A REVIEW OF FOLATE INTAKE, METHODOLOGY, AND STATUS

November 1981

Prepared for

BUREAU OF FOODS
FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES
WASHINGTON, D.C. 20204

under

Contract Number FDA 223-79-2275
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by

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FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. Reports are based on comprehensive literature reviews and the scientific opinions of knowledgeable investigators engaged in work in specific areas of biology and medicine.

This technical report was prepared for the Bureau of Foods, Food and Drug Administration (FDA), by Sue Ann Anderson, Ph.D., R.D., Staff Scientist, and John M. Talbot, M.D., Senior Medical Consultant, LSRO, FASEB, in accordance with the provisions of Contract No. FDA 223-79-2275.

The LSRO acknowledges the contributions of the investigators and consultants who assisted with this study. The report reflects the opinions expressed by participants in an ad hoc study group that met at the Federation on March 23-24, 1981. A judicious attempt has been made to incorporate the various viewpoints and opinions. The report was reviewed by these consultants; however, listing of their names in Section XI does not imply that they endorse the study. The authors and LSRO accept responsibility for the contents of the report.

The report was reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent Society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures, the report was approved and transmitted to FDA by the Executive Director, FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of each individual member of the FASEB constituent Societies.

Kenneth D. Fisher, Ph.D.
Director
Life Sciences Research Office
SUMMARY

Folacin, one of the B complex vitamins, occurs naturally in several forms: the reduced dihydro- and tetrahydro- forms, unsubstituted or bearing one of several one-carbon moieties, and/or conjugated in peptide linkage to a poly-γ-glutamyl chain occur in a variety of foods. The vitamin is a coenzyme for the transfer of one-carbon units including certain reactions in nucleic acid and amino acid metabolism.

Estimates of dietary intakes of folates for large numbers of individuals are limited, but available figures indicate that average per capita disappearance of folate in foods is about 225 μg/d for the United States population. Although dietary intake is the main determinant of folate status in healthy individuals, in certain conditions other factors influence folate status. For example, absorption of the vitamin may be impaired by alcohol, drugs, inhibitors of intestinal folate conjugases, and diseases of the gastrointestinal tract. Folate metabolism may be adversely affected by a deficiency of iron, zinc, or vitamin B₁₂, as well as by ingesting alcohol or certain drugs. Periods of increased cellular proliferation or metabolic activity and conditions causing increased body losses of the vitamin also increase the need for folate.

Bioavailability of the vitamin depends on the specific forms of folate, the pH in the small intestine, and the efficiency of hydrolysis of folyl polyglutamates by intestinal conjugases. Relative bioavailability of folates in single foods has been estimated, but, for the most part, such studies have provided only approximations. Knowledge of the absorption and biological activity of the mixtures of folates present in foods consumed under normal dietary conditions is limited.

Analysis of folate content of foods and biologic materials as well as assessment of folate status of individuals is difficult because of the numerous forms of the vitamin having nutritional and metabolic significance and because of limitations of available methodology. At present, the Lactobacillus casei assay, including conjugase treatment, is the most widely accepted method for assaying the vitamin. Despite large variations in results, these L. casei values may be correct and may simply reflect differences in folate content among samples. A radiometric binding assay provides a rapid, accurate, and technically simple alternative analysis of folate.

Folates differ in their stability to conditions encountered during storage and processing of foods such as changes in pH, presence of oxidizing agents, and exposure to heat and light. In general, total food folate content and polyglutamate chain length decrease under such conditions. Methodologies such as high performance liquid chromatography show promise in determinations
of the forms of folates in samples. These data will be helpful in
determining stability and relative availability of the vitamin in
foods. Current sensitivity of this method does not allow analysis
of samples with low concentrations of folate, such as sera, but
progress in derivitization may soon permit use of this method for
their analyses.

Definitions of compromised folate status in the literature
vary. In this report, frank folate deficiency is defined as less
than 3 ng folate per ml serum and less than 140 ng/ml red cells,
and marginal folate status is defined as 3-6 ng folate per ml serum
and approximately 150-160 ng/ml red cells.

Correlation between folate intake and overall body status
is extremely difficult. Blood is considered the best tissue avail-
able for determination of folate status in humans. Serum folate
levels are indicative of immediate past intakes of the vitamin,
but red cell folate concentrations are thought to provide a more
reliable measure of folate status over time. Peripheral blood
smears can be used to detect megaloblastic changes characteristic
of folate deficiency, although further tests are needed to identify
the cause of the megaloblastosis. Hypersegmentation of neutrophils
is an early hematologic sign of folate deficiency and occurs in
only a few instances not associated with deficiency of the vitamin.
Abnormal deoxyuridine suppression of DNA synthesis in bone marrow
cells and phytohemaglutinin-stimulated lymphocytes is a useful
laboratory research procedure, but this method is not practical
for large numbers of samples. Additional valuable tests include
bone marrow smears, measurement of organ tissue folate levels,
and urinary formiminoglutamic acid excretion. However, these
procedures are not amenable to survey protocols.

In many cases, clinical evaluation on an individual basis
is required. Because some apparently healthy people have low or
borderline folate status, more data are needed to characterize
conditions leading to folate deficiency, to refine norms, and to
account for the seeming lack of impairment among such individuals.

In contrast to problems of deficiency, excessive intake
of folate may mask the hematologic manifestations and neurologic
complications of pernicious anemia. Evidence of excessive folate
intakes from food sources has not been documented. While there is
limited evidence that repeated oral doses of folic acid of 400 μg/d
may mask the development of pernicious anemia in some individuals,
it is not known whether long-term consumption of this amount of
folic acid added to foods would produce a similar effect.

Interpretation of folate status in the population of
the United States based on data from large-scale surveys has been
difficult and equivocal. Population groups who may be at risk of
developing folate deficiency include premature infants, adolescents,
pregnant or lactating women, nonlactating women during the first
2-3 years postpartum, and the elderly. In the United States, substantial percentages of such groups have been shown to have serum and red cell folate values at deficiency levels, but no causally related illnesses have been reported. In addition, available evidence of compromised folate status based solely on mean values of serum and red cell folate from large-scale surveys is insufficient to determine whether a medical problem of public health significance exists in this country.

Addition of folic acid to staple foods in South Africa and to wine in the United States has been an effective means of increasing serum and red cell folate in certain groups at risk of developing folate deficiency. Calculation of the possible folate intake by use of selected foods containing added folic acid also suggests that such foods can contribute significantly to folate intake in the United States. However, the actual patterns of use of such products by individuals in population groups considered at risk of folate deficiency have yet to be determined. These data are essential to any consideration of folate fortification of foods in this country.
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I. INTRODUCTION

Folacin is one of the essential nutrients of the B complex of vitamins. It occurs naturally in many commonly consumed food staples in the form of folates. However, recent data suggest that dietary intakes of folate in certain North American population groups such as adolescents, pregnant women, and the elderly, may be insufficient to maintain health. Conversely, some authorities are concerned about possible excessive intakes of folate as a result of consumption of folate-containing foods and the use of over-the-counter supplements containing folic acid. The principal concern about excessive folate consumption relates to temporary masking of pernicious anemia by intakes of folate in amounts not strikingly greater than those in the diet that are necessary to maintain normal health.

The Food and Drug Administration is responsible for evaluating and monitoring the safety and adequacy of foods, establishing regulations, and providing nutrition information to consumers. The major goal of this study is to provide the Office of the Associate Director for Nutrition and Food Sciences, Bureau of Foods, Food and Drug Administration, with comprehensive information to assist in decision-making relative to folate in the food supply, specific diets, and food fortification practices. An essential part of achieving this objective is the collection and analysis of available data to develop a sound scientific basis for dealing with folate safety and nutriment.

The purpose of the study is to examine the influence of dietary intake of folate on nutritional status, including consideration of the following questions:

- Can compromised folate status be defined biochemically and clinically?
- Does chronic dietary intake below recommended levels occur in certain population subsets?
- Does chronic dietary intake below recommended levels lead to clinically identifiable compromised folate status?
- What are the most appropriate methodologies to measure folate status in survey or case-controlled investigations?
- What types of nutrient-nutrient and drug-nutrient interactions affect folate status?
- Can food composition tables be developed that allow more accurate estimates of folate intake?
- What losses of folate occur in foods during storage and processing?
- What are the advantages and disadvantages of addition of folate to specific dietary items?
- Is folate fortification needed?

Scope. This report analyzes available data on folate nutriture, bioavailability and stability of food folates, biomedical aspects of folate deficiency or excess, and the influence of drugs, alcohol, and various nutrients on folate availability and metabolism. In addition, the study focuses on current and developing analytic methods for folates, and the clinical and biochemical characterization of compromised folate status. Critical issues are identified, and suggestions for future research are provided.
II. BACKGROUND

In the early 19th century Channing (1824) recognized that inadequate diet coupled with a succession of pregnancies produced a life-threatening anemia. More than a century later, Wills (1933) noted that diets consisting largely of polished rice and bread which caused megaloblastic anemia in pregnant women in India produced the same condition in monkeys. Supplementation of the monkeys' diet with marmite, a yeast product, improved their hematologic condition. The unidentified component of marmite became known as the "Wills factor." Subsequently, the active principle was purified (Stokstad, 1943), crystallized (Pfiffner et al., 1943), and its structure elucidated and verified by total synthesis (Angier et al., 1946). The term "pteroylglutamic acid" was introduced by the chemists who determined the vitamin's structure; however, the trivial name "folic acid," proposed by Mitchell et al. (1941) after isolation of the compound from spinach, has persisted as the common name of the parent compound.

Folic acid consists of a pteridine nucleus, p-aminobenzoic acid, and glutamic acid:

\[
\begin{align*}
\text{NH}_2-C_2 & \quad N_3 \quad N_8 \quad \text{CH} \\
N_4 \quad N_5 \quad C_4 - C & \quad N_6 \quad \text{CH}_2 - \text{NH} \quad \text{CH} \quad \text{COOH} \\
| & \quad \text{OH} \\
\end{align*}
\]

\[
\begin{align*}
\text{Pteridine nucleus} & \quad p\text{-Aminobenzoic acid} & \quad \text{Glutamic acid} \\
\hline
\text{Pteroic acid} & \\
\end{align*}
\]

Folic acid (Pike and Brown, 1975)

This oxidized parent form of the vitamin is found in foods and in mammalian tissues only as a product of oxidation of the naturally occurring, active, reduced dihydro- and tetrahydro- forms.

\[
\begin{align*}
\text{NH}_2-C_2 & \quad N_3 \quad N_8 \quad \text{CH} \\
N_4 \quad N_5 \quad C_4 - C & \quad \text{H} \quad \text{H}_2 \\
| & \quad \text{OH} \\
\end{align*}
\]

\[
\begin{align*}
\text{COOH} & \quad \text{CH}_2 \\
\text{CH}_2 & \\
\end{align*}
\]

\[
\begin{align*}
\text{C} \quad -\text{NH}\quad \text{CH} \quad \text{COOH} \\
\end{align*}
\]

Tetrahydrofolic acid (Pike and Brown, 1975)
Tetrahydrofolic acid, unsubstituted or with one of several possible single carbon substituents (5-formyl, 10-formyl, 5-formimino, 5,10-methylene, or 5-methyl) and bearing a poly-Y-glutamyl chain attached in peptide linkage to the gamma carboxyl group of the glutamyl residue of the tetrahydrofolic acid moiety, is the active coenzyme form of the vitamin. The term "free folate" has been used to designate the various forms that are biologically active as growth factors for microorganisms without prior treatment to remove glutamate moieties; those forms containing additional glutamic acid units in gamma peptide linkage and activated by enzyme digestion are known as "bound" or "conjugated" folates. Baugh and Krumdieck (1971) have estimated that at least 150 different forms of the vitamin could exist on the basis of the various combinations of one-carbon substituents, state of oxidation, and length of side chain. The IUPAC-IUB designated collective descriptor for folic acid and its biologically-active related compounds is the generic term "folacin(s)" (IUPAC-IUB Commission on Biochemical Nomenclature, 1966). However, since the synonym, folate, is more widely used, both terms are used in this report.

In mammalian systems folacin derivatives function as coenzymes for the transfer of one-carbon units, i.e., formyl, methyl, or formimino groups. Such transfers occur in de novo purine synthesis, thymidylate synthesis, certain pathways of amino acid metabolism, generation and utilization of formate, and methylation of transfer RNA in some bacteria (Blakley, 1977; Herbert et al., 1980). A direct role of folic acid polyglutamates in heme formation has been suggested recently (Piper et al., 1979).

In 1968 the Food and Nutrition Board of the National Research Council (National Research Council, 1968) established a recommended dietary allowance (RDA) for adults at 400 µg/d total folate. This level has been retained in the current recommendations (National Research Council, 1980). This value, or the appropriate value for other age and sex groups, is considered the standard against which food folacin intakes of the United States population are evaluated. The RDA is established for total food folate and the amount needed may be less, if pure folic acid meets part of the requirement. Clinical and biochemical indicators of folate status, frank deficiency, and compromised folate status are defined on page 27. The minimal amount of the pure vitamin form of folic acid (pteroylglutamic acid) necessary to maintain serum levels around 5 ng/ml in healthy adult women consuming a folate-deficient diet was suggested to be about 50 µg/d (Herbert, 1962a). This estimate was based on studies of only a few subjects using three dosage levels and estimated only the minimum amount necessary to evoke a hematologic response in deficiency. The Food and Nutrition Board was hindered in developing its recommendations by limitations of the available clinical and epidemiological data and estimates of dietary intakes. Such uncertainties together with problems in assay methodology make it difficult to correlate measurements of folate content of foods, intake, and status with clinical findings and public health evaluations.
III. FOOD FOLATES

A. FOOD COMPOSITION TABLES

Accurate folate values of foods have only recently been included in USDA tables of food composition. Establishment of the RDA for folacin (National Research Council, 1968) stimulated a greater demand for reliable information on its content in foods. Although not complete, the most extensive compilations of total food folate concentrations are the USDA Agricultural Handbooks 8-1 through 8-7 (U.S. Department of Agriculture, 1976a,b; 1977; 1978; 1979a,b; 1980a), the USDA provisional table for food folate (Perloff and Butrum, 1977), the supplement to the provisional table (Perloff, 1979), and the Canadian (Hoppner et al., 1972, 1973) and British (Paul and Southgate, 1978) tables of food folates. Total folate content of foods appears to be the best index presently available; however, Dong and Oace (1973) caution that such values should not be used indiscriminately since preferential utilization of different folate forms may occur.

Problems with methodology (Section VI) have been a major deterrent to wide acceptance of the folate values in food composition tables. Food folate levels reported in earlier tables (Toepfer et al., 1951) are considered too low because ascorbate had not been used to prevent oxidation (Herbert, 1963), because the Streptococcus faecalis assay did not measure 5-methyl tetrahydrofolate (a predominating form in many foods), and because a conjugase enzyme had not been used to make polyglutamyl folates available to the test organisms (Butterworth et al., 1963). Values of total folate content currently supplied to USDA by university, government, and industry laboratories are based on the Lactobacillus casei assay with conjugase pretreatment. Large variations among samples and lack of agreement among laboratories have been recorded. Butterworth and Krumdieck (1975) pointed out that complete conjugase hydrolysis and extraction procedures often are not performed by laboratories because of the complex methodology involved. Such inadequate practices may contribute substantially to the variability among samples reported by different laboratories, as may bacterial synthesis of folate or inhibition of folate deconjugation by fiber, etc. (Santini and Corcino, 1974). It is understood that large-scale analyses of folate in foods are not planned by USDA until analytical differences are resolved or more consistent methodology becomes available.

The values now available, however, may actually be correct and merely reflect the large variations in folate content present among food samples. The L. casei assay is probably the best method available for total food folates. The radiometric binding assay has recently been used to measure food folate (Graham et al., 1980; Tigner and Roe, 1979) and the values obtained correlate reasonably well with the L. casei assay. Development of chemical methods
such as high performance liquid chromatography (HPLC) may not reduce significantly the variability reported with the L. casei assay. On the other hand, the L. casei assay does not yield any information regarding the chain length distribution of the polyglutamyl folates or the relative proportions of the various one-carbon-substituted forms present in the sample. Data concerning this may become available through the application of newer HPLC methodologies. The more detailed knowledge concerning the forms of folate present in specific foods may be helpful in determining stability and relative availability of folates in foods.

B. STABILITY OF ENDOGENOUS FOLATES DURING STORAGE AND PROCESSING

During storage and processing of foods endogenous folates are present mainly as polyglutamates of tetrahydrofolate with one-carbon substituents at the formyl (5-formyl, 10-formyl, 5,10-methenyl, and 5-formimino) and methyl (5-methyl) oxidation levels. These forms of folate differ in their stability to oxidants, pH, and buffer ions (O'Broin et al., 1975) as well as to heat, light, and oxygen, thereby presumably causing differences in the stability of total food folate content during storage and processing. Reed and Archer (1979) demonstrated that 10-nitrosofolic acid is formed by a reaction in vitro of folic acid with sodium nitrite under acidic conditions. Effects of the presence of such folate compounds in foods have not been investigated.

Several studies have measured loss of folates in foods or solutions subjected to conditions likely to be encountered in storage, processing, or home preparation (Augustin et al., 1978; Cooper et al., 1978; Day and Gregory, 1981a; Keagy et al., 1975; Leichter et al., 1978; Moscovitch and Cooper, 1973; Reed et al., 1976; Shinton and Wilson, 1975). In general, total folate content and polyglutamate chain length decreased under the test conditions, but analyses of changes in specific reduced one-carbon-substituted folate monoglutamates and polyglutamates were not consistent from study to study. Hurdle et al. (1968) reported that as much as 50-95% of folate activity may be lost during food processing and home preparation. The thermal stability of folacin derivatives is strongly influenced by dissolved oxygen concentration (Chen and Cooper, 1979). However, when processed under conditions of sufficiently low oxygen content, even labile reduced folates are well retained (Day and Gregory, 1981a). Uncontrollable differences in storage and processing of foods constitute a confounding factor for studies relying on food composition tables for estimating dietary folate intakes.
C. BIOAVAILABILITY OF FOLATES IN FOODS

Studies of the bioavailability of folate are of relatively recent origin. It is recognized that many factors influencing absorption and bioavailability of folates have yet to be determined and that methods used to estimate bioavailability of food folates need further development.

Absorption of folic acid and reduced folates has been studied in human subjects primarily by jejunal perfusion techniques (Gerson et al., 1971; Halsted, 1980), by urinary excretion of labeled folic acid following a flushing dose of unlabeled folate (Rosenberg, 1975; Russell et al., 1979), and by a fecal excretion method (Chanarin and Perry, 1977). Such studies show that absorption of folic acid itself is greatest in the jejunum (Hepner et al., 1968). Absorption increases in the presence of glucose (Gerson et al., 1971) and in a slightly acidic medium (pH 6.3) (Russell et al., 1979; Smith et al., 1970). It decreases in an alkaline or markedly acidic pH (Benn et al., 1971; MacKenzie and Russell, 1976; Tamura et al., 1976); after administration of pancreatic extract (Russell et al., 1980); or during drinking episodes in malnourished alcoholics (Halsted et al., 1967, 1971).

Absorption efficiency differs among the various folate forms. Following administration of tritium-labeled preparations to human subjects, 79% of folic acid (Anderson et al., 1960), 90-98% of tetrahydrofolic acid and 95-99% of 5-methyl tetrahydrofolic acid (Chanarin and Perry, 1977) were absorbed. Differences in plasma or urinary folate levels following oral doses of folic acid, one-carbon-substituted reduced folate monoglutamates, or folate polyglutamates suggested that all forms of folate may not be absorbed equally by the human (Brown et al., 1973; Tamura and Stokstad, 1973). Studies with radiolabeled pteroylheptaglutamate indicated that polyglutamates were cleaved in the gut to the monoglutamate form; 50-70% of the heptaglutamate-derived portion was not recovered in the feces and was presumably absorbed (Butterworth et al., 1969). Urinary excretion and jejunal perfusion studies gave similar results; polyglutamates were absorbed 70-90% as efficiently as the monoglutamates (Godwin and Rosenberg, 1975; Halsted et al., 1975; Rosenberg and Godwin, 1971; Tamura and Stokstad, 1973). Further investigations have located two separate folate conjugases in human jejunal mucosa: one with a pH optimum of 6.5 in the brush border and the other with a pH optimum of 4.5 in the cytoplasm of mucosal cells (Reisenauer and Halsted, 1981; Reisenauer et al., 1977).

Investigations on transport of folate across the mucosal cell have produced divergent results. Perfusion studies indicate that folate is transported against a concentration gradient by a saturable process in man (Hepner et al., 1968). On the other hand, similar percentages of labeled folate are absorbed over a dose range of 25-3000 µg, suggesting a passive diffusion process.
(Chanarin, 1979a). In light of such evidence, Lindenbaum (1979) has postulated that a dual transport mechanism is involved, consisting of a carrier-mediated, energy-requiring, saturable system operable at low concentrations and a passive diffusion mechanism operating at high concentrations. Two lines of evidence strongly suggest that folate absorption in the human involves a carrier mechanism: the selective absorption of the natural isomer of 5-methyl tetrahydrofolate over the unnatural isomer and the identification of children with congenital folate malabsorption who absorb no folate (Cooper, 1977). The transport system carries reduced folates and folic acid, and polyglutamate absorption shares at least a portion of this system.

Less is known about the availability of folates in foods consumed under normal dietary conditions than absorption from solutions of single folate compounds. For example, much of the folacin in food is present as mixtures of polyglutamates (Butterworth et al., 1963; Scott and Weir, 1976) and absorption of such mixtures by humans has been difficult to evaluate. In addition, foods such as yeast (Swendsen et al., 1947) and a variety of legumes (Krumdieck et al., 1973) contain conjugase inhibitors which may prevent cleavage of folate polyglutamates to monoglutamates by enzymes of the gastrointestinal tract.

The availability of food folates has been estimated from hematologic responses, changes in blood folate levels, urinary folate excretion, intestinal perfusion studies, and growth responses (Chanarin, 1979a). Hematologic responses have been measured after feeding raw lettuce (Baumslag and Metz, 1964), a soup containing asparagus, lettuce, and spinach (Butterworth, 1968), or yeast extract (Wills, 1931) to patients with megaloblastic anemia. Measurement of red cell or plasma folate after consumption of grain products containing added folic acid indicated that added folic acid in bread produced about 30%, and that in maize and rice produced 50%, of the rise in blood folates observed with a folic acid standard (Colman et al., 1975a,b; Margo et al., 1975). Similarly, the rise in plasma folates from wine fortified with folic acid was about 70% that of a standard folic acid solution (Kaunitz and Lindenbaum, 1977). Plasma folates have responded variably to oral administration of yeast polyglutamates or folic acid. Grossowicz et al. (1975) reported similar increases in plasma folate with doses of folic acid or yeast folate as high as 300 µg, but other investigators reported a greater rise in plasma folates with folic acid than with yeast polyglutamates (Perry and Chanarin, 1970; Spray, 1952; Spray and Witts, 1952). Red cell folate levels also showed a lower response to yeast than to folic acid over a 3-mo period (Perry and Chanarin, 1970).

Bioavailability of food folates has been tested most often by urinary excretion of the vitamin following ingestion of various foods. Retief (1969) compared urinary excretion of folates after ingestion of several foods to urinary excretion following administration of graded amounts of folic acid. This study suggested that
folates in calves' liver, spinach, and peas were well absorbed, while those in tomatoes, pumpkin, and cauliflower were poorly absorbed. Such comparisons between excretion of folates following administration of folic acid and folates in foods may not be reliable because mixtures of reduced folates may not be absorbed and metabolized in the same way as fully oxidized folic acid (Chanarin, 1979a). Tamura and Stokstad (1973) and Babu and Srikanthia (1976) measured urinary excretion of the vitamin in response to administration of a number of test foods. Subjects were made folate-replete by oral administration of folic acid on alternate days. Availability of folates was low in orange juice, romaine lettuce, and egg yolk, but high in bananas and in dried and frozen lima beans. Excretion of tritium-labeled folic acid following a flushing dose of unlabeled folate has also been used as a test method for absorption of folic acid in bread (Anderson et al., 1960). In general, the urinary excretion methods to determine folate bioavailability from foods require consumption of huge amounts of the test foods to ensure detectable urinary losses. These methods are also subject to extreme variability in data collected on the same food.

A few bioavailability studies have utilized intestinal perfusion. Nelson et al. (1975) measured disappearance of folates from orange juice and a folic acid solution using this technique. They reported equal absorption of folate from both sources, a finding which differed from that of Tamura and Stokstad (1973) who reported poor folic acid absorption from orange juice. Later Tamura et al. (1976) indicated that this discrepancy may have been caused by differences in the pH of the administered solutions. Tamura and Stokstad (1973) had measured absorption of orange juice folacin administered as a concentrate at pH 3.7 whereas Nelson et al. (1975) had measured absorption following perfusion at pH 7.0.

Availability of dietary folate has also been measured by animal growth responses. The usual model has been the chick (Jukes, 1955), although a rat growth assay was recently described by Keagy and Oace (1980). The chick growth assay correlates well with total food folates as determined by the L. casei assay but Chanarin (1979a) cautions that avian folate conjugase differs markedly from the mammalian enzyme.

The chick assay has been recently employed to examine dietary fiber effects on folic acid absorption. Cellulose, pectin, bran, lignin, and sodium alginate were found to have no effect on folic acid absorption as indicated by plasma and liver folacin data (Ristow et al., 1981). These results were in agreement with those of recent rat (Keagy and Oace, 1981) and human studies (Keagy et al., 1979), although limited data suggest that certain fiber components may retard the utilization of folate polyglutamates (Keagy et al., 1979). The chick bioassay has yielded results which correlate well with previous human bioavailability data for cabbage folacin, i.e. approximately 40% availability by the chick assay (Ristow, 1981) and approximately 47% availability by the human bioassay (Tamura and Stokstad, 1973).
A potential in vitro bioassay of pteridine substituted monoglutamates, using the survival and growth of primary monolayer cultures of hepatocytes in chemically defined media has been reported by Crane (1980). In this assay 5-formyl tetrahydrofolate supported only 50% as much cell attachment and growth as did folic acid. While these data suggest differences among monoglutamates in supporting important cell functions which may correlate with biological potency, additional studies are needed to determine the value of this approach.
IV. FOLATE STATUS IN THE NORTH AMERICAN POPULATION

A. FACTORS AFFECTING FOLATE STATUS

1. Dietary intake

Accurate estimates of dietary intakes of folates for large numbers of individuals are limited. A WHO study group (Joint FAO/WHO Expert Group, 1970) estimated the per capita free folate intake to be 37-279 μg/d in the United States. Tamura and Stokstad (1981) calculated folate availability to be 227 μg/capita/d, based on the average per capita disappearance of principal U.S. foods. Similarly, Hoppner et al. (1973) estimated that in Canada the intake was 210 μg/d, based on the per capita disappearance of folate in foods. Such estimates are likely to be high since they do not account for food wastage or nutrients lost, for example in home food preparation and by deterioration during storage. It should also be noted that these estimates of per capita folate intake fall short of the RDA for adults.

Ranges of free and total folate intakes determined in several studies are shown in Table 1. In most studies the lower limit of the range is less than the RDA for the group studied (National Research Council, 1980) and in several studies does not reach the minimum dietary requirement for the adult (50 μg/d, as determined by Herbert, 1962a). However, these data yield only crude comparisons. For example, the use of Streptococcus faecalis to determine folate content of the diet or the failure to use ascorbate to protect the folate from oxidation could result in serious underestimates of the dietary folate (Section III. A). Similarly, limitations of the recall procedure for determining dietary intakes are recognized (Pike and Brown, 1975; U.S. Department of Health, Education, and Welfare, 1972), but better methods for large studies have not been developed. The determination of actual folate intake is further complicated because little is known about the bioavailability of the many folate derivatives in foods (see pages 7-10).

Dietary analyses in large-scale nutrition surveys in the United States (Abraham et al., 1979; U.S. Department of Health, Education, and Welfare, 1972) have not included calculation of folacin intakes from dietary recalls of the participants because of uncertainties and incompleteness of the data in food folate tables and because of problems concerning the microbiological assay for folate (see Section VI. A.1). Similarly, the U.S. Department of Agriculture Nationwide Food Consumption Survey (U.S. Department of Agriculture, 1980b) and the Food and Drug Administration (1979) Total Diet Study have omitted analysis of folates.
Table 1. Content of Folate in Various Diets

<table>
<thead>
<tr>
<th>Study Information</th>
<th>Folic Acid, µg/d</th>
<th>Investigators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low- to high-cost food plans, calculated</td>
<td>Free: 11-33</td>
<td>Total: 30-120</td>
</tr>
<tr>
<td>Low- to high-cost food plans, analyzed</td>
<td>15-65</td>
<td>47-193</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 diets, S. faecalis</td>
<td>24-78</td>
<td>80-308</td>
</tr>
<tr>
<td>4 diets, Lactobacillus casei</td>
<td>37-297</td>
<td>379-1,097</td>
</tr>
<tr>
<td>Calculated from tables; diets typical of pregnant clinic population</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young controls</td>
<td>-</td>
<td>161-297</td>
</tr>
<tr>
<td>Free living elderly</td>
<td>-</td>
<td>95-250</td>
</tr>
<tr>
<td>Hospitalized elderly</td>
<td>-</td>
<td>41-190</td>
</tr>
<tr>
<td>L. casei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low folate diets</td>
<td>&lt;5</td>
<td>17-30</td>
</tr>
<tr>
<td>Control diet, L. casei</td>
<td>163-253</td>
<td>332-429</td>
</tr>
<tr>
<td>Pregnant and nonpregnant teenagers, calculated from USDA tables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Home diets, L. casei</td>
<td>63-586</td>
<td>69-601</td>
</tr>
<tr>
<td>4-day dietary record, analyzed</td>
<td>-</td>
<td>92-656</td>
</tr>
<tr>
<td>4-day dietary record, calculated</td>
<td>-</td>
<td>39-138</td>
</tr>
<tr>
<td>7-day dietary record, analyzed</td>
<td>-</td>
<td>183-697</td>
</tr>
<tr>
<td>7-day dietary record, calculated</td>
<td>-</td>
<td>39-224</td>
</tr>
<tr>
<td>High-cost diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-cost diet, L. casei</td>
<td>37-130</td>
<td>1,588-3,156</td>
</tr>
<tr>
<td>Low-Income white adolescent males</td>
<td>-</td>
<td>0-722</td>
</tr>
<tr>
<td>Low-Income white adolescent females</td>
<td>-</td>
<td>4-536</td>
</tr>
<tr>
<td>Low-Income black adolescent males</td>
<td>-</td>
<td>0-529</td>
</tr>
<tr>
<td>Low-Income black adolescent females</td>
<td>-</td>
<td>3-488</td>
</tr>
<tr>
<td>Middle-Income white adolescent females</td>
<td>-</td>
<td>10-167</td>
</tr>
<tr>
<td>Calculated from USDA tables</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The data are not strictly comparable because of differences in methodology and population samples examined. See original references for details.
† Blanks signify that no values were reported.
Analysis of dietary folate intake based on microbiological assay of foods obtainable in Canada (Hoppner et al., 1972) was included in the Nutrition Canada Survey (Health and Welfare Canada, 1973). These calculations indicated mean and range values for the "free folate" intakes in each province, by social groups. The values for the Indian and Eskimo populations in several provinces were considerably below the Canadian Dietary Standard of 200 μg/d of "free folate" for adults (Health and Welfare Canada, 1975a,b). These calculated low intakes were in accord with the low mean serum folate concentrations found in much of the general population (12% in the high-risk category, <2.5 ng/ml; and 40% in the moderate-risk category, 2.5-5.0 ng/ml) (Health and Welfare Canada, 1975a,b). However, analysis of folate concentrations in 560 postmortem liver samples from Canadian subjects failed to show evidence of folate deficiency (Hoppner and Lampi, 1980). Based on the concentrations in the livers of 90 of the subjects who died accidentally, the authors concluded that less than 10% of Canadians have low folate reserves. In addition, only in 9.9% of a group of Canadian blood donors was serum folate in the range associated with folate deficiency (Cooper, 1978).

Food intakes were recorded of pregnant adolescents and mature women (Bailey et al., 1980), elderly subjects (Bailey et al., 1979; Wagner et al., 1981), and adolescents (Bailey et al., 1981a,b) participating in nutrition surveys in Florida, but folate intakes have not been calculated yet from these dietary recalls. Biochemical findings (serum and red cell folate levels) in these studies indicate that folate status may be compromised in significant portions of these groups. Low dietary folate would seem to be the most likely cause, although other factors, as noted below, cannot be ruled out.

2. Other factors

Factors other than inadequate dietary intake may influence the folate status of individuals. Herbert et al. (1980) discuss five additional factors that may affect folate status: inadequate absorption, impaired metabolism, increased requirement, increased body losses, and increased destruction of the vitamin. In addition, certain clinical conditions that may influence folate status are reviewed in Section IV. B.2.

a. Inadequate absorption. Aspects of folate bioavailability and absorption were noted previously (see Section III. C). However, inadequate absorption may be related to factors disrupting the absorptive process. For example, malabsorption may be induced by substances inhibiting the cleavage of folate polyglutamates by an intestinal conjugase or decreasing transport of folate from the intestinal lumen into the portal circulation. As mentioned before, acidic conditions in the small intestine (Tamura et al., 1976) and intestinal conjugase inhibitors in foods such as yeast (Swendseid et al., 1947) and pulses (Krumdieck et al., 1973)
may limit the amount of folate monoglutamate available for absorption. Such factors in foods may impair absorption of folates added to foods (Colman et al., 1975a) as well as endogenous folates. Preliminary data suggest that the intestinal conjugase is zinc-dependent in man (Tamura et al., 1978) and compromised zinc status in the elderly has been associated with low serum and red cell folate levels (Wagner et al., 1980). Alcoholics who are already nutritionally depleted demonstrate impaired folate absorption (Halsted et al., 1971), but the mechanism is unclear. Use of drugs such as diphenylhydantoin (Chanarin, 1980; Gerson et al., 1972; Rosenberg et al., 1979), sulfasalazine (Franklin and Rosenberg, 1973; Reisenauer and Halsted, 1981; Rosenberg and Dyer, 1979), and barbiturates has also been shown to impair folate absorption. Absorption of folate, as well as uptake of other nutrients, is adversely affected by diseases of the gastrointestinal tract including tropical sprue, celiac disease, Crohn's disease, and ulcerative colitis (Carcino et al., 1976; Franklin and Rosenberg, 1973; Halsted et al., 1977). (See also pages 22-25 for additional information on illnesses and drugs associated with folate deficiency).

An autosomal recessive inborn error of metabolism resulting in folate malabsorption and consequent central nervous system disorders has been described (Luhby and Cooperman, 1967; Rosenblatt and Erbe, 1972).

b. Impaired metabolism. Imbalance in the proportion of folate coenzymes may result from defective interconversions of folates. Maintenance of specific proportions of folate coenzymes has been suggested as a critical factor in the control of folate metabolism. Central to the metabolism of folic acid is the vitamin B₁₂-dependent conversion of 5-methyl tetrahydrofolate to unsubstituted tetrahydrofolate and hence to other coenzyme forms. In vitamin B₁₂-deficient animals, blocking of this pathway results in methyl folate accumulation, the "methyl folate trap" (Herbert and Zalusky, 1962; Noronha and Silverman, 1962), which is reversed upon oral administration of vitamin B₁₂ (Metz et al., 1968; Smith and Osborne-White, 1973; Thenen and Stokstad, 1973).

Folate metabolism is also impaired by use of a number of drugs. In particular, dihydrofolate reductase inhibitors such as methotrexate, aminopterin, and others (Herbert et al., 1980) have been associated with development of megaloblastic anemia. Cyclic changes in levels of female steroid hormones have been reported to affect the activity of uterine folate conjugase and the concentration of serum folate in experimental animals (Boots et al., 1975; Krumdieck et al., 1976). Such effects may partially explain the occurrence of megaloblastic anemia in some users of oral contraceptives (see also page 24). Use of alcohol has also been implicated in impaired folate utilization, possibly by disrupting the enterohepatic cycle of folate metabolism (Steinberg et al., 1979). As with impaired absorption, genetic anomalies of metabolism involving folate-dependent enzymes may illustrate the consequences
of a total lack of folate at specific metabolic sites. Congenital deficiencies of four folate-dependent enzymes have been identified and studied (Arakawa et al., 1965; Cooper, 1976; Erbe, 1975; Rowe, 1978).

c. Increased requirement. One of the most common causes of folate deficiency, or risk of deficiency, is an increased requirement for the vitamin. This condition has been described during periods of increased cellular proliferation (and therefore nucleic acid synthesis) such as pregnancy (Rothman, 1970) and in premature infants (Dallman, 1974; Hoffbrand, 1970; Strelling et al., 1979). Similarly, conditions of increased metabolic activity including neoplastic disease (Butterworth, 1980; Butterworth et al., 1980) and hyperthyroidism (Herbert et al., 1980) increase the requirement for folate. Folate-deficient animals demonstrate impaired immune response to experimental infections which may indicate an increased requirement during infections (Beisel et al., 1981). Rowe et al. (1979) have described a striking increase in the activity of eight folate-related enzymes in human lymphocytes following blast transformation, associated with a marked increase in cellular uptake of folate. Blast transformation and cellular survival were impaired in cultures having a limited supply of folate in the medium. Beard et al. (1980) observed the acute onset of folate depletion in bone marrow cells using the deoxyuridine suppression test in a series of patients following massive blood loss and surgery. They postulated the existence of a localized folate deficiency in these subjects because of increased demand that could not be met adequately by tissue reserves and transport systems.

d. Increased body losses. Folate deficiency may occur when body losses of the vitamin are increased. Lactation lowers the maternal folate supply and can prevent repletion of reserves following pregnancy (Metz, 1970). Colman et al. (1975c) indicated that effects of such losses of maternal reserves in rural, South African black women consuming diets low in folate were prolonged, tending to persist throughout the child-bearing years. Significant urinary losses of folates were reported in patients with liver disease (Tamura and Stokstad, 1977) and those undergoing kidney dialysis (Svitt and Hoffbrand, 1969; Whitehead et al., 1968). Increased levels of folate in bile were reported in pernicious anemia (Herbert, 1965) but it was not determined whether this folate was excreted or recycled through the enterohepatic circulation. The enterohepatic cycle of folates appears to be a major pathway for attaining appropriate balance among the various coenzyme forms of the vitamin (Steinberg et al., 1979). Additional folate is also necessary to compensate for continuous losses associated with increased turnover in various forms of hemolytic anemia.

e. Increased destruction. Folates are susceptible to oxidation, which, if excessive, may lead to deficiency. Some investigators suggest that megaloblastic anemia observed in scurbutic patients may be caused by insufficient ascorbate to protect
folate from oxidation (Asquith et al., 1967). However, other work suggests that the megaloblastic anemia in these patients is due to a folate deficiency because scorbutic diets contain almost no folate (Zalusky and Herbert, 1961). Effects of other antioxidants on folate metabolism have not been examined.

An animal (mouse) model for assessing folate catabolism has recently been developed. Use of this model has shown that hydantoins accelerate folate catabolism whereas alcohol and barbiturates do not influence this aspect of folate metabolism (Kelly et al., 1979, 1981).

B. EVIDENCE OF COMPROMISED STATUS

1. Epidemiological studies

Herbert (1972) estimated that up to one-third of all pregnant women in the world experience folate deficiency. Chanarin (1979b) ranked folate deficiency with iron deficiency as one of the common nutritional deficiencies seen in clinical practice. The difference between frank folate deficiency and being at risk of deficiency should be recognized. Few cases of frank folate deficiency, as defined by morphologic changes in blood and bone marrow, have been documented in the general population. However, in some populations such as hospitalized patients in London (Hoffbrand et al., 1966), folate deficiency manifesting macroovalocytosis and megaloblastic bone marrow is reportedly not uncommon.

Although definitions vary, compromised folate status, in terms of blood values, is generally expressed as a serum folate concentration of less than 6 ng/ml and a red cell concentration of less than 160 ng/ml; and folate deficiency or high risk of deficiency, as a serum value of less than 3 ng/ml and a red cell value of less than 140 ng/ml (Sauberlich et al., 1974). It is recognized that some laboratories define normal ranges quite differently from these values.

It is noteworthy that large-scale surveys using serum folate determinations identify percentages of tested populations who may be at risk of folate deficiency. When red cell determinations are included, better estimates of folate status are possible because red blood cell folate represents liver stores more closely than serum folate values (Wu et al., 1975). However, a firm diagnosis of folate deficiency in an individual may require additional laboratory and clinical procedures (see pages 28-32) that are beyond the scope of large surveys. For instance, certain hematologic procedures and measures of iron status appear practical for groups of moderate size such as the 193 elderly subjects studied by Bailey et al. (1979).
The prevalence of folic acid deficiency and associated megaloblastic anemia, on a global basis, has probably been underestimated; among pregnant women in developed countries, the estimated frequency of megaloblastic anemia resulting from folate deficiency has ranged from 2.5 to 5%, with a higher prevalence among pregnant women in developing countries (Sauberlich, 1977). Colman et al. (1974a) reported that megaloblastic anemia of pregnancy is common among South African blacks and listed economic status, adverse diet, poverty, and prolonged breast feeding as contributory factors. In the same population source, about 45% of women 16–40 years old and about one-third of persons older than 60 years had low red cell folate levels (Colman et al., 1975c); approximately 18% of males 16–60 years old and women 40–60 years old had red cell folate levels below 160 ng/ml. The entire increase in prevalence of folate deficiency among women 16–40 years old was attributable to effects of pregnancy and lactation and thus would be more important in populations with high birth rates. Other examples of folate deficiency in developed countries presumably resulting from inadequate dietary intake involved primarily the elderly, but also younger persons at risk because of pregnancy or illness (Anonymous, 1968; Hurdle and Williams, 1966; Read et al., 1965; Varadi and Elwis, 1964, 1966).

Limited data suggest that folate status is suboptimal in certain population groups in the United States such as adolescents, poorly nourished pregnant women, the elderly, and users of such commonly prescribed drugs as oral contraceptives, anticonvulsants, and sulfasalazine (Bailey et al., 1979, 1981a; Chai et al., 1980; Giles, 1966; Herbert et al., 1975; Lawrence and Klipstein, 1967). However, data from the Health and Nutrition Examination Survey II (HANES II)* should provide more accurate information on the frequency of marginal and deficient folate status in segments of the population. If data from HANES II and other sources, including a 1981 FDA telephone survey of nutrient supplement intakes, support the observations of earlier surveys with respect to folate nutrition, the aggregate evidence may indicate that compromised folate status is sufficiently widespread to constitute a public health problem. Folate deficiencies as measured by serum and red cell folate levels have been identified in several population groups in the United States. However, most subjects with serum folate levels in the range defined as deficient show no clinical manifestations, and folate deficiency has not been clearly identified as a medical problem in the general population.

Attempts to correlate folate status of pregnant women in Canada and England and perinatal morbidity or mortality have been unsuccessful. Delivery and health of infants and mothers were identical in patients without evidence of folate deficiency and

* These data will be available in late 1981 or early 1982.
in those with mild megaloblastic changes in bone marrow, without anemia. Thus, although it is prudent to prevent such deficiency in pregnant women in Western populations, its occurrence has not been shown to affect the health of either infant or mother (Cooper et al., 1970).

Folate status of a variety of population groups of different sizes, ages, locations, ethnic origins, and economic status is presented in Table 2. The folate status in most of these studies was based upon serum folate values; however, both serum and red cell values were obtained in the Ten-State Nutrition Survey (U.S. Department of Health, Education, and Welfare, 1972). Bailey et al. (1979, 1980, 1981b) added hematologic parameters, iron, and vitamin B₁₂ status to aid in evaluating the frequency and degree of compromised folate status (Table 3). Additional information on the prevalence of compromised folate status is presented on page 13.

Attempts to reach firm conclusions on the folate status of populations from mean values of serum and red cell folate levels such as reported in the Ten-State Nutrition Survey (U.S. Department of Health, Education and Welfare, 1972), and the Nutrition Canada Survey (Health and Welfare Canada, 1973), have been criticized (Butterworth, 1977; Herbert et al., 1975; Thompson and Hoppner, 1979). For example, the Ten-State Nutrition Survey suggested that folate nutriture in the tested population was adequate based on the mean serum and red cell data. However, Herbert et al. (1975) cautioned that "mean values may obscure the existence of a substantial number of actual values which are sufficiently below the mean as to suggest widespread folic acid deficiency", and noted that the Massachusetts section of the sample yielded an incidence of folate deficiency of 25% among pregnant women.

Survey data are accumulating that suggest compromised folate status may exist in several susceptible population groups. Bailey et al. (1979, 1981a,b) and Wagner et al. (1981) reported a high prevalence of folacin deficiency as defined by subnormal serum and red cell folate levels in adolescents and elderly persons from urban and rural, low-income households in Florida. The iron status of the elderly subjects was normal; however, transferrin saturation was less than normal (<20% M, <15% F) in 10-32% of the people in adolescent groups. The prevalence of anemia was variable, depending on the norms used; however, it was evident in all groups of adolescents with the exception of the Spanish-American adolescents (Table 3). Inadequate dietary folate was considered a key etiologic factor in these cases of folate deficiency.

An earlier study of adolescents from low-, medium-, and high-income families (total subjects, 459) showed that 9.4% of the boys and 4.7% of the girls from low-income families had plasma folate levels below 2 ng/ml (Daniel et al., 1975). As the adolescents approached sexual maturity, folate status tended to decline.
<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Frequency of Compromised Status</th>
<th>Comment</th>
<th>Source and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,900</td>
<td>15% white (n=1550) 30% black (n=1580) 30% Spanish-American (n=432)</td>
<td>With few exceptions, the mean values were: serum &gt;6, red cell &gt;300 ng/ml</td>
<td>Sauberlich, 1977; U.S. DHEW, 1972 (from Ten-State Nutrition Survey)</td>
</tr>
<tr>
<td>12,795%</td>
<td>~ 25%</td>
<td>60% elderly at risk of folate deficiency; liver folate in 560 autopsy cases showed little evidence of severe folate deficiency</td>
<td>Health and Welfare Canada, 1973 (from Nutrition Canada: National Survey); Hoppen and Lampi, 1980; Thompson and Hoppen, 1979</td>
</tr>
<tr>
<td>1,087 F</td>
<td>25.6%</td>
<td>Included 115 pregnant subjects</td>
<td>Edozien, 1972 (from Ten-State Nutrition Survey)</td>
</tr>
<tr>
<td>459 (202 M; 257 F)</td>
<td>9.4% M; 4.7% F</td>
<td>deficient subjects were from low-income families; sexual maturation associated with low folate</td>
<td>Healthy adolescents from low-, medium-, high-income families; Daniel et al., 1975</td>
</tr>
<tr>
<td>362** (UB--161; USA--32; RB--58; RW--111)</td>
<td>UB--42% USA--52% RB--63% RW--45%</td>
<td>(see Table 3 for anemia and iron status)</td>
<td>Adolescents from urban and low-income households; Bailey et al., 1981a,b</td>
</tr>
<tr>
<td>300 M and F up to 6 yr</td>
<td>~ 10% (serum folate); 1% (red cell folate)</td>
<td>common were: retarded stature; dental caries; upper respiratory, epidermal, conjunctival infections</td>
<td>Mexican-American children of migrant farm workers; Colorado; Chase et al., 1971</td>
</tr>
</tbody>
</table>
Table 2. (continued)

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Frequency of Compromised Status</th>
<th>Comment</th>
<th>Source and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deficient* 26% (serum values)</td>
<td>Serum iron &lt;50 g/dl in 8%; transferrin sat. &lt;16% in 6%; hemoglobin &lt;13 g/dl in 14%</td>
<td>White subjects &gt;59 yr old, living in Missouri; Chal et al., 1980</td>
</tr>
<tr>
<td>200</td>
<td>Marginal† 3% (red cell values)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified 3% (red cell values)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>193 (137 F; 56 M)</td>
<td>60% 11%</td>
<td>Iron status normal, all subjects; 14% had anemia</td>
<td>Elderly persons from urban, low-income households; Bailey et al., 1979</td>
</tr>
<tr>
<td>106 (77 black; 29 white)</td>
<td>12% black M; 28% black M; 20% black F; 35% black F</td>
<td>Instances of borderline anemia; no sign of effect on hematopoiesis</td>
<td>Apparently healthy adults; migrant farm workers, hospital/clinic personnel; Hall et al., 1975</td>
</tr>
<tr>
<td>97 (F)</td>
<td>unspecified % below acceptable values</td>
<td>Mean serum folate 8 ng/ml; range 2.4 to 16.9 ng/ml</td>
<td>Healthy 9-yr old black and white girls from low- and high-income families; Riester and Wa llen, 1975</td>
</tr>
<tr>
<td>94 (black and white F)</td>
<td>30% (serum values); 11% white, 3% black (serum and red cell)</td>
<td>Estimated 82% ingested 2/3 or less of RDA for folate; 30% &lt;1/3 RDA</td>
<td>Age: 14 ± 0.5 yr; Dawson and Disney, 1981</td>
</tr>
</tbody>
</table>

* Serum folate <3 ng/ml; red cell folate <140 ng/ml.
† Serum folate 3.0-5.9 ng/ml; red cell folate 150-159 ng/ml.
‡ Expressed as being below acceptable levels.
†† Selected persons from the 10 provinces, excluding Inians and Eskimos.
** UB—urban blacks; USA—urban Spanish Americans; RB—rural blacks; RW—rural whites.
Table 3. Prevalence of Anemia and Subnormal Iron Status in Urban and Rural Adolescents*

<table>
<thead>
<tr>
<th>Criterion for low value</th>
<th>Urban black n=161</th>
<th>Urban Spanish-American n=32</th>
<th>Rural black n=58</th>
<th>Rural white n=111</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dl</td>
<td></td>
<td>14%</td>
<td>19%</td>
<td>5%</td>
</tr>
<tr>
<td>M &lt;13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F &lt;11.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration, %</td>
<td>&lt;32</td>
<td>19%</td>
<td>0</td>
<td>15%</td>
</tr>
<tr>
<td>Mean cell volume, fl</td>
<td>&lt;81</td>
<td>18%</td>
<td>13%</td>
<td>34%</td>
</tr>
<tr>
<td>Red blood cells X 10^6</td>
<td>&lt;4.7</td>
<td>19%</td>
<td>0</td>
<td>13%</td>
</tr>
<tr>
<td>Serum iron, μg/dl</td>
<td>M &lt;60</td>
<td>5%</td>
<td>0</td>
<td>10%</td>
</tr>
<tr>
<td>F &lt;40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>M &lt;20</td>
<td>10%</td>
<td>13%</td>
<td>32%</td>
</tr>
<tr>
<td>F &lt;15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Modified from Bailey et al., 1981b. Folate values of the subjects of these studies are presented in Table 2.
In a group of 97 apparently healthy, 9-year-old girls from low- and medium-income families, mean serum folic acid was reported as 8 ng/ml, with a range of 2.4-16.9 ng/ml (Riester and Waslien, 1975). Economic status appeared to have no influence on folate status in these subjects. Approximately 10% of 300 young children of Mexican-American migrant farm workers in Colorado had subnormal serum folacin levels (Chase et al., 1971) and a racially mixed group of migrant farm workers, clinic and hospital personnel in the State of New York showed serum folate values of <3 ng/ml in 12% of the black men and 30% of the black women (Hall et al., 1975). The authors noted "some instances" of borderline anemia, but no evidence of disturbed hematopoiesis associated with the low folate values.

Investigations in Germany that are of interest from an epidemiologic standpoint concern the relationship between human serum and red cell folate concentrations and the degree of neutrophil segmentation in ostensibly normal subjects. Pietrzik et al. (1978), using serum folate concentrations of >4.5 ng/ml and the number of neutrophil segments <3.30 as optimal values, reported 71% of 130 student volunteers had adequate folate status, 13% were marginal (3.5-4.5 ng/ml and 3.30-3.65 segments/neutrophil), and 11% were deficient (<3.5 ng/ml and >3.65 segments/neutrophil). In an extension of these studies, Urban (1980) measured serum and red cell folate concentrations, urinary formiminoglutamic acid excretion, blood cell differential count, and neutrophil segmentation in 420 industrial workers. She reported folate status as normal in 40%, marginal in 45%, and deficient in 15%. In this study, red cell folate concentrations <250 ng/ml corresponded with increased neutrophil segmentation.

2. **Clinical studies**

Rosenberg and Dyer (1979) noted that four target populations are at risk of developing folate deficiency: "... pregnant women; patients with gastrointestinal diseases, particularly those on restrictive diets or with malabsorption; alcoholics; chronic users of drugs such as anticonvulsants and sulfa salazine." To these should be added lactating women, premature infants, persons with hemolytic anemias, and persons undergoing dialysis.

a. **Pregnant women.** Lack of proper antenatal care, even in developed countries, has been identified as a major factor in the genesis of folate deficiency. For instance, one study indicated that nearly half the women who bore children in municipal hospitals in New York City had no antenatal care (Schoeneck, 1964). Even when prophylactic care is provided, the data in one British study suggested extremely low compliance (Bonnar et al., 1969). Herbert et al. (1975) reported that 18 of 110 women, mostly black or Puerto Rican, from low-income families had red cell folate levels below 150 ng/ml at the time of their first visit to the antenatal clinic; an additional 15 had red cell folate levels
between 150 and 199 ng/ml, which was regarded as suggestive, but not conclusive evidence of tissue folate depletion. Serum folate levels correlated well with depressed red cell levels; however, low serum levels were also observed in the presence of normal red cell levels. The authors noted a frequent association between poverty and compromised folate status and suggested that the diets of their patients contained insufficient green, leafy vegetables and that their foods tended to be overcooked, depleting the available folate.

Bailey et al. (1980) reported that 29% of a group of 269 low-income pregnant women had red cell folacin levels <140 ng/ml. An additional 11% had values ranging from 140-160 ng/ml. Sixty-two percent of this group were in their second trimester of pregnancy at the time of their initial prenatal visit, which illustrates the common practice of delaying prenatal care, including routine folacin supplementation.

b. Illnesses associated with folate deficiency.
Alcohol abusers are estimated to exceed ten million in the United States (Rosenberg and Dyer, 1979), and, according to Halsted (1980), chronic alcoholism is probably the leading cause of folate deficiency in this country. Folate malabsorption apparently occurs in alcoholics who have the intestinal abnormalities associated with alcoholism, undernutrition, and folate deficiency (Rosenberg et al., 1979). The etiology of folate deficiency in chronic alcoholism is complex and appears to be multifactorial. Persons diagnosed as being alcoholics, but who otherwise consume an adequate diet, are not likely to become folate-deficient. However, the "dedicated" alcoholic ingesting a diet lacking sufficient folate-containing foods for approximately one month would be expected to become folate-deficient (Herbert et al., 1963). Halsted (1980) estimates that about 40% of derelict alcoholics are likely to have megaloblastosis, with higher percentages manifesting folate deficiency, based on serum and red cell concentrations.

The malabsorption syndrome of gluten-sensitive enteropathy is commonly associated with folic acid deficiency. Patients with Crohn's disease and ulcerative colitis who are treated with the preferred drug, sulfasalazine, frequently develop folate deficiency. The incidence of Crohn's disease in the United States in the mid-1970s was two to three cases per 100,000 (Glickman and Isselbacher, 1980); of ulcerative colitis, about 10 per 100,000 in white adults (LaMont and Isselbacher, 1980). The population of the United States in 1975 was approximately 215 million. Rosenberg and Dyer (1979) reported that two-thirds of their patients with Crohn's disease and ulcerative colitis developed folate deficiency, with megaloblastic changes occurring in 10%.

It has been estimated that at least one million persons in this country have recurrent seizures, requiring anticonvulsant therapy (Salam-Adams and Adams, 1980). Anticonvulsants such as diphenylhydantoin, primidone, and phenobarbital interfere with
folate metabolism so that low serum, red cell, and cerebrospinal fluid values commonly accompany chronic anticonvulsant therapy (Smith and Obbens, 1979). Reynolds (1973) estimated that folate-responsive megaloblastic anemia occurred in about 0.75% of epileptic patients treated with anticonvulsants while low serum folate levels were thought to be present in about 50% of such patients.

c. Use of oral contraceptives. Women using birth control pills constitute a substantial percentage of the population of women of child bearing age in this country (Mishell, 1977). Folate deficiency has been observed among women taking such substances. However, Paine et al. (1975) indicated no difference in folate status between a group of women taking oral contraceptives and a matched control group not taking the medication. Preparations low in estrogen (≤0.05 mg) may be less likely to influence folate metabolism adversely than those containing larger amounts of estrogens, but this has not been shown. While some evidence suggests that use of oral contraceptives interferes with folate metabolism and leads to folate deficiency, the question is unsettled.

Alter et al. (1971) reported that long-term aspirin therapy in patients with rheumatoid arthritis was associated with low serum folate levels and rapid plasma clearance of tritium-labeled pteroylglutamic acid. The results of this study suggested that low serum values represented aspirin-induced alterations in folic acid binding to protein rather than deficiency. Chanarin (1980) described the apparent effect of aspirin (and a variety of other drugs) as coincidental and not causally related in some reported cases of megaloblastic anemia.

C. EVIDENCE OF EXCESSIVE INTAKES

There is little documented or circumstantial evidence of excessive folate intake from food sources. A few reports claim that large amounts of folic acid taken as supplements may produce adverse effects but little is known about possible subclinical effects of long-term, high dietary intakes of the vitamin. Intravenous LD<sub>50</sub> doses for folic acid were 600 mg/kg in mice, 500 mg/kg in rats, 410 mg/kg in rabbits, and 120 mg/kg in guinea pigs (Harned et al., 1946). For comparison, the RDA is approximately 5.7 μg/kg for a 70 kg adult man. Acute renal failure resulting from tubular obstruction by precipitated folate has been reported in animals (Schmidt and Dubach, 1976; Schubert, 1976). Sensitivity to folic acid has been reported with oral doses as large as 20 mg in man (Chanarin et al., 1957; Mathur, 1966; Mitchell et al., 1949; Woodliff and Davis, 1966). Signs and symptoms included a generalized pruritic erythematous rash, malaise, and bronchospasm. Intradermal tests for hypersensitivity were positive to folic acid but negative to 10-formyl tetrahydrofolate (Chanarin et al., 1957). In an uncontrolled study (Hunter et al., 1970), a majority of 14 healthy
subjects given pharmacological doses (15 mg/d) of folic acid for 1 mo had sleep disturbances, gastrointestinal disturbances and/or behavioral changes which Chanarin (1979a) believed were probably psychological in nature. Double-blind controlled studies (Hellström, 1971; Richens, 1971) failed to confirm these findings of Hunter et al. (1970), and it has been pointed out that large numbers of patients have received such doses of folic acid without manifesting the effects described by Hunter et al. (1970) (Grant and Stores, 1970; Jensen and Olesen, 1970; Mattson et al., 1973; Ralston et al., 1970).

Long-term supplementation with folic acid of epileptic patients being treated with anticonvulsants reportedly increased frequency of fits in some isolated cases (Chanarin et al., 1960; Reynolds, 1973; Smith and Obbens, 1979) but controlled studies have failed to demonstrate a consistent decrement of seizure control among folate-supplemented, drug-treated epileptic patients (Bowe et al., 1971; Houben et al., 1971; Jensen and Olesen, 1970; Mattson et al., 1973; Norris and Pratt, 1971; Spaans, 1970). A subsequent study (Ch'ien et al., 1975) of electroencephalographic responses to large (75 mg) intravenous infusions of folic acid in eight epileptic patients treated with anticonvulsants precipitated a tonic-clonic seizure in one patient and electroencephalographic abnormalities in another. A marked variability among the patients' responses was indicated by these studies. It seems improbable that oral folate administration will cause such severe adverse effects. However, folate supplementation of drug-treated epileptic patients should be undertaken with caution.

Large doses of folic acid mask the development of pernicious anemia, thereby delaying diagnosis of the disease and allowing neurological degeneration to progress. Early studies on treatment of pernicious anemia with large amounts of folic acid (0.5 to 50 mg/d) for varying periods (10 d to 10 yr) indicated that hematologic changes were temporarily reversed while neurologic manifestations continued to develop (Marshall and Jandl, 1960; Schwartz et al., 1950; Vilter et al., 1947; Will et al., 1959). These investigators noted no other adverse effects of such large doses of folic acid. The smallest oral dosage of folic acid causing a hematologic response in one pernicious anemia patient was 400 μg/d (Herbert, 1963). While doses of folic acid of 400 μg/d to some individuals might mask pernicious anemia, it is unknown whether long-term consumption of this level in foods would have a similar effect. Absorption studies have shown that food folates are less well absorbed than oral doses of pure folic acid (see Section III. C). However, the question of effects in man of long-term, low level folic acid supplementation of foods cannot be resolved experimentally because of ethical constraints.
V. IDENTIFICATION OF COMPROMISED FOLATE STATUS

Experts differentiate between individuals with frank folate deficiency and those at risk of folate deficiency whose marginal stores of folate may be depleted by intervening events such as pregnancy, poverty-induced undernutrition, alcoholism, and development of malabsorption disorders. Folate deficiency is not necessarily accompanied by overt clinical manifestations.

While several possible definitions of compromised folate status exist, commonly accepted biochemical criteria, as noted on page 16, are: serum folate <3 ng/ml and red blood cell folate <140 ng/ml to denote frank deficiency with a high risk of developing megaloblastic anemia, and serum folate of 3.0–5.9 ng/ml and red cell folate of 150–159 ng/ml to indicate low or marginal folate status (Christakis, 1973; Herbert, 1975, 1977; Sauberlich et al., 1974). Compromised folate status signaled by these blood folate values may be confirmed by other procedures (see pages 28–32). In addition, the use of proper clinical protocols will assure follow-up to detect or rule out possible underlying causes or complicating factors such as pernicious anemia, mineral and vitamin imbalances, malabsorption syndromes, malignancies, and drug interactions. Authoritative papers on these subjects include those of Babior and Bunn (1980); Chanarin (1979a,b; 1980); Colman (1977); Herbert (1975); Hoffbrand (1976); National Research Council (1977); and Shojaiania (1980).

An unknown proportion of the population has marginal or subclinical folate status, is at medium risk of developing folate deficiency, and may represent a significant public health problem. Of special importance in this otherwise healthy population are those with more than average susceptibility to development of compromised folate status including pregnant and lactating women, particularly those from low-income households, low-birth-weight infants, adolescents, and elderly persons. Thus, the detection of compromised folate status from the point of view of epidemiology via health and nutrition surveys is of importance. Whether available methods of assessment are sufficiently accurate to detect marginal folate status in large-scale health and nutrition surveys is an open question (see pages 16–18). In addition, an understanding of the causes and effects of compromised folate status requires consideration of the clinical disorders associated with altered folate nutrition and of the related diagnostic methods.

A. BIOCHEMICAL INDICATORS

Because of difficulties in diagnosing folic acid deficiency solely by clinical means and the limited usefulness of available methods of estimating dietary intakes as well as bioavailability of food folates, health and nutrition surveys have
relied primarily on serum and red blood cell folate assays for estimating folate status (Sauberlich, 1977) (see Section VI). Ancillary biochemical procedures useful in limited surveys, which may aid in detecting and differentiating compromised folate status from other altered states, include blood hemoglobin, serum iron, total iron binding capacity, and serum vitamin B\textsubscript{12} determinations (Christakis, 1973).

Folate deficiency is sometimes masked by concomitant nutrient imbalances, metabolic derangements, or diseases that influence nutriment; thus, differential diagnosis of folate deficiency may prove difficult. Under such circumstances, aids to diagnosis of folate deficiency include such clinical biochemical determinations as iron status and serum vitamin B\textsubscript{12} as well as the clinical protocols described below. Additional tests for confirming folate deficiency and its effects include the urinary formiminoglutamate (FIGLU) excretion test and the in vitro deoxyuridine (dU) suppression test using bone marrow cells or transformed lymphocytes.

The appearance of FIGLU in the urine after an oral histidine load occurs in folate deficiency as well as in vitamin B\textsubscript{12} deficiency and in liver disease (Herbert, 1975) (see also page 45). The dU suppression test is considered useful in detecting folate deficiency (Colman and Herbert, 1980). A defect in the dU suppression of incorporation of radiolabeled thymidine into DNA by bone marrow cells or transformed lymphocytes is said to be diagnostic of folate deficiency if this suppression can be corrected by in vitro folate supplementation (Herbert, 1975; Metz et al., 1968; Waxman et al., 1969) (see also page 44). Another aid to differential diagnosis is elevated urinary excretion of methylmalonic acid, with or without oral loading with valine or isoleucine, which occurs in vitamin B\textsubscript{12} deficiency, but not in folate deficiency (Carmel, 1978).

B. CLINICAL INDICATORS

According to Carmel (1978), the diagnosis of megaloblastic anemia, one of the eventual manifestations of folate deficiency, can usually be established by blood count and blood cell morphology. Suspicion of compromised folate status is likely to arise from a traditional diagnostic approach; that is, a careful history, physical examination, and routine laboratory procedures including hemoglobin estimation, complete blood count, and examination of a blood smear. The Coulter Counter has proved to be highly sensitive for measuring mean cell volume (MCV). From these procedures, the presence may be established of macrocytosis and hypersegmented polymorphonuclear neutrophils, and in megaloblastic anemia, of pancytopenia. The foregoing findings should suggest the need for serum and red cell folate and serum vitamin B\textsubscript{12} determinations for the biochemical confirmation of folate deficiency.
Positive findings resulting from the foregoing tests and procedures should lead to further questions regarding possible inadequate dietary folate as well as a history of manifestations of folate deficiency, of the use of medicinal drugs, or of gastrointestinal disorders, and a more detailed physical examination to elicit additional signs of folate deficiency and megaloblastic anemia. Signs and symptoms which may aid in the diagnosis include:

- excessive fatigability
- anorexia
tiredness
- glossitis
- loss of energy and drive
- recurrent aphthous ulcers
- weakness
- pallor of skin and mucous membranes
- insomnia
- membranes

Establishing folate deficiency as the cause of megaloblastic anemia requires the exclusion of vitamin B₁₂ deficiency by finding normal serum B₁₂ values or by demonstrating normal B₁₂ absorption (Chanarin, 1980). Other valuable confirmatory tests are an elevated FIGLU excretion in the urine and a positive hematologic response to daily therapeutic doses of folate.

Whether folate deficiency, per se, results in neurologic or psychiatric disorders is controversial (Colman, 1977; Colman and Herbert, 1977; Reynolds, 1976). Nevertheless, evidence is increasing that folate deficiency may, indeed, affect the nervous system (Anonymous, 1981; Botez and Bachevalier, 1981; Chanarin, 1980). Botez et al. (1979) recommend a careful neurologic evaluation in suspected folate deficiency to elicit a history of forgetfulness, irritability, insomnia, the restless legs and the burning feet syndromes, and to detect locomotor ataxia, diminished vibration sense, and weakness or minor paralysis of the lower extremities. After evaluating 84 consecutive cases of megaloblastic anemia, Shorvon et al. (1980) reported that approximately one-third of both folate- and vitamin B₁₂-deficient patients had no neurologic manifestations; affective disorders were more frequent in folate- compared with vitamin B₁₂-deficiency, and subacute combined degeneration of the spinal cord and optic atrophy were found only in the vitamin B₁₂-deficient subjects. Peripheral neuropathy was a feature of folate deficiency, but was much more frequent in vitamin B₁₂ deficiency.

Diagnosis of folate deficiency may be complicated by such factors as iron, zinc, or vitamin B₁₂ deficiency, malabsorption, and drug interference. Table 4 lists some factors that may influence interpretation of laboratory results and clinical recognition of folate deficiency.

A practical clinical approach for evaluating folate status that does not require excessive testing would include a dietary history (including cooking methods), mean red cell volume, blood smear, and hemoglobin estimation. If these procedures identify or suggest megaloblastic anemia, the serum and red cell folate values should be obtained. However, some authorities consider that blood
<table>
<thead>
<tr>
<th>False positives/negatives</th>
<th>Applies to some degree to all test methods</th>
<th>Example: spuriously elevated serum folate from non-fasting or hemolyzed sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faulty Interpretation</td>
<td>Low serum folate may mean recent dietary Insufficiency, not folate deficiency</td>
<td></td>
</tr>
<tr>
<td>Hemolytic anemias</td>
<td>Microcytosis may confuse blood picture; megaloblastic marrow increases folate requirement</td>
<td>Example: sickle cell anemia</td>
</tr>
<tr>
<td>Alcoholism</td>
<td>May cause: acute decrease in serum folate, megaloblastic marrow, macrocytosis with increased MCV, and may increase folate requirement</td>
<td>Common</td>
</tr>
<tr>
<td>Imbalance or deficiency of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>Inhibits methyl folate uptake by cells; RBC folate uptake may be B₁₂-dependent</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>In scurvy, bleeding reduces folate stores</td>
<td>Severe undernutrition</td>
</tr>
<tr>
<td>Iron</td>
<td>Deficiency causes microcytosis; needed for ribonucleotide reductase (cytidylic to deoxycytidylic acid); Iron deficiency may induce megaloblastosis and/or neutrophil hypersegmentation</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>May be needed in intestinal folate conjugase; low zinc status associated with compromised folate status</td>
<td></td>
</tr>
<tr>
<td>Physiologic changes in blood during pregnancy</td>
<td>Increased plasma and red cell volume; decreased hemoglobin, red cell count, and hematocrit</td>
<td></td>
</tr>
<tr>
<td>Folate antagonists and antibiotics</td>
<td>May cause megaloblastic marrow, but falsely low serum folate via inhibition of L. casei in microbial assay</td>
<td>Examples: methotrexate, pirmethamine, trimethoprim, triamterine</td>
</tr>
</tbody>
</table>
Table 4. (continued)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
<th>Other Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequently used medications</td>
<td>low serum folate from aspirin interference with folate binding capacity of serum; folate deficiency common with sulfasalazine</td>
<td>anticonvulsants and oral contraceptives†</td>
</tr>
<tr>
<td>Uremia</td>
<td>results in low serum folate in assays that do not separate folate from binding protein; hypersegmented neutrophils in renal failure</td>
<td></td>
</tr>
<tr>
<td>Sudden onset of megaloblastic anemia</td>
<td>red cell folate may be in normal range</td>
<td>examples: in pregnancy, prematurity, parenteral nutrition; acute catastrophic illnesses</td>
</tr>
</tbody>
</table>

* The material in this table was derived from the following references: Beard et al., 1980; Carmel, 1978; Chanarin, 1980; Colman, 1977; Herbert, 1975; Shojania, 1980; Waxman, 1973.

† See also page 24.
folate assays should be done routinely in folate evaluation, at least in selected groups of subjects. That is, in order to evaluate totally the folate status, it is necessary to measure blood folate levels since folate deficiency can still occur with a normal hemoglobin and red cell volume. Although the most sensitive indicator of folate deficiency in the blood smear is hypersegmentation of the neutrophil, this requires an expert opinion for evaluation. Therefore, in selected groups, such as patients with borderline folate deficiency, it is necessary to obtain a serum and red cell folate level since these are sensitive indicators of folate deficiency, and are reproducible among well qualified laboratories.

C. APPLICABILITY TO MEDIUM AND LARGE POPULATION GROUPS

The practicality of using biochemical and clinical laboratory procedures to estimate folate status in selected groups of the population has been demonstrated by a number of investigators such as Bailey et al. (1979; 1981a), Colman et al. (1974a; 1975a), Herbert et al., (1975), and Wagner et al. (1981). Table 5 lists the methods of assessment used by these investigators.

Health and nutrition surveys of larger populations have used the serum folate assay (Health and Welfare Canada, 1973) or the serum and red cell folate assays in the Ten-State Nutrition Survey (Sauberlich, 1977) to estimate folate status and to identify population groups at risk of folate deficiency. When the related hematologic and biochemical measurements listed in Table 5 are added to the folate assays, fairly reliable assessment of folate status is possible. As already noted, the absence of dependable data for estimating bioavailability and dietary intakes of folates from food sources and the difficulty of diagnosing compromised folate status solely on the basis of clinical evaluation emphasize the importance of the biochemical methods for determination of folate nutritional status. According to some investigators, such techniques are considered reasonably reliable for the detection of folate deficiency in large population samples (Sauberlich, 1977).
Table 5. Practical Methods for Assessing Folate and Iron Status and Associated Hematologic Parameters in Selected Population Groups

<table>
<thead>
<tr>
<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Microbiol. assay for serum folate</td>
<td>x</td>
<td>x</td>
<td>x*</td>
<td>x</td>
</tr>
<tr>
<td>Microbiol. assay for red cell folate</td>
<td>x</td>
<td>x</td>
<td>x*</td>
<td>x</td>
</tr>
<tr>
<td>Serum Vitamin B₁₂</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Mean cell hemoglobin</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Red cell count</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Mean red cell volume</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Serum iron</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Total iron binding capacity</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Neutrophil lobe average</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Neutrophils with &gt;5 lobes (%)</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

* Used chloramphenicol-resistant strain of _L. casei_ rather than the conventional strain.
VI. CURRENT STATUS OF FOLATE METHODOLOGY

Folates are present in foods and organ tissues in numerous forms having nutritional and metabolic significance for man. This heterogeneity of folates leads to considerable difficulties in analyzing the folates in foods and biological materials. Several methods, including microbiological assays, radiometric binding assays, and high performance liquid chromatography (HPLC) are useful systems for measuring folate concentrations in biological materials. In addition, assays of folate function such as the deoxyuridine suppression test and measurement of hematologic parameters are valuable in determining folate status. Strengths and weaknesses of these methods are outlined in Table 6.

A. TECHNIQUES FOR FOLATE ANALYSIS

1. Microbiological assay

Microbiological assays utilizing folate-responsive organisms (Streptococcus faecalis, Pediococcus cerevisiae, and Lactobacillus casei) provided an early means for measuring folate levels in foods and biological samples. The growth response of these microorganisms is usually measured as turbidity after incubation under controlled conditions with a folic acid standard or an extract containing endogenous folates. Table 7 indicates the response of these organisms to the various folates found in biological materials. S. faecalis was used as the major test organism until it was determined that this bacterium utilized the folate antagonist, pteroic acid, while it did not utilize 5-methyltetrahydrofolate, a predominating form of folate in grain products and in red blood cells and plasma (Stokstad and Koch, 1967). Despite this limitation, S. faecalis remains the official A.O.A.C. method of folic acid assay (Horwitz, 1980). The second microorganism, P. cerevisiae, utilizes only reduced monoglutamyl tetrahydrofolates with the exception of 5-methyl tetrahydrofolate. Thus, although each may be used to distinguish between different forms of folates and thereby help to establish a profile of the types of folates in different sources (Dong and Oace, 1973), these organisms have limited use in the analysis of total folates in biological and food samples.

The third microorganism, L. casei, responds to the numerous biologically active forms of folate (Baker et al., 1959; Stokstad and Koch, 1967). Originally it was thought that L. casei responded only to folates conjugated to three or fewer glutamate residues. Later it was shown by Tamura et al. (1972) that, at the dosage used, tetraglutamate produces 66%, pentaglutamate 20%, hexaglutamate 4%, and heptaglutamate 2% of the response of the monoglutamate in L. casei. Of the three test organisms employed for folate analysis, only L. casei is capable of utilizing N-5-methyl
Table 6. Procedures for Measuring Folates and Folate Status

<table>
<thead>
<tr>
<th>Method</th>
<th>Appropriate samples</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Techniques for folate content</td>
<td>Food, tissues</td>
<td>Measures biologically active folate</td>
<td>Inhibited by drugs; unexplained variability</td>
<td>Baker et al., 1959; Butterworth et al., 1963</td>
</tr>
<tr>
<td>Microbiological assay (L. casei; conjugase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbiological radiometric assay</td>
<td>Tissues, foods*</td>
<td>Ease of handling; measures $^{14}$CO$_2$ production</td>
<td>Inhibition by drugs; cost</td>
<td>Chen et al., 1978</td>
</tr>
<tr>
<td>Radiometric binding assay</td>
<td>Serum, tissues*, foods*</td>
<td>Ease of method; good sensitivity; less interference from antibiotics</td>
<td>Lack of standardization; needs correlation with microbiological assay</td>
<td>Waxman et al., 1971</td>
</tr>
<tr>
<td>High performance liquid chromatography</td>
<td>Foods, tissues*</td>
<td>Identifies folate forms in food and organ tissue samples</td>
<td>In early stages of development. Not yet sufficiently sensitive for status determinations</td>
<td>Cashmore et al., 1980; Clifford and Clifford, 1977; Day and Gregory, 1981b; Eto and Krumdeleck 1981; Krumdeleck et al., 1981</td>
</tr>
<tr>
<td>Techniques for folate status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum folate</td>
<td></td>
<td>Indicates immediate past intake</td>
<td>May not reflect tissue stores</td>
<td>Herbert, 1962b</td>
</tr>
<tr>
<td>Red blood cell folate</td>
<td></td>
<td>Indicates long-term status (3 mo); often indicates tissue stores</td>
<td>Vitamin B$_12$ deficiency also lowers red blood cell folate</td>
<td>Hoffbrand et al., 1966</td>
</tr>
<tr>
<td>Peripheral blood smear (erythrocyte size and shape; macroovalocytosis)</td>
<td></td>
<td>Early sign</td>
<td>Similar morphology caused by conditions other than folate deficiency</td>
<td>Chanarin, 1976; Herbert, 1975</td>
</tr>
<tr>
<td>Method</td>
<td>Advantages</td>
<td>Disadvantages</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Hypersegmented neutrophils</td>
<td>Early sign. Usually present despite concomitant iron deficiency</td>
<td>Tedious procedures</td>
<td>Herbert, 1962b, 1970; Lindenbaum and Nath, 1980</td>
<td></td>
</tr>
<tr>
<td>Bone marrow smear</td>
<td>Early sign; megaloblastic erythropoiesis</td>
<td>Requires needle biopsy</td>
<td>Chanarin, 1979a</td>
<td></td>
</tr>
<tr>
<td>Deoxyuridine suppression test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. PHA-stimulated lymphocytes</td>
<td>Tests folate function; may indicate past status or covert deficiency</td>
<td>Time consuming; may not indicate current status</td>
<td>Das et al., 1978</td>
<td></td>
</tr>
<tr>
<td>2. Bone marrow</td>
<td>Good correlation with megaloblastosis. Differentiates between folate and vitamin B₁₂ deficiencies</td>
<td>Not amenable to field work</td>
<td>Metz et al., 1968; Waxman et al., 1969</td>
<td></td>
</tr>
<tr>
<td>Organ tissue folate</td>
<td>Shows individual tissue status</td>
<td>Requires biopsy</td>
<td>Wu et al., 1975</td>
<td></td>
</tr>
<tr>
<td>Urinary FIGLU</td>
<td>Non-invasive procedure; screening test</td>
<td>May not differentiate between folate and vitamin B₁₂ deficiencies; requires histidine load; histidine load does not increase FIGLU excretion in presence of anticonvulsants</td>
<td>Herbert, 1967</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates that the method may be adapted for measurement of folate in these samples.
Table 7. Relative Activity of Folate Compounds for Microorganisms*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lactobacillus casei (ATCC 7469)</th>
<th>Streptococcus faecalis (ATCC 8043)</th>
<th>Pediococcus cerevisiae (ATCC 8081)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>+†</td>
<td>+</td>
<td>-§</td>
</tr>
<tr>
<td>Pteroyldiglutamic acid</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pteroyltriglutamic acid</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pteroylheptaglutamic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetrahydrofollic acid</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5-Formyl tetrahydrofollic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10-Formyl tetrahydrofollic acid</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5,10-Methylene tetrahydrofollic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5-Methyl tetrahydrofollic acid</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pterolic acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10-Formyl pterolic acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5-Formyl tetrahydropterolic acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Table adapted from Stokstad and Koch (1967).
† 70-100% of folic acid activity on a molar basis for L. casei and S. faecalis and 70-100% of 5-formyl tetrahydrofollic acid for P. cerevisiae.
§ Minus sign indicates activity of less than 5%.
tetrahydrofolate and the triglutamate form as a growth factor. Coupling of the \textit{L. casei} assay with a conjugase treatment to hydrolyze the polyglutamyl chain, a modification considered to measure total folates (Butterworth et al., 1963), has become the most widely used and broadly accepted method of measuring folate content of biological materials and foods.

Day-to-day shift in baseline growth response of \textit{L. casei} represents a source of variation of unknown magnitude common to all microbiological assays. Some investigators relate slope of the growth response curve to a constant prior to determining the baseline response. Use of a uniform inoculum derived from lyophilized cultures of \textit{L. casei} may reduce day-to-day variations in growth response (Grossowicz et al., 1981).

There is some question whether oxidized folic acid is the most appropriate standard for the \textit{L. casei} assay of folates in foods and biological samples. However, there are problems of oxidation when reduced one-carbon-substituted folates are used as standards. For example, 10-formyl tetrahydrofolate would be oxidized to 10-formyl folic acid during the assay and methylene tetrahydrofolate would decompose in an acidic medium. A more stable reduced folate to use as a standard is 5-methyl tetrahydrofolate, but whether the response of \textit{L. casei} to 5-methyl tetrahydrofolate is more representative of its response to other forms than its response to folic acid has not been determined. Quantitative differences in growth responses of \textit{L. casei} to various folate forms were not found (Tamura et al., 1980) and advantages of routine use of forms other than folic acid as standards have not been determined.

Use of chloramphenicol in the growth medium for a chloramphenicol-resistant strain of \textit{L. casei} (Scott et al., 1974) decreases problems of contamination of the assay but the use of chloramphenicol in such laboratory procedures has been criticized because of the potential for producing aplastic anemia (Williams et al., 1977). Modifications of the technique for the chloramphenicol-resistant organism have been adapted for use with the standard \textit{L. casei} strain, ATCC 7469 (Cooper, 1973).

A second microbiological method for folate in human plasma and red cells has been developed (Chen et al., 1978). This radioisotopic microbiologic assay measures $^{14}$CO$_2$ evolved from metabolism of [1-$^{14}$C]gluconate through the hexose monophosphate shunt in \textit{L. casei}. Production of $^{14}$CO$_2$ depends upon the amount of 5-methyltetrahydrofolate present in the system. This modification overcomes the problem of sample turbidity but problems of bacterial contamination and presence of antibiotics in blood or tissue samples still remain. This modification of the microbiological method has excellent potential as a sensitive assay for folate in foods but, currently, cost is a problem as it requires sophisticated instrumentation as well as radioisotopes.
2. Radiometric binding assay

The radiometric binding assay was developed as an alternate procedure to microbiological assays. This method, used more widely in clinical laboratories than the microbiological assay, is technically uncomplicated and is not affected by sample turbidity, bacterial contamination, or presence of antibiotics (Waxman and Schreiber, 1977). The method is based on the principle of binding of radio-labeled folate by relatively specific protein binders (Waxman et al., 1971). Many modifications of this assay have been developed, including significant differences in assay pH (Givas and Gutcho, 1975), differences in folate compounds and radiolabels employed for standard curves (Johnson et al., 1977; Mitchell et al., 1976), use of preservatives during sample storage (Kerkay et al., 1977; Lindemans et al., 1975), sequential vs. simultaneous addition of sample and labeled folate compound (Rothenberg et al., 1974; Waxman and Schreiber, 1973), and source of protein binders (Waxman and Schreiber, 1980). Proteins used in the assay bind folic acid and 5-methyl tetrahydrofolate tightly, but have a low affinity for 5-formyl tetrahydrofolate. This may prove to be a serious limitation of these procedures in view of the recent demonstration (Krumdieck et al., 1981) that folates bearing one-carbon substituents at the formyl oxidation level constitute the major fraction of the folates in some tissues such as liver.

Some uncertainty exists concerning differences of binder affinity for folate monoglutamates and polyglutamates. Some investigators have claimed no differences in binder affinity for mono- and polyglutamates (Schreiber and Waxman, 1974) while others have observed a significant increase in binding activity for polyglutamates (Colman and Herbert, 1979; Shane et al., 1980). As with the microbiological assay, the most appropriate compound to use as a standard has not been identified. The radiometric binding assay correlates well with the microbiological assay for serum folates but correlation coefficients between this method and the microbiological assay for red cell folate vary (Longo and Herbert, 1976; Schreiber and Waxman, 1974).

Recent work by Graham et al. (1980) indicated excellent correlation ($r = + 0.99$) between total folate content as determined by the L. casei assay and folate content as measured by radiometric binding assay for a limited number of foods fortified with folic acid. Similar results ($r = + 0.87$) were reported by Tignor and Roe (1979) for tissues autolyzed for 5 h preceding analysis. Overall, good correlation of the radiometric binding assay with the L. casei assay is possible and, once such a correlation is obtained in a particular laboratory, the radiometric binding assay may be the method of choice for serum folate analysis. Additional experience will be required to confirm the usefulness of this method for food and tissue folate analysis.
Radiometric binding assays utilizing kits have not generally correlated as well with the microbiological assay as the laboratory radiometric binding assay. Comparisons of microbiological and radiometric binding assays have emphasized the need for more extensive testing of such kits (Baril and Carmel, 1978; Jones et al., 1979; Longo and Herbert, 1976; McGown et al., 1978; Shane et al., 1980).

3. **High performance liquid chromatography**

Separation of folate compounds by conventional chromatographic methods including thin layer chromatography, gel filtration, and ion exchange chromatography has led to improved differentiation of folate forms. HPLC techniques have recently been developed and appear to have the most potential as a sensitive method for resolution of folate compounds. Oxidized and reduced one-carbon-substituted folate monoglutamates have been separated and quantified (Allen and Newman, 1980; Branfman and McComish, 1978; Cashmore et al., 1980; Chapman et al., 1978; Clifford and Clifford, 1977; Day and Gregory, 1981b; Reed and Archer, 1976; Reingold et al., 1980a; Stout et al., 1976) and folate polyglutamates have been separated and identified (2 to 8 glutamate residues) by HPLC (Cashmore et al., 1980). Profiles of folates in foods have been successfully analyzed by such systems (Clifford and Clifford, 1977; Day and Gregory, 1981b; Gregory et al., 1981; Reingold et al., 1980b) and HPLC has also been recently used to provide a rapid separation of tissue folates prior to *L. casei* analysis of collected fractions (McMartin et al., 1981). Results to date with the HPLC method exhibit variability analogous to that reported with the *L. casei* assay. Further studies may show this variation is related to the sample content of folate and not the method.

Current sensitivity of HPLC does not permit analysis of samples such as sera with low concentrations of folates, but formation of derivatives of compounds separated by chromatographic techniques such as those reported by Day and Gregory (1981b) may enhance the sensitivity of the analysis of folate compounds. Eto and Krumdieck (1980, 1981) and Krumdieck et al. (1981) have recently developed procedures that permit the quantitation of three different one-carbon-substituted "pools" of reduced folates with the simultaneous determination of their corresponding polyglutamyl chain lengths. The method is based on the different susceptibility to oxidative cleavage of the C9-N10 bond of the various forms of reduced one-carbon-substituted folates present in biological samples. Conditions have been developed that permit the selective cleavage of the folates of pool 1 (which includes 5,10-methylene- and unsubstituted tetra- and dihydrofolates), of pools 1 and 2 (pool 2 is made up by 5-methyltetrahydrofolates), and of pools 1, 2, and 3 (pool 3 includes the folates with one-carbon substituents at the formyl oxidation level: 5,10 methenyl-, 10- and 5-formyl- and 5-formimino- tetrahydrofolates). The cleaved folates yield
p-aminobenzoylpolyglutamates which are derivatized to intensely colored azo dyes which can be spectrally quantitated. HPLC of the azo derivatives permits the determination of the polyglutamyl chain length of the folates of each of the pools that the method distinguishes. So far, these procedures have been applied to a limited number of tissues (rat liver, brain, and kidney) with remarkably reproducible results. Application to food analysis will require some modification to allow inclusion of fully oxidized folates which might otherwise escape detection.

B. TECHNIQUES FOR MEASURING FOLATE STATUS

Assessment of folate status in humans has been limited by lack of development of methodology, lack of reliable determinations of the folate content of foods and biological materials, and therefore inability to correlate folate intake and overall body status. While recognizing that deficiencies in select cell lines, for example, cervical cells and lymphocytes (Butterworth et al., 1980; Das and Herbert, 1978; Das et al., 1978; Whitehead et al., 1973) may not always be detected by analysis of blood, most investigators consider that blood and its components are the best tissues for clinical or survey determination of folate status. Serum and red cells are the samples most commonly analyzed. Leucocyte folate levels (Swendseid et al., 1951) are rarely used because of difficulties in separating leucocytes from other blood components (Sauberlich et al., 1974) and because they correlate well with lymphocyte duU suppression which is less laborious (Colman and Herbert, 1980). Assay of the folic acid content of urine is unreliable because of potential problems with bacterial overgrowth, inaccurate collection, and variation in recent dietary intake.

1. Serum folate

Hematologic changes occurring during folate depletion of one male subject were systematically described by Herbert (1962b) and have provided the basis for assessment of folate status in humans. Of these changes, serum folate was shown to fall after about 21 days on a diet low in folates (containing about 5 μg folate per day as determined by the L. casei assay). Depletion of serum folates has thus been generally considered to reflect the first stage of development of a folate deficiency. However, such a sensitive response to variation in dietary folate intake, based on a single serum sample, may not be a reliable indicator of the folate status of an individual.
2. Red cell folate

In contrast to the immediate fluctuations reflecting previous folate intake indicated by serum folate concentrations, red cell folate concentrations reflect tissue status over a longer period of time, 90 days, (Herbert, 1962b) and are more reliable for indicating risk of development of folate deficiency. Wu et al. (1975) have shown that red cell folate levels accurately represent levels of the vitamin in the liver. Although small amounts of folate may be transported by red cells (Branda, 1981), effects of such transport on red cell folate levels would be minimal and would not influence the interpretation of folate status. Determining vitamin $B_{12}$ status together with folate status is necessary clinically because low concentration of folate in red cells may be caused by lack of vitamin $B_{12}$ as well as by an inadequacy of dietary folate. Determination of vitamin $B_{12}$ status is less important in large population surveys because the prevalence of vitamin $B_{12}$ deficiency in the population is quite small. Vitamin $B_{12}$ is necessary to convert 5-methyl tetrahydrofolate to tetrahydrofolate. In vitamin $B_{12}$ deficiency this conversion is impaired and there are accumulations of 5-methyl tetrahydrofolate in the serum. In addition, red cell folate is decreased possibly due to a deficiency of a vitamin $B_{12}$-dependent red cell folate conjugase (Chanarin, 1979a).

The microbiologic assay (Cooper and Lowenstein, 1964; Grossowicz et al., 1962; Hoffbrand et al., 1966) is widely used for determination of red cell folate levels. In this assay, the folate content of samples of whole blood and serum is measured, and the red cell folate calculated on the basis of the packed cell volume. Alternatively, red cell folate may be determined by radiometric binding assays (Schreiber and Waxman, 1974).

3. Erythrocyte size and shape

Certain changes in red cell morphology, particularly macrocytosis, are indicative of megaloblastic anemia and may provide an indication of folate deficiency. Herbert (1962b) reported that these changes were observed at 127 days in a subject consuming a diet low in folate. While macrocytosis is indicative of megaloblastic anemia, it occurs in too many other conditions to be considered as a diagnostic criterion for folate deficiency without additional supporting information.

4. Hypersegmented neutrophils

Hypersegmentation of neutrophils was detected after 49 days of consumption of a low folate diet (Herbert, 1962b). This change is considered to be the earliest hematologic sign of folate deficiency, and occurs even with concurrent iron deficiency that may mask megaloblastic red cell changes. An exception to its
occurrence with low serum and red cell folate is during pregnancy so that it is an unreliable indicator of folate status at this time (Herbert et al., 1975). In addition, it has been reported that about 1% of normal adults have congenital polymorphonuclear leucocyte hypersegmentation (Herbert, 1964) and that hypersegmented neutrophils may possibly occur as a result of iron deficiency alone (Chanarin, 1979a). Hypersegmentation of neutrophils occurs during granulopoiesis rather than as addition of lobes in previously formed cells (Friedner et al., 1964) and also persists for about 2 weeks following folate therapy in folate-depleted patients having megaloblastic anemia (Nath and Lindenbaum, 1979).

Several criteria have been used to assess hypersegmentation, including a lobe average > 3.5 lobes/cell or presence of > 5% of five-lobe polymorphonuclear neutrophils. Recently Lindenbaum and Nath (1980) reported that 351 of 357 patients having megaloblastic anemia in relapse had at least one six-lobe polymorphonuclear neutrophil per 100 cells. Some investigators consider this occurrence as a sensitive and definitive screening test for megaloblastic anemia, but not as a specific diagnostic criterion of the disorder. The large number of blood smear slides available from nutritional surveys might provide materials for verification and correlation with other methods.

5. Deoxyuridine (dU) suppression test

Impairment of DNA synthesis, as measured by suppression by deoxyuridine of 3H-thymidine incorporation into DNA in bone marrow cells and phytohemagglutinin (PHA)-stimulated lymphocytes, is a major biochemical abnormality in folate- and vitamin B12-deficient subjects (Das et al., 1978). In bone marrow samples, the dU suppression test correlates well with megaloblastosis. Vitamin treatment (folate and/or vitamin B12) in patients with abnormal bone marrow dU suppression tests (Metz et al., 1968) allows differentiation between folate and vitamin B12 deficiencies. However, the bone marrow test is not adaptable to determination of folate status in nutrition survey studies because it is an invasive procedure.

The dU suppression test using PHA-stimulated lymphocytes has been shown to correlate well (r = 0.64) with lymphocyte levels of folate and to reliably predict low folate levels (Colman and Herbert, 1980). Work by Das and Herbert (1978) and Das et al. (1978) indicates that the persistence of an abnormal lymphocyte dU suppression test following therapy may be utilized as an indicator of past, but not necessarily current, folate deficiency and that it may reveal hidden folate deficiency in iron-deficient subjects. Modifications of this technique such as use of whole blood rather than separated lymphocytes (Das et al., 1980) may allow it to be simplified sufficiently for routine clinical assays or nutrition survey work.
Despite a considerable number of studies, such as those cited above, which indicate that dU suppression of tritiated-thymidine incorporation into DNA is a reliable and sensitive indicator of folate status, questions remain concerning the biochemical mechanisms that form the basis of the test. Pelliniemi and Beck (1980) report that mechanisms other than those corrected by administration of folate and vitamin B_{12} may be responsible for effects of exogenous deoxyuridine on thymidylate synthesis, and caution that interpretation of dU suppression test results should recognize the more complex role of exogenous deoxyuridine on DNA synthesis.

6. **Formiminoglutamic acid excretion test**

Increased urinary excretion of FIGLU following a histidine load may indicate deficiency of folic acid (Luhby, 1957; Luhby and Cooperman, 1964) or vitamin B_{12} (Chanarin, 1980). The test is compromised in pregnant women (Chanarin et al., 1963) and in persons taking anticonvulsants (Reynolds et al., 1966) because the expected rise in FIGLU excretion following histidine administration does not occur. The test may have some merit as a screening mechanism to detect folate- and/or vitamin B_{12}-deficient persons but hematologic measurements discussed previously are more useful indicators of folate status and this test is not practical for use as a part of survey protocols.
VII. ADDITION OF FOLATE TO FOODS

Addition of synthetic folic acid to foods has been investigated as a means of increasing dietary folate intake. The most comprehensive evaluation was a series of studies assessing folate status in rural South African black adults (men and non-pregnant, pregnant, and lactating women) having a high incidence of folate deficiency (Colman et al., 1974a,b,c; 1975a,b,c; Margo et al., 1975). Addition of folic acid to staple foods in amounts supplying 300 to 1000 µg/d did not adversely affect organoleptic or cooking properties of these items (Colman, 1977). Overall, food fortification proved a feasible means for increasing serum and red cell folate in these groups. Seven subjects having saturated folate stores ingested a folic acid solution or fortified maize meal, rice, or bread. Mean absorption as reflected by summated serum increases at 1 h and 2 h was about 55% from maize porridge and boiled rice and about 30% from whole wheat bread, compared with the increase in serum folate produced by the same dose of folic acid in solution (Colman et al., 1975a).

Supplementation of 104 South African pregnant women with maize meal containing 300, 500, or 1000 µg folic acid or tablets containing 300 µg folic acid daily for approximately 1 month before delivery caused significant increases in red cell and serum folate in the mothers at the time of delivery (Colman et al., 1975b). The rate of increase was dependent upon the dose administered in the maize meal; 500 µg/d of the vitamin in maize meal produced increments in serum and red cell folate concentrations similar to those of 300 µg folic acid in tablet form, which correlated well with prior absorption tests. Maize meal fortified with 300 µg folic acid also prevented the folate depletion which is characteristic of late pregnancy and was observed in the control group receiving no additional folate (Colman et al., 1975b). In four lactating women with severe megaloblastic anemia caused by folate deficiency, daily consumption of maize meal containing 300 or 500 µg folic acid produced an optimal hematologic response while daily consumption of maize meal containing 100 µg folic acid produced a slower and smaller hematologic response in a fifth subject (Colman et al., 1974b).

A South African field trial was also conducted in six families given a 6 wk supply of maize meal fortified to supply each adult with 500 µg/d folic acid. In each family the red cell folate level was monitored in a pregnant or lactating female and in the eldest member. Red cell folate levels rose significantly in 9 of the 12 persons monitored and indicated that folate fortification of maize meal could be an effective means to increase dietary folate intake (Colman et al., 1974c). Examination of the heat stability of the folic acid used to fortify the maize meal,
rice, and wheat flour for these studies indicated that boiling for as long as 2 hours or baking at 230°C for up to 1 hour did not decrease the amount of microbiologically available folic acid (Colman et al., 1975a). Colman reported further that the folic acid in the fortified maize meal was stable for as long as 18 months during storage at ambient temperatures (Colman, 1977), probably partly because all but the outer layer of maize was protected from light.

In the United States one study has assessed the effects of adding folic acid (10 μg/ml) to wine (Kaunitz and Lindenbaum, 1977). Serum folate levels in six normal volunteers and seven recently intoxicated chronic alcoholics were markedly increased over fasting levels when the subjects consumed intoxicating doses of the wine (4 ml/kg body weight). Appearance and taste of the wine were not detectably changed by this addition. The folic acid content of the wine dropped to 86-90% of the initial activity after 1 month and to 61-66% after 3 months storage at 27°C and exposure to fluorescent and natural light (Kaunitz and Lindenbaum, 1977). Although a positive response to the fortified wine was reported, widespread application appears unlikely. Fortification of potentially harmful substances, which may be used in excess, is a practice discouraged by the Joint FAO/WHO Expert Committee on Nutrition (1971).

Currently in the United States folic acid is added to some breakfast cereals, instant breakfast pastries and drinks, and diet foods (40 to 400 μg/serving) and to infant formulas (27 μg/l). One serving of such fortified foods would supply 10% to 100% of the RDA for the adult and between 13 and 400% of the RDA for children less than 10 years of age. One liter of the infant formula provides 60-90% of the RDA for infants less than 1 year of age. Since the folic acid added to these products is in the pure form, it may be more effective than the naturally-occurring food folate, upon which the RDA is based (National Research Council, 1980). Thenen (1981) has reported that folacin-fortified breakfast cereals can be a major contributor of folate in the Special Supplemental Food Program for Women, Infants and Children (WIC). By selection of foods allowed in the WIC Program that were the highest in folate by analysis, as much as 69% of the RDA for folacin for pregnant women (800 μg) can be obtained in only 34% of the energy allowance. Although research would suggest that fortification of breakfast cereals might be an effective means to increase the folate intake of the general population, the pattern of use of these products by groups at risk of developing folate deficiency and the prevalence of masking pernicious anemia are not known.

Herbert (1980) has suggested that daily consumption of a raw fresh fruit or vegetable would be the "ideal nutritional therapy" for folate deficiency. However, in populations where inclusion of foods high in folates cannot be assured, fortification of specific dietary items might be a more practical approach
to increase folate intake. The extensive work in South Africa of Colman and associates, mentioned above, has demonstrated that addition of folic acid to staple food items is a feasible means of improving folate status in a population having a diet limited to a few foods.

Data from studies of limited population samples show that compromised folate status exists in significant numbers of adolescents, pregnant women, and elderly persons in the United States. Analysis of data collected in the HANES II survey may indicate whether these preliminary observations apply only to isolated groups and regions, or throughout the United States. Thus, analysis of extant epidemiologic data and possibly collection of additional data are critical to a sound determination of the folate status of the population of the United States.

If forthcoming data warrant consideration of public health measures, the fortification programs that have demonstrated their efficacy in other countries should be considered as a possible model of nutritional intervention. These trial programs have documented that the addition of physiologic amounts of folic acid to carefully selected food items resulted in improvement in the folate status of groups known to be at high risk of folate deficiency.
VIII. CONCLUSIONS

FOOD FOLATES

- Estimates of dietary folate intakes in subsets of the United States population are limited. An estimate of the folate available from principal folate-containing foods indicates that the total amount available in average daily diets is about 225 μg. Studies of nutritional status suggest that levels commonly consumed by certain groups of adolescents, pregnant women, and the elderly may not be sufficient to maintain adequate serum and red cell folate levels.

- Although more data are needed, the most accurate and extensive compilations of total folate contents of foods are contained in the USDA Agricultural Handbooks 8-1 to 8-7, the USDA provisional table for food folate and its supplement, and the Canadian and British tables of food folates.

- Numerous reports indicate loss of food folates under conditions of storage, processing, and home preparation. Little is known of the losses of specific folates in individual foods.

- Although studies indicate intestinal conditions influence absorption, and possible mechanisms of transport have been identified for folic acid and polyglutamyl folates, little is known about absorption of food folates consumed in a normal diet.

- Addition of folic acid to foods is a feasible means of improving folate status. This was convincingly demonstrated in South African populations consuming a diet consisting primarily of one specific fortified food.

FOLATE STATUS

- Uncompensated increases in metabolic requirement for folate, as in adolescence and pregnancy, enhance the risk of developing compromised folate status. Conditions influencing absorption, utilization, breakdown and losses of the vitamin have been shown to be important in maintaining adequate nutritional status. One or more of these factors in addition to marginal folate intake is associated with almost all cases of frank folate deficiency.
Available evidence documents the existence of significant folate deficiency throughout the world in population groups that are at special risk such as undernourished adolescents, pregnant and lactating women, women of child bearing age for 2-3 years after lactating, and the elderly. In addition, folate deficiency is common among alcoholics and patients using certain drugs and/or suffering from malabsorption or other diseases.

Data from investigations of special target populations of approximately 100-400 noninstitutionalized subjects in the United States, such as adolescents, pregnant women and the elderly, strongly suggest a high prevalence of compromised folate status in such groups, but evidence that this causes illness has not been presented.

The number of alcoholics and patients at risk of folate deficiency in this country probably totals in the millions. These include poorly nourished alcoholics and patients on long-term anticonvulsant therapy and those taking sulfasalazine for inflammatory bowel diseases.

Whether the use of oral contraceptives results in a significant risk of folate deficiency is unsettled.

Interpretation of folate status in the population of the United States based on data from large-scale surveys has been difficult and equivocal. Available evidence of compromised folate status based solely on mean values of serum and red cell folate from large-scale surveys is insufficient to determine whether a medical problem of public health significance exists in this country.

Excessive intake of folic acid may mask the hematologic manifestations and delay the recognition of neurologic complications of pernicious anemia. Excessive intakes of folate from dietary sources have not been documented.

IDENTIFICATION OF COMPROMISED STATUS

Serum and red cell folate determinations, despite their diagnostic limitations, are currently the most practical methods of assessing folate status in large-scale population studies.

A practical approach to the clinical assessment of folate status in individuals that minimizes the number of laboratory procedures might include a dietary history, mean red cell volume, blood smear, and hemoglobin estimation followed by serum and red cell folate.
Diagnosis of folate deficiency can be complicated by coexisting conditions such as iron, zinc, or vitamin B₁₂ deficiency, malabsorption syndromes, other diseases, and drug interference. In such cases, clinical evaluation on an individual basis is required.

The practicality of determining serum and red cell folate values, iron status, and selected hematologic measures to estimate folate status in target groups of moderate size such as 100–400 subjects has been amply demonstrated.

FOLATE METHODOLOGY

The microbiological assay with *Lactobacillus casei* plus conjugase treatment to hydrolyze the polyglutamyl chain is the most widely accepted method currently available for determination of total folate concentrations in foods and in biological materials.

The radiometric binding assay for folate is well suited for serum samples and additional experience may demonstrate its usefulness for food and tissue folates. Correlation of the radiometric binding assay with the *L. casei* assay in individual laboratories should be obtained before relying solely on the results of the radiometric binding assay.

High performance liquid chromatography shows promise as a method for determining folate profiles in biological materials.

Considerable variance is evident in the results of assays of folate in foods, regardless of the methodology used. Some evidence suggests the apparent discrepancies result from actual variation in folate content of different samples of the same foods.

Neutrophil hypersegmentation, an early hematologic indicator of folate deficiency, is a key diagnostic aid that is underutilized in determining risk of folate deficiency. Other hematologic changes such as macrocytosis indicate megaloblastic anemia but occur in many conditions other than folate deficiency.

In phytohemagglutinin-stimulated lymphocytes, the deoxyuridine (dU) suppression test may reveal past folate deficiency or folate deficiency otherwise hidden by concurrent iron deficiency.
IX. SUGGESTIONS FOR FUTURE CONSIDERATION

- More reliable information is needed for the folate content of foods and the bioavailability of the several forms of the vitamin. Development of newer technologies to measure folate concentrations, bioavailability, and status should be encouraged.

- The HANES II data on folate status should be analyzed to determine whether data reported from less extensive nutrition surveys can be confirmed.

- Valuable data might be obtained from analysis of the folate status of persons consuming foods already fortified with folate compared with the status of persons not consuming these products.

- Better estimates of folacin intakes and correlation of these intakes with biochemical indicators of folate status are needed.

- Because the clinical significance of low serum and red cell folate values measured in large-scale studies is unknown, more sophisticated studies of individual subjects from such groups may be desirable.

- Future health and nutrition surveys should be designed to facilitate data processing so that individuals below the norms for serum and red cell folate levels may be readily identified for additional evaluation.

- In view of the evidence that some apparently healthy people have low or borderline folate status, more data are needed to characterize the condition of being at risk of folate deficiency, to refine norms, and to account for the seeming lack of impairment among such individuals. This kind of information would assist, as well, in efforts to define the daily folate requirements for various segments of the population.

- Because masking of vitamin B_{12} deficiency is generally regarded as the most serious adverse effect of excessive folate intake and because the prevalence of masking is unknown, additional epidemiologic studies including evaluation of persons using over-the-counter folate supplements without medical supervision should be undertaken.

- There is insufficient information concerning the effects on the fetus of folate deficiency. Degrees and duration of deficiency as well as time of onset during pregnancy
may be important variables. The effects of alcoholism on folate metabolism during pregnancy should be considered. In addition, possible teratogenic effects of dietary deficiency should be investigated.

- The potential importance of reliable data on the effects of folate deficiency on the nervous system justifies further investigation including epidemiological studies, clinical observations, and animal experiments.

- In view of the equivocal data suggesting that use of oral contraceptives may be associated with compromised folate status, further clinical investigations are indicated. If possible, large-scale health and nutrition surveys should also include provisions for examining this question. Future clinical studies should include an evaluation of possible differential effects of low-estrogen, low-progestogen formulations compared with standard products.

- The value of hypersegmented neutrophils as predictors of risk of folate deficiency might be established as a useful procedure in large-scale surveys by examination of slides available from previous surveys.

- The relationship between serum and red cell folate needs further elaboration as does the relationship between serum folate and tissue folate levels in other target organs.

- Standardization among laboratories of the L. casei assay is essential to provide the best possible values for folate levels in foods and biological samples.

- The relationship between the deoxyuridine suppression test in megaloblastic bone marrow versus lymphocytes from subjects with folate deficiency and megaloblastic changes should be investigated in an effort to establish possible practical advantages of either test. Where possible, laboratory research methods should be adapted to use in field work and surveys.
X. LITERATURE CITED


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