that provision of 500 µg/d synthetic folic acid, in addition to low dietary folate intake, may be above the minimal daily requirement since higher serum folates were observed in pregnant women compared to nonpregnant controls (11.0, 7.02 ng/mL, respectively). Because the effects of oversupplementation with folic acid on the developing fetus are unknown (Scott et al. 1991), high supplemental doses (>1000 µg/d) should be avoided under normal circumstances.

In conclusion, 450 µg/d consumed as a combination of synthetic folic acid and dietary folate or approximately 600 µg/d dietary equivalents is sufficient to maintain normal folate status throughout the second trimester. The first and third trimesters or postpartum period were not investigated under controlled conditions in the current study, therefore future areas of research should encompass these time frames under controlled conditions as well as the response of previously unsupplemented pregnant women to defined folate intakes.
CHAPTER 6
SUMMARY

An 84 d metabolic study was conducted in order to assess the folate status response of pregnant women and nonpregnant controls to folate intakes comparable to the current and former RDA for pregnant women, 400 and 800 μg/d, respectively. The overall goal of this investigation was to provide metabolic data that may assist in the interpretation of current dietary recommendations. Subjects consumed either 450 or 850 μg/d of folate derived from both synthetic and dietary sources. A series of folate response parameters were then evaluated in relation to folate intake in both pregnant subjects and nonpregnant controls. Following sample analyses, the data indicated that the response of pregnant women was equivalent to that of nonpregnant controls in either the 450 or 850 μg/d supplementation groups. In addition, normal folate nutriture was maintained by subjects in all groups. These results suggested that 450 μg/d was adequate during the second trimester of pregnancy. Since our overall goal was to assist in the interpretation of current dietary recommendations, it was necessary to consider the bioavailability of the folate consumed by our subjects which was a mixture of synthetic folic acid and dietary folate. It is generally agreed that dietary folate is approximately 50% available and that synthetic folic acid, consumed under fasting conditions, is approximately 100% available. Our subjects consumed the
synthetic folic acid (330 μg/d) along with food thus we conservatively estimated it was 75% available. Following adjustments for bioavailability, subjects in the 450 μg/d consumed approximately 600 μg/d dietary equivalents. In conclusion, our data suggests that 600 μg/d dietary folate is adequate in maintaining normal folate status throughout the second trimester of pregnancy.
Microbiological Assay Procedures

Microplate Medium Preparation for *L. casei*  
Folic acid medium-Difco (dehydrated) (230.3 g) was weighed into a tared plastic weigh boat. The dehydrated medium was added to a glass flask to which 2,450 mL of Milli-Q water was added. The flask was covered with foil and the media mixed and brought to a boil. After one to two minutes, the media was removed from heat and cooled to room temperature. The media was then dispensed into disposable glass tubes, capped and placed into a metal rack holder. The tubes containing the media were covered with foil and autoclaved for 5 minutes. After cooling to room temperature, the medium was stored in the refrigerator at 4 °C. The microorganism, *L. Casei*, was added to the media just prior to use.

Folic Acid Standard Preparation  
Ten milligrams of folic acid (Sigma F-7876) was weighed and dissolved in 0.1 N NaOH. The dissolved folic acid solution was transferred to a 100 mL volumetric flask and filled to 100 mL with Milli-Q water to a final concentration of 100 μg/mL. A 1:10 dilution was performed by taking 100 μL of folate solution and adding 900 μL of 0.1 M KH₂PO₄ (pH 7). The exact
concentration of the folate solution was determined spectrophotometrically using 0.1 M KH$_2$PO$_4$ (pH 7) as the blank.

Concentration by weight: 

\[
0.1 \text{ mg/ml} \times \text{mmol/441.4mg} = 0.2266 \times 10^{-3} \text{ mg/mmol} \\
= 226.6 \times 10^{-3} \mu g\text{/mmol} = 226.6 \mu g/L = 226.6 \mu M.
\]

Expected Absorbance for folic acid at pH 7.0 and 282 nm with an extinction coefficient of 27,600 is: 

\[
226.6 \mu M \times 27,600 \times 10^{-6} \times 10^{-1} = 0.6253
\]

A 1:10 000 dilution of the folate solution was performed after adjusting for actual concentration. Sodium ascorbate (0.1%; w/v) was added to the folate standard (1 L). One milliliter portions were aliquoted while gently mixing to ensure homogeneity. The standards were stored at -20°C. The remaining folate stock solution with a concentration of 100 µg/mL was stored at -20 °C.
LITERATURE CITED


Marie Anne Caudill was born on November 3rd, 1967, in Sarnia, Ontario, Canada. At 11 years of age, her goal was to obtain a tennis scholarship at a university located in the southern United States. In 1987, she accepted a tennis scholarship at Lamar University in Beaumont, Texas. Two years later, she transferred to the University of North Florida where she received a Bachelor of Art in Physical Education, a Bachelor of Science in Health Science and a Master of Science in Health Science and also met her husband, Tony. During her fifth year on athletic scholarship, she competed in the NAIA women’s marathon for the University of North Florida and became national champion. In May 1994, she completed a dietetic internship at Emory University Hospital in Atlanta, Georgia. Three months later, she entered the doctoral program in the Food Science and Human Nutrition Department at the University of Florida, Gainesville. She graduated with her doctoral degree in August, 1997 and has accepted a faculty position in nutrition at California State Polytechnic University located in Pomona.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of Nursing
This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1997

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