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Folate Status in Adolescent Girls

Hugh Wyatt Dawson
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Hugh Wyatt Dawson entitled "Folate Status in Adolescent Girls." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Gail W. Disney, Major Professor

We have read this thesis and recommend its acceptance:

Edward T. Howley, John T. Smith

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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John T. Smith

Accepted for the Council:

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Vice Chancellor
Graduate Studies and Research

X

FOLATE STATUS OF ADOLESCENT GIRLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Hugh Wyatt Dawson

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ABSTRACT

This nutrition status survey was conducted at The University of Tennessee, Knoxville in conjunction with a southern regional nutrition project operative in ten sites throughout the southeast. The purpose of this study was to assess folate nutriture of the subjects using both biochemical and dietary indicators. The sample group was comprised of 94 adolescent females 14 $\pm$ 0.5 years of age, from two race groups (black and white) and two income categories ($\leq$ \$1,200/capita/year and $\geq$ \$2,000/capita/year). Fasting blood was collected and analyzed by radioassay methods for both serum and red cell folate concentrations. Two 24-hour dietary recalls were also obtained from each subject. Eighty-two percent of the girls consumed 2/3 or less of the RDA for folate, with 36% taking in less than 1/3 of the RDA. Among the white, black and low-income groups there was a statistically significant ($P<0.05$) correlation between dietary folate and kilocalories consumed. Only 6.5% of all subjects (11% of the whites and 3.0% of the blacks) were marginal or deficient in both serum and red cell folate levels. Thirty percent of the subjects had marginal or deficient serum folate levels and 8% of all subjects had deficient folate stores as indicated by deficient red cell folate levels. Implications of presented results regarding adolescent folate status and Recommended Dietary Allowances for folate are discussed.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Adolescence is a stage of life marked by accelerated physical growth and maturation. The rapid linear and weight growth, and organ development, create increased nutritional demands on the individual. Proper nutrition is paramount to this developmental sequence in life, which is neither preformed nor automatic. It is, rather, dependent upon an interaction between biological (genetic) capacity and environmental stimulation. Sound physical maturation then is contingent upon adequate physical (cellular) nutrition. Folic acid is one nutrient that is needed during this stage of rapid growth, but folate intake can be inherently low in the adolescent diet due to poor dietary choices and habits.

Occurring simultaneously during this adolescent growth spurt is a unique period of intellectual/ emotional awareness. The teenage adolescent faces increasing mental pressure from the realization that certain behavior patterns and responsibilities are now expected of him/her. Peer group acceptance and compliance at this point seem the only security against this anxiety. One of the primary familial or adult norms often rejected as a result of this identity crises is a nutritionally adequate diet. This occurs as a result of peer group pressure and an independence from the family unit. Fashionable fast or convenience foods with low nutrient densities characterize the mainstay of the adolescent's diet, with balanced meals at home consumed

on an irregular basis. Because folate is predominately found in the fresh vegetable, fruit and whole grain foods, it is one of the nutrients often consumed in low quantities in the adolescent diet.

Together these two circumstances, increased requirements and decreased intake of folate, constitute the basis for suspecting the teenage adolescent of poor or inadequate folate nutriture.

In this study, a follow-up to a two-year longitudinal study, the folate status of adolescent girls was evaluated. The objectives of this study were:

1. to evaluate folate status as indicated by serum and red cell folate levels.

2. to study the relationship of folate status to factors that may directly or indirectly influence these values: income, race and dietary intake.

CHAPTER II

REVIEW OF LITERATURE

A. ADOLESCENT FOLATE NUTRITURE

The incidence of poor folate nutriture during adolescence has not been well documented. Indications from the few studies done (1-5) are that serum and red cell folate levels in adolescent girls are generally at acceptable levels. However, in two recent studies, over 90% of the girls studied consumed less than 50% of the RDA (1,2).

Further research needs to be done to assess our approach to adolescent folate nutriture so that we are confident with this aspect of their nutrition. The major factors which must be kept in mind that influence folate status in adolescence are the rate of body growth and folate loss. Also, recent research indicates that amount and availability of folate derivatives ingested, and stress situations such as pregnancy in adolescence, must be considered (6-13).

Rate of Growth

The adolescent stage of life is one showing accelerated growth and development in the human being (14,15). It is the combination of linear/weight growth and gonadal organ development that accentuates physiological demands for essential nutrients (14-17). The velocity of this physical growth in adolescence is second only to the rate of growth during infancy, manifested by an increase in total body mass as well as reproductive maturity (14,17). Because folic acid coenzymes are required for one-carbon fragment transfers necessary in the

biosynthesis of purines and pyrimidines, components of RNA and DNA molecules, increases in total cell number and/or rate of cellular synthesis will create greater folate needs (6-8).

Studies by Daniel et al. (1) indicated that males had plasma folate concentrations lower than females even though their intake was greater. Daniel et al. attributed this paradox to the greater need for folate in rapid cell growth, especially seen in males who may double their muscle mass during adolescence (14). The same study also noted that girls exhibited a negative slope for plasma folate concentrations as related to increasing developmental maturity during adolescence. They postulated that this decrease may also be related to increased cellular growth, with tissue storage and changes associated with the menstrual cycle as other possible influences.

Rate of Loss

There is little information available concerning body turnover rates of folate compounds as they would be estimated by metabolic balance studies. Because the sources of fecal folacin activity, the metabolic end products of folate metabolism and the route(s) of excretion are still uncertain, the factorial method of ascertaining human folate requirements is currently not feasible; however, many investigators have contributed to various pieces of the puzzle concerning the metabolic fate of body folacins.

It is known that normally nourished individuals excrete 5-40 µg of free (folate activity for L. casei growth) folate in the urine each day (18). It is not known, however, what other body pteridines, ultimately excreted in the urine, are initially derived from folacin

or synthesized de novo (19). Cooperman et al. (19) found low urinary folacin activity (1-12 μg L. casei or S. faecalis folacin activity per day) which remained constant over a wide range of daily dietary intakes.

Since bacteria in the colon synthesize folate, fecal excretion is not a reliable indicator of dietary folate absorption. The fecal measurement of folate compounds has been reported to be 5-15 times the amount of ingested folacin activity. Possible sources of this folacin activity include unabsorbable dietary folate compounds, folate compounds synthesized by bacteria in the colon and oral cavity, intestinal mucosal slough-off, endogenous salivary sources and bile. Baker et al. (20) found that bile contains approximately five times the folacin activity of serum. These authors reported a range of 10-89 μg L. casei activity/ml bile, suggesting that enterohepatic recirculation is a factor that would tend to conserve the body pool of folate compounds, since most bile folate is believed to be reabsorbed.

Other reported levels of measured folacin loss from the body are salivary secretions (3.8-40 ng/ml/day folacin activity) (21) and dermal losses of 30 μg/day folacin activity (22).

Food Sources

Naturally occurring folates in the diet are derivatives of pteroylglumate (PteGlu), conjugated with up to seven γ-glutamyl residues, and usually containing either a formyl or methyl derivative on the pteridine ring (7,23-25). The polyglutamates ($PteGlu_x$) comprise

the majority of naturally occurring folates, accounting for more than 70% of dietary compounds (25-28). The polyglutamates are principally 5-CH_3-H_4-$PteGlu_x$, while the remaining monoglutamates are mainly 10-CHO-H_4-$PteGlu_1$ (23,25).

The determination of the types and amounts of folate compounds in biological materials has been reviewed (25,27-29,30) indicating the problems of obtaining accurate assays of foods for folate derivatives. These problems include low concentrations of folate compounds in most food samples, and their susceptibility to destruction or change in structure by light, heat, pH, oxygen and endogenous food folate conjugases. Hurdle et al. (31) have reported that as much as 50-95% of the folacin activity in food may be destroyed by cooking and/or processing. It is obvious when referring to the literature that no extensive lists of food folate contents are available, whether as measured raw or cooked. The few studies done that are reliable (26,27,29), indicate that total folacin content of foods is the best way to report content for usage in intake studies because it is a consistent and easy to use figure. Further improvements on the method of assay for folates in food and their availability, as eaten, are needed.

Folate derivatives are present in a wide variety of foods (28,31). Good sources include toasted wheat germ, having 420 µg total folacin activity/100gms; almonds, 96 µg/100gms; all dry legumes except lentils, greater than 100 µg/100gms; green leafy vegetables like spinach, 193 µg/100gms; and beets, 133 µg/100gms.

Availability

To determine folate requirements, an estimation of the availability of the ingested dietary folate derivatives is necessary. Babu and Srikantia (29) recently found the mean availability of folate compounds in seven different foods, determined by urinary excretion after dose food loads, to be close to 50%, ranging from 37-72%. Babu and Srikantia (29) noted a wide variation among individuals and foods eaten. Other authors, compared in a recent review (27), also agree with Babu on the relative availability of folate compounds.

Clinical and experimental evidence (32-36) indicate that folate compounds are absorbed preferentially in the proximal jejunum. The relatively unknown effect of conjugase inhibitors, as well as the effect of the intragastric and intraintestinal environment on the stability of various folates has made it difficult to determine bioavailability of food folates.

It has been adequately demonstrated that folic acid and its monoglutamyl derivatives (including diglutamates), are more available than the polyglutamyl forms (37-40). Polyglutamyl derivatives undergo hydrolysis to predominately the monoglutamate form (or di/tri-glutamate forms) before uptake into the mesenteric circulation (41-47). Monoglutamyl derivatives, including diglutamates, can enter and traverse the intestinal mucosal cell into the mesenteric circulation without any chemical modification (39), although significant carbon-unit addition to and reduction of PteGlu does occur in the jejunal mucosal cells (41,43). Folate conjugases found in the intracellular lysosomal fractions (47,48) and brush border regions (49) of the mucosal cells

have been identified. The overall rate of transport of folate derivatives into the mesenteric circulation, however, is postulated to be controlled by the movement of monoglutamates alone (38).

Although conjugase inhibitors are present in certain foods and do reduce the absorption of polyglutamyl derivatives (50), no case of conjugase deficiency resulting in a folate deficiency has been reported. Since the rate of hydrolysis of polyglutamyl folated substantially exceeds overall transport rates in the intestinal mucosal cell (41), only a severe effect on the hydrolytic process would result in a slowing of transport, whereas any direct effect on the transport process would cause malabsorption of dietary folates (33,34). In diseases such as celiac or tropical sprue where loss of normal intestinal mucosa is prevalent, malabsorption of folate compounds is indeed linked to transport defects (34,36).

The intestinal pH is also a factor which is important in the utilization of ingested folates. Folate conjugases are pH dependent enzymes which exhibit reduced activity in alkaline pH (51,52).

A recent study by Tamura et al. (53) has shown the effect of zinc deficiency in man on polyglutamyl folate absorption. In this study it was not ascertained whether the conjugases were zinc metalloenzymes, however, in a zinc-depleted state decreased intestinal hydrolysis of the polyglutamates was found, indicating that zinc was essential for maintaining normal conjugase activity.

B. RECOMMENDED DIETARY ALLOWANCES (RDA) FOR FOLIC ACID

The majority of research evidence suggests that 25-50% of dietary folates are nutritionally available (26,29,35,44), and that 100-200 µg of PteGlu are needed daily to maintain an adequate store of folic acid (54-57). The Food and Nutrition Board (56) has set the RDA at 400 µg of total folacin activity per day. This figure is exclusive for normal, non-pregnant, non-lactating adults and adolescents. The 400 µg/day figure is an expression of total $PteGlu_1$ equivalents assayable with L. casei in extracts of diets treated with conjugase. It is important to note that the range of variability among individuals has not been established. Findings by Babu and Srikantia (29) support this fact, indicating that there are considerable variations between persons in absorption of folates from foods, and that in some people marked variations were noticed in absorption from different foods.

C. FOLIC ACID DEFICIENCY

Studies by Sullivan and Herbert (12) have suggested that the minimal amount of folic acid required from the diet to maintain a normal hematological picture is approximately 50 µg/day. Other studies agree with this estimate (57-60). Estimates of daily dietary intakes of folate compounds in healthy adults consuming a mixed diet have been shown to be between 129-300 µg/day (61), which is in agreement with others (13,26).

Recent studies by Elsborg and Rosenquist (2), and similar studies done by others (1,4,5), revealed that a low dietary intake of

folic acid was found in a high percentage of adolescent girls. Elsborg and Rosenquist found that 90% of the 110 girls consumed less than 100 µg folate/day. The mean intake level in this study was 70 µg (range 21-121 µg), with 16% of the subjects consuming less than 50 µg/day. In comparison, Daniel et al. (1) showed that 95% of the girls studied (n=257) had intakes of less than 200 µg folate/day (50% of RDA). Both studies concluded that because of possible low values in food tables and limited lists of foods containing folate derivatives, these percentages might not be truly indicative of poor intake.

Determination of Folate Status

Measurement of serum and red cell folate levels is the most practical way to assess folate nutriture for application to population studies. Sauberlich et al. (62) have reviewed the diagnostic capabilities of these measurements, indicating that low values for both serum and red cell folate are strong evidence that a folic acid deficiency exists. Sauberlich et al. (62) found, and others agree (63-66), that red blood cell folate levels are a more accurate and less variable quantitative index of folate status than serum folate. The advantage of red blood cell folate measurements over those of serum folate in the diagnosis of folate deficiency is explained by the relative rates of change with subnormal dietary intake. The drop in serum folate to below normal levels occurs well before the appearance of abnormal hematological signs, whereas low red cell folate levels are measured only just before the diagnosis of a megaloblastic bone

marrow (63,67). Thus low serum levels may be just a reflection of recent low intakes or negative folate balance, neither of which is a single positive predictor of poor folate status.

Microbiologic assay procedures using L. casei have been the most widely used method to measure folate levels in both serum and red blood cells. Folate measured by radioisotope dilution and binding methods have also been reported, with comparable results and accuracy to microbiological methods (68-70).

Diagnosis

Parameters used for assessing folate status by either method of assay have been described by Sauberlich et al. (62). For serum levels, less than 3.0 ng/ml is considered deficient, 3.0-5.9 ng/ml low, and 6.0 ng/ml and above considered normal. A deficient red cell level is less than 140 ng/ml folate, whereas 140-159 ng/ml is low and above 159 ng/ml considered acceptable.

Clinical assessment of folate deficiency is difficult because the megaloblastosis caused by vitamin B_{12} deficiency and folate deficiency is indistinguishable except through direct measurement of the respective vitamin. A depletion of the tetrahydrofolate pool occurs if a deficiency of the B_{12} coenzyme methylcobalamin exists, which would be explained by the methylfolate-trap concept of their relationship (65). People with primary B_{12} deficiency often have elevated serum folate levels, while low levels may be encountered in the red cell (59). Other clinical observations that are indistinguishable except by direct vitamin measurement are neutrophil hypersegmentation, urinary formiminoglutamate excretion and

megaloblastic bone marrow resulting in megaloblastic anemia (67,71). Hoffbrand et al. (65) have reported a correlation between hemoglobin and red cell folate levels when both are at low levels in patients studied.

Other methods for diagnosing folate deficiency also exist. The types and/or ratio of the folates in serum and red cells may possibly be a better indicator of folate status than total serum values (43,72). Dawson (72) found that following the absorption of folic acid, a rise in 5-CH_3-H_4-PteGlu in the systemic circulation was directly related to the folate content of the liver. Hoffbrand et al. (65) have reported that red cell levels paralled formiminoglutamate excretion in the patients examined and that this was an indirect measure of liver folate concentration. One author has suggested measurement of serum 10-CHO-H_4-PteGlu, which normally is relatively constant, to ascertain cell replication rate and hence folacin sufficiency (73). Also the amount of free versus protein-bound folate, the amount of unsaturated folate binding protein in the serum, and the distribution of folacin activity among folate-binding proteins may be useful in evaluating folate status (70). Recent reports (67,74) indicate that reticulocytopenia and macroreticulocytosis, and neutrophil hypersegmentation are among the earliest hematological occurrences in the development of folate deficiency. These conditions, which are present before the mature red cells become large and immediately follow the appearance of low serum folate levels, precede the lowering of mean red blood cell folate levels and could, therefore, be useful as an early index of folate deficiency.

Complications

The role of folacin compounds involved in single carbon-unit transfers throughout the body have been thoroughly described (6-8), indicating the importance of this vitamin in the synthesis of the purine nucleus, the synthesis of n-formyl methionine t-RNA, interconversions of glycine from serine, the methylation of homocysteine to form methionine, and in the synthesis of thymidylic acid. A deficiency of folic acid is generally recognized morphologically by megaloblastic changes in the most rapidly proliferating cells, namely erythrocytes, granulocytes and platelets. Hence, the hematological system is generally considered to be damaged first and most drastically by a folacin deficiency. Epithelial cells in the gastro-intestinal and genito-urinary tracts also turnover rapidly, showing megaloblastosis when deprived of folic acid compounds (63,67,71,73).

The course of folate deficiency in humans has been thoroughly reviewed (8,11,75). As outlined (6,67,75,76), the biochemical and hematological sequence of events in dietary folic acid deficiency are as follows: low serum folate for 3 weeks; followed by hypersegmentation of polymorphonuclear cells at 7 weeks; high urinary formiminoglutamate excretion at 13.5 weeks; low red blood cell folate at 17.5 weeks; elliptical erythrocytes at 18 weeks; megaloblastic bone marrow at 19 weeks; and anemia at 19.5 weeks. These clinical signs appear in patients that were either followed in the laboratory and on folate deficient diets, or from actual cases.

Complications from folacin deficiency resulting from megaloblastic anemia have been reviewed (11,54), revealing that periods

of growth, pregnancy and lactation manifest the most detrimental effects of poor status. Daniel et al.'s (4) study regarding obstetric and fetal complications in folate deficient girls concluded that low dietary intake and low blood folate levels are not significant factors in obstetric complications of young (pregnant) adolescent girls. However, the actual time period of deficiency was not stated, nor any fetal complications. Complications identified by other investigators to be due to severe folate deficiency are neurologic and psychiatric disorders (77,78), low birth weight infants (79) and GI-tract disorders (80).

Prevalence

Studies conducted in the early 1900's formed the basis for the history of folate deficiency. A crude commercial yeast extract which was found to contain good quantities of folacin activity was used successfully in the treatment of a macrocytic anemia found in the course of pregnancy and distinct from pernicious anemia. Folate deficiency has been most commonly recognized in pregnant women (55,59,81). A 30-50% incidence of serum or red cell deficiency has been reported among females who are pregnant and not taking supplements containing folic acid (60,82,83). Lactation, rapid growth, alcoholism and malabsorption syndromes are other factors contributing to folate deficiency (16,34,54,61,67,73).

Very few studies of adolescent folate status are available in the literature. Daniel et al. (1) recently studied adolescent folate nutriture and found that 4.7% of the 108 low-income girls studied had subnormal plasma folate (2.0 ng/ml). When the girls were evaluated

according to race, 10% of the white girls and 9.0% of the black girls had subnormal plasma folate concentrations at adolescent maturity (approximately 14 years of age). Other studies (3-5) report that adolescent girls generally have sufficient folate levels, either serum, plasma or red cell.

It is important to consider the possibility of adolescent pregnancy (84) which would cause additional stress on folate stores. In 1977 the birth rate of adolescent girls under 15 years of age was 1.2/1000 live births, of which 15% were low-birth weight infants; the 15-17 year old rate was 34.5/1000 live births (85).

CHAPTER III

EXPERIMENTAL PROCEDURE

A. GENERAL PLAN

The S-87 Regional Nutrition Project began in 1974 with approximately 120 pre-adolescent girls age 9 $\pm$ 0.5 years. Data were again collected on the girls in 1975 and 1976. The present restudy carried out in 1979 involved 94 of these girls, now adolescents, age 14 $\pm$ 0.5. The racial distribution of subjects in the original group was chosen to be 50 percent white and 50 percent black, with an equal distribution of low income families ($\leq$ \$1,200/capita/year) and middle income families ($\geq$\$2,000/capita/year) within each of these groups. The subjects were selected initially through the city and county school systems of Knox County, Tennessee, and had no known metabolic disorders. Informed written consent was obtained from the parents or guardians. Dietary intakes and fasted blood levels of folic acid were evaluated to determine folate status.

B. COLLECTION METHODS

24-Hour Dietary Recalls

Dietary information was obtained on two occasions from each subject using 24-hour recalls of food intake. The first 24-hour recall was completed approximately two weeks prior to the day of blood collection, and the second taken the same day as blood drawing. The recalls were obtained by trained interviewers using food models to

assist in determining accurate food portion sizes. Nutrient composition of the summarized dietaries was calculated using the Extended Table of Nutrient Values.[1]

Venous Blood Samples

Following an overnight fast, 13 ml of venous blood were drawn from each subject by a nurse or medical technician from the University of Tennessee Memorial Hospital. All subjects received written instructions prior to the day of collection concerning the fast and blood collection. Three milliliters of blood were drawn into heparinized vacutainer tubes to facilitate the hematocrit determinations. Ten milliliters of blood were drawn into non-heparinized vacutainer tubes and allowed to coagulate. Following coagulation the blood was centrifuged at 664 x g for 10 minutes. Each serum sample was extracted from the clotted blood using a Pasteur pipet, placed in a test tube, stoppered, and stored at -12^{o} until it was needed for analyses.

C. EQUIPMENT

The Damon/IEC Division Centrifuge Model No. HN-S[2] was used for centrifugation in all analyses. All radiation evaluation was measured with the Beckman Bio-Gamma II® Spectrophotometer.[3]

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[1]International Dietary Information Foundation, Inc., P.O. Box 38143 Atlanta, GA. 30334.

[2]Damon/IEC Division, Needham Hts., MA 02194.

[3]Beckman Instruments, Inc., Fullerton, CA 92634.

D. ANALYSES OF BLOOD

Serum Folic Acid

Serum folic acid was measured using the Bio-Rad[4] method of radioassay, based on the principles of folate radioassay described by Waxman and Schreiber (68) and Dunn and Foster (69). Measurement of folate in serum and red cell was done according to the Quanta-Count® folate procedure. A measured amount of patient serum is mixed with a folate protecting buffer and radioactively labeled folate ^{125}I (PGA) derivative. Under the influence of heat (water-bath at 100^{o}), folate binding proteins are inactivated while the protective agent (borate buffer and dithiothreitol) stabilizes the folate.

The mixture is incubated with a measured amount of binding protein (folate binding milk factor). The amount of folate binding protein is sufficient to bind some, but not all of the labeled and unlabeled folate present in the mixture. During this incubation period, unlabeled and labeled folate are competing for the available binding sites of the binding protein on the basis of concentration; the more unlabeled folate in the mixture, the less binding of labeled folate occurs. After incubation, the bound folate is separated from the unbound folate by charcoal absorption and centrifugation processes. The degree to which the binding of labeled folate is inhibited by the unlabeled folate is a measure of the endogenous folate concentration in the serum.

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[4]Bio-Rad Laboratories, Richmond, CA 94804.

Reagents

1. Folate zero standard: a lyophilized folate-free protein base and preservative.

2. Folate zero standard stock solution: zero standard reconstituted by adding 2.0 ml of distilled water to the vial and stored at 2-8°.

3. Folate standards: equivalent to 1.0, 2.5, 5.0, 10.0 and 20.0 nanograms of folate (PGA), lyophilized, in a protein base (phosphate-buffered albumin saline, pH 7.4) and preservative (mercaptoethanol).

4. Folate standard stock solutions: standard equivalents reconstituted by adding 1.0 ml of distilled water to each vial and stored at 2-8°.

5. Folate binding protein: a lyophilized folate binding protein (beta-lactoglobulin) and preservative (mercaptoethanol).

6. Folate binding protein stock solution: binding protein reconstituted by adding 1.0 ml of distilled water to the vial and stored at 2-8°.

7. Folate binding protein working solution: 1.0 ml of stock solution mixed with 4.0 ml of distilled water.

8. Adsorbent tablets: hemoglobin coated charcoal tablets (1:20) stored at 20-25° in a dry place.

9. Borate buffer: 120 ml of borate-saline buffer and stabilizer stored at 2-8°.

10. Dithiothreitol (DTT): 240 mg of lyophilized dithiothreitol stored at 2-8°.

11. Dithiothreitol-buffer solution: DTT combined with 4.0 ml of borate buffer and agitated gently; then quantitatively mixed with the rest of the borate buffer and stored at 2-8°. The pH of this solution was 9.4.

12. Folate ^{125}I (PGA) tracer stock solution: one vial with less than 10 µCi of lyophilized and radioactively labeled PGA ^{125}I derivative and preservative with 1.3 ml of distilled water added to it, mixed gently, and stored at 2-8°.

13. Folate ^{125}I (PGA) working solution: prepared by diluting the stock solution 1:100 with the DTT-Borate buffer mixture. One milliliter of this working solution was required for each assay and was prepared only immediately prior to each assay run.

Method

12x75 mm glass reaction tubes were labeled appropriately, two for each blank, standard and patient's blood. One microliter pipettes were used to deliver the necessary amounts of reagents and blood. To two reaction tubes labeled "blank", 100 µl of zero standard was added. To each of duplicate standard tubes, 100 µl aliquots of the respective standard was added. One hundred microliters of patient serum was pipetted into duplicate tubes for each subject. One milliliter of working tracer solution was pipetted into all tubes, mixed gently by hand afterwards. At this point, a total counts tube was prepared by adding 1.0 ml of working tracer solution directly into polypropylene counting vials designed specifically for use with the Beckman Bio-Gamma II® spectrophotometer. These were set aside until counting commenced.

All tubes were placed in test tube racks and set in a 98° water bath for 15 minutes. Each tube was capped loosely with rubber stoppers. After 15 minutes all tubes were cooled to room temperature (20-25°).

To the blank tubes, 1.0 ml of distilled water was added. To all remaining tubes, the standards plus patient samples, 1.0 ml of folate binding protein working solution was added. These tubes were mixed well manually and then incubated at approximately 23° for 30 minutes.

At the end of the incubation period one adsorbent tablet was added to each tube which was then allowed to stand for 5 minutes. The tubes were then mechanically mixed[5] for 10 seconds and allowed to stand for another 5 minutes. All tubes were then centrifuged[1] at 664 x g for 10 minutes. The supernatant fluid was then decanted into polypropylene counting vials, which were placed in the spectrophotometer for a period of 100 minutes or 10,000 radiation units, whichever came first.

Calculations

The recorded counts per minute (cpm) of the blanks were subtracted from the uncorrected cpm of the standards and patient samples to obtain a corrected cpm. The corrected cpm of each standard and patient sample (B) was then divided by the cpm of the total counts

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[5]Vortex-genie, Scientific Industries, Springfield, MA 01103.

tube (T). This quotient was multiplied by one hundred to obtain a percentage of bound labeled folate as a function of the total counts;

uncorrected cpm - average blank cpm = corrected cpm (B)

$$\frac{(B)}{(T)} \times 100 = \% \; B/T$$

A standard curve was plotted on linear graph paper, with %BT on the ordinate against concentration of folate in ng/ml on the abcissa. A smooth curve was drawn through these points and each patient sample's %B/T was interpolated from the curve into ng/ml.

Red Cell Folate

The procedure for the determination of red cell folate utilizes the same reagents and methodology as that used for serum folate measurements. However, because whole blood is used, a dilution has to be made since whole blood contains about 22 times the folate found in serum. Also, the sample has to be preserved with an ascorbic acid solution.

Reagents

All reagents required for serum folate assays were necessary for this measurement. In addition, the following reagents were needed:

1. Whole blood diluent: lyophilized folate-free protein base (phosphate-buffered albumin saline, pH 7.4) and preservative (mercaptoethanol).

2. Red cell folate diluent: whole blood diluent reconstituted by adding 5.0 ml of distilled water to the vial, which was then allowed to stand for 30 minutes and stored at -12^{o}.

3. Ascorbic acid: one gram per vial of crystalline ascorbic acid U.S.P., stored at room temperature.

4. Ascorbic acid solution: two-tenths of a gram crystalline ascorbic acid quantitated to 15.0 ml with sufficient distilled water and then mixed by gentle inversion to make a 0.4% mixture.

Method

After mixing thoroughly, 100 µl of each patient sample was pipetted into a reaction tube which contained 1.0 ml of the ascorbic acid solution. The contents were mixed mechanically[5]. The hemolysate was allowed to stand for 10 minutes at room temperature.

At this point 100 µl of each patient's hemolysate was pipetted out and added to another tube. One-hundred microliters of the red cell diluent was then added to this tube and the contents were mixed well. The original whole blood was now diluted 1:22. One-hundred microliters of the diluted blood were then added to a labeled tube. The same procedure followed for the serum assays began at this point.

Calculations

Calculation of the ng/ml of red cell folate in each dilution of whole blood was done the same way as for serum folate determination; however, the value obtained from interpolation from the standard curve was multiplied by 22 to account for the dilution of the whole blood. This value was then divided by the hematocrit obtained previously[6], to calculate the red cell folate concentration.

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[6]B. Thurman (1980), Iron Status of Adolescent Girls. Unpublished Master's thesis, The University of Tennessee, Knoxville, TN.

$$\text{Red cell folate} = \frac{\text{Whole blood folate - serum folate}}{\text{Hematocrit/100}}$$

F. STATISTICAL ANALYSES

Data collected were analyzed at The University of Tennessee Computing Center using the Statistical Analyses System (SAS79) developed by Barr et al. (86) and the Statistical Package for the Social Sciences (SPSS) developed by Nie et al. (87). Least squares means and standard errors of the least squares means were determined for dietary intake and biochemical measurements by race, income and race/income categories. The least squares means were used because they allow a better comparison between groups of different sizes by accounting for the greater or smaller variances within these unequal samples. Pearson correlation coefficients, analysis of variance and Scheffe tests of least squares mean differences among dietary and biochemical data were calculated by computer also.

CHAPTER IV

RESULTS

A. DIETARY FOLATE INTAKE

Mean dietary intakes of folate calculated for the eight race and/or income groups are shcwn in table 1. Means are shown for diets including supplements and exclusive of supplements. An analysis of variance was performed to test the effects cf race and/or income groups on mean intake levels; the conclusions were that the means are not significantly different. Across all groups the mean intake of folate, whether with supplements or without, was below 200 µg/day (50% of RDA).

The mean intakes of folate per 1000 kilocalories are reported in table 2. Nutrient density of the diets for folate was calculated from dietary intake without supplements containing folate included. The low-income blacks (LIB) had the highest overall mean folate intake per 1000 kilocalories, and the low-income whites (LIW) had the lowest mean.

A comparison of the dietary intake of folate to the 1980 Recommended Dietary Allowances (RDA) for folate is shown in figure 1. The four levels of comparison, expressed as precentages of the RDA, are 33%, 33-66%, 67-100% and >100%. Bar heights represent the percentage of subjects in each of the race or income groups that fell into the specified categories of the RDA. The distribution of all girls among the four categories was similar to the race classification

TABLE 1

Effect of race and income levels on dietary intakes of folate in 14 ± 0.5 year old girls

Parameters	Group							
	White	Black	Low Inc.	Middle Inc.	LIW	MIW	LIB	MIB
	µg/day							
Dietary								
Total folate With supplements	179.9 ± 23.7[1,2]	172.9 ± 19.1	181.1 ± 20.1	171.0 ± 22.8	184.6 ± 29.8	175.3 ± 36.9	179.1 ± 27.0	166.8 ± 27.0
Total folate W/out supplements	160.6 ± 20.8	164.9 ± 16.8	173.0 ± 17.7	152.5 ± 20.1	175.9 ± 26.2	145.3 ± 32.4	170.1 ± 23.7	159.6 ± 23.7
No. of subjects	38	56	51	43	23	15	28	28

[1] Means ± SEM

[2] Analysis of variance showed no effect of race or income on mean differences.

TABLE 2

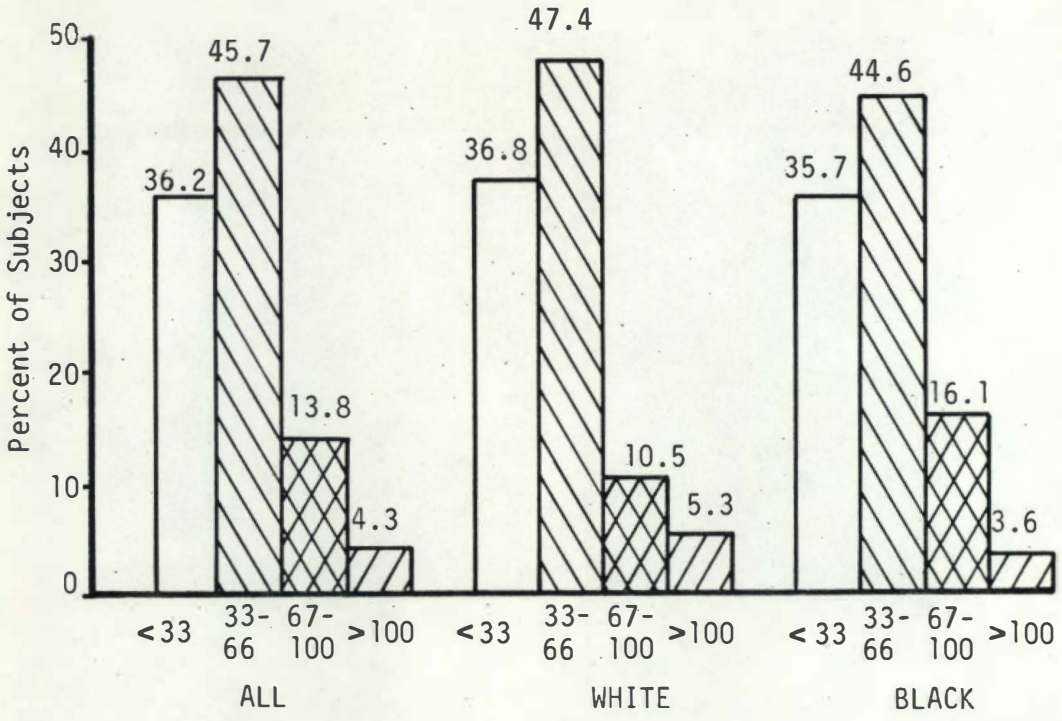
Dietary intakes of folate per 1000 kilocalories[1]

	Group[2]			
	LIW	MIW	LIB	MIB
	µg/1000 Kcals			
Folate	81.5 ± 17.3 [3,a]	83.6 ± 10.3 [a]	93.4 ± 11.8 [a]	91.0 ± 13.2 [a]
No. of subjects	23	15	28	28

[1]Data are without supplements.

[2]LIW = low-income whites, MIW = middle-income whites, LIB = low-income blacks, MIB = middle income blacks.

[3]Means ± SEM. [a]Means not sharing common superscript are significantly different ($P<0.05$).



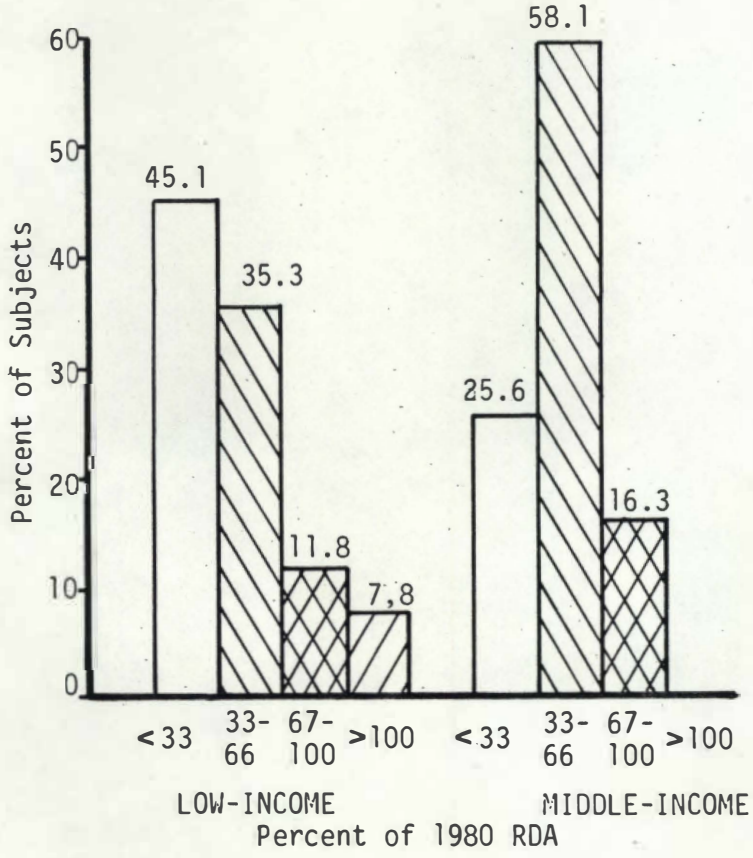


Figure 1. Comparison of dietary intakes as percentages of the Recommended Dietary Allowances 1980, by race (A) and income (B) groups. Data are with supplements.

distribution. Only 4.3% of all subjects (n=94) consumed over 100% of the RDA for folate, with only 18.1% meeting at least two-thirds of the RDA or greater. Clearly the majority of subjects (82% of total) took in less than two-thirds of the RDA.

B. VITAMIN/MINERAL SUPPLEMENTATION

The dietary data were recorded with and without supplements. Percentages of girls consuming vitamin/mineral supplements are recorded in table 3. The percentage of girls consuming folate-containing supplements is also reported in table 3. Approximately 14% of the subjects took vitamin/mineral supplements. Fifty-four percent of those subjects took supplements containing folate. The greatest percentage of girls consuming supplements was in the middle-income white (MIW) group, with 40% of the 15 girls taking supplements; in contrast, only 4.3% of the LIW group took supplements.

C. BIOCHEMICAL MEASUREMENTS

Means of serum and red cell folate for race, income and race/income groups are presented in table 4. A comparison of means by a regression analysis revealed no significant influences or effects of these population categories on mean values. The mean values for both serum and red cell folate levels were within normal limits in all groups.

The percentage of subjects from each race and/or income group that were either marginal or deficient in serum or red cell folate levels is shown in table 5. Overall 30% of those measured for serum folate (n=77) had either marginal or deficient values, while only 12%

TABLE 3

Percentage of subjects taking vitamin/mineral supplements with folate and without

	Group[1]				
	LIW	MIW	LIB	MIB	ALL
	%	%	%	%	%
Supplements	4.3[2] (1)	40.0 (6)	10.7 (3)	10.7 (3)	13.8 (13)
Supplements With folate	4.3 (1)	20.0 (3)	7.1 (2)	3.6 (1)	7.5 (7)
No. of subjects in study	23	15	28	28	94

[1]LIW = low-income whites, MIW = middle-income whites, LIB = low-income blacks, MIB = middle-income blacks.

[2]Values in parenthesis are the number of subjects represented by each percentage.

TABLE 4

Effect of race and income levels on biochemical measures of folate status in 14 ± 0.5 year old girls

Parameters	Group[1]							
	White	Black	Low Inc.	Middle Inc.	LIW	MIW	LIB	MIB
	ng/ml							
Biochemical								
Serum folate	9.9 ± 0.9[2,3]	10.4 ± 0.7	10.2 ± 0.7	10.0 ± 0.9	9.2 ± 1.1	10.5 ± 1.5	11.3 ± 1.0	9.5 ± 1.1
No. of subjects	(31)	(46)	(44)	(33)	(20)	(11)	(24)	(22)
Red cell folate	340.3 ± 32.8	349.2 ± 27.5	337.0 ± 28.5	352.5 ± 31.9	316.2 ± 40.8	364.5 ± 51.3	357.8 ± 39.8	340.6 ± 37.9
No. of subjects	(31)	(42)	(39)	(34)	(19)	(12)	(20)	(22)

[1] LIW = low-income whites, MIW = middle-income whites, LIB = low-income blacks, MIB = middle-income blacks.

[2] Means ± SEM.

[3] Analysis of variance showed no effect of race or income on mean differences.

TABLE 5

Percentage of marginal and deficient biochemical measurements of folate status by race and income level for 14 ± 0.5 year old girls

Parameters	Criteria	Group[1]								
		White	Black	Low Inc.	Middle Inc.	LIW	MIW	LIB	MIB	All
	ng/ml					%				
Serum folate	Marginal (3.0-5.9)	32.2	19.6	22.7	27.3	35.0	27.3	12.5	27.3	24.7
	Deficient (3.0)	9.7	2.2	4.5	6.1	5.0	18.0	4.2	0.0	5.2
	N=	(31)	(46)	(44)	(33)	(20)	(11)	(24)	(22)	(77)
Red cell folate	Marginal (140-159)	6.4	2.4	2.6	5.9	5.3	8.3	0.0	4.5	4.1
	Deficient (140)	9.7	7.1	7.7	8.8	15.8	0.0	0.0	13.6	8.2
	N=	(31)	(42)	(39)	(34)	(19)	(12)	(20)	(22)	(73)
Serum and red cell folate	Marginal or Deficient	11.1	2.9	5.5	8.0	11.1	11.1	0.0	6.3	6.5
	N=	(27)	(34)	(36)	(25)	(18)	(9)	(18)	(16)	(61)

[1]LIW = low-income whites, MIW = middle-income whites, LIB = low-income blacks, MIB = middle-income blacks.

of the girls tested for red cell folate (n=73) were classified as being marginal or deficient. White girls had a greater percentage of subjects at a potential nutritional risk from marginal or deficient serum and red cell levels (42% and 16% of total, respectively) than blacks (22% and 10%, respectively). Comparison by income indicates greater percentages of middle-income girls (32% and 14%) in these two categories for serum and red cell measurements compared to low-income girls (27% and 10%). Combined race/income groups further highlighted the trend of whites having greater percentages of subjects marginal or deficient in serum and/or red cell folate. The subjects who were marginal or deficient in both serum and red cell folate represent the group at greatest nutritional risk from poor nutriture; only 6.5% of all subjects were classified as such. Whites had the highest percentage (11.1%) in this high-risk category, and middle-income girls, with 8% of total subjects, were the next highest and most likely to exhibit poor folate nutriture.

D. RELATIONSHIPS AMONG DIETARY AND BIOCHEMICAL DATA

Investigation of the relationships among dietary folate values and biochemical determinations of folate status was another objective of this study. Pearson correlation coefficients were calculated and are presented in tables 6 through 9 for the two race and income classifications in this group of 14 $\pm$ 0.5 year old girls. Data reported in these tables include supplements. Significant positive correlations existed between serum and red cell folate ($P<0.0001$), red cell folate and dietary folate ($P<0.01$) and dietary folate and kilocalories ($P<0.05$) in white girls (table 6). Table 7 represents

TABLE 6

Pearson correlation coefficients among dietary and biochemical variables for white girls age 14 ± 0.5 years[1]

	Serum Folate	Red Cell Folate	Hemoglobin	Dietary Folate[2]
Red cell folate	0.70[a]			
Hemoglobin	-0.22	-0.11		
Dietary folate	0.17	0.50[b]	0.16	
Kilocalories	-0.14	0.02	0.33	0.42[c]

[1] [a](P<0.0001) [b](P<0.01) [c](P<0.05)

[2] With supplements

TABLE 7

Pearson correlation coefficients among dietary and biochemical variables for black girls age 14 ± 0.5 years[1]

	Serum Folate	Red Cell Folate	Hemoglobin	Dietary Folate[2]
Red cell folate	0.65[a]			
Hemoglobin	0.06	0.14		
Dietary folate	-0.07	0.07	0.43[b]	
Kilocalories	-0.13	0.02	0.12	0.28[c]

[1] [a](P<0.0001) [b](P<0.005) [c](P<0.05)

[2] With supplements

TABLE 8

Pearson correlation coefficients among dietary and biochemical variables for low-income girls age 14 ± 0.5 years[1]

	Serum Folate	Red Cell Folate	Hemoglobin	Dietary[2] Folate
Red cell folate	0.60[a]			
Hemoglobin	-0.33	-0.07		
Dietary folate	0.12	0.51[b]	0.31[c]	
Kilocalories	-0.04	0.11	0.20	0.38[c]

[1] [a](P<0.0001) [b](P<0.001) [c](P<0.05)

[2] With supplements

TABLE 9

Pearson correlation coefficients among dietary and biochemical variables for middle-income girls age 14 ± 0.5 years[1]

	Serum Folate	Red Cell Folate	Hemoglobin	Dietary[2] Folate
Red cell folate	0.77[a]			
Hemoglobin	0.17	0.05		
Dietary folate	-0.04	-0.02	0.21	
Kilocalories	-0.29	-0.13	0.23	0.17

[1] [a](P< 0.0001)

[2] With supplements

correlations among various parameters measured for black girls. In this group significant correlations are shown between serum and red cell folate ($P<0.0001$); dietary folate and hemoglobin[6] ($P<0.005$); and dietary folate and kilocalories ($P<0.05$). The low-income category (table 8), showed significant correlations between serum and red cell folate ($P<0.0001$); red cell folate and dietary folate ($P<0.001$); hemoglobin[6] and dietary folate ($P<0.05$); and dietary folate and kilocalories ($P<0.05$). Finally, in the middle-income group (table 9), there was a statistically significant correlation between serum and red cell folate ($P<0.0001$).

CHAPTER V

DISCUSSION

A. DIETARY FOLATE INTAKE

Results of the 24-hour diet recall evaluations showed mean intakes of folate (table 1) across all race and income categories to be less than 50% of the RDA. Recent studies by Elsborg and Rosenquist (2) reported that 90% of the girls studied between the ages of 13 and 15 (n=110) had consumed less than 100 µg folate/day (25% of RDA). Earlier dietary evaluations of this age group of girls (1,4,5) produced similar results. Some of these authors (1,4) have suggested, and others agree (25-30), that there is a need to establish more extensive tables of food folate contents. Evidence in support of this need is shown in the present study, as well as previous studies (1,4) which reported a high incidence of low dietary intakes yet very low percentages of subjects with actual low serum, plasma, or red cell folate levels. Also, it must be kept in mind that the RDA's are guidelines designed to meet the known needs of practically all healthy persons, and are estimated to exceed the requirements of most individuals. Since therapeutic studies (12,57-60) have indicated that 50-100 µg folate/day was sufficient to maintain a normal hematological picture, then the present adolescent recommendation of 400 µg perhaps needs to be re-evaluated to facilitate a realistic assessment of adolescent folate nutriture.

LIB girls had the greatest mean dietary intake of folate per 1000 kilocalories (table 2) suggesting that choice of foods in this

group of adolescent girls tends to be, in general, more folate rich. However, no relationship existed between folate densities and combined race/income groups as the means were not significantly different. There are no similar studies which provide a comparison with these findings.

When intake was compared to the RDA for various levels of the RDA (fig. 1A and 1B), approximately 36% of all girls (fig. 1A) in the present study consumed less than 133 µg folate/day (one-third of RDA). The white and black groups were similar in their distribution of subjects between various RDA levels as compared to the total population of girls (fig. 1A). However, middle-income adolescents (n=43) had approximately 23% more girls consuming between one-third and two-thirds of the RDA and 20% fewer girls taking in less than one-third of the RDA compared to the low-income population (n=51). It should be noted that 9.3% of the middle-income group consumed vitamin/mineral supplements with folate contrasted to 5.8% in the low-income girls (see table 3). Overall, 82% of the 94 girls studied consumed two-thirds or less of the RDA for folate (fig. 1A). These observations, as previously stated, must be considered with recognition of possible incomplete food folate content tables and excessive folate recommendations.

B. VITAMIN/MINERAL SUPPLEMENTATION

The percentage of subjects using vitamin/mineral supplements (shown in table 3) according to combined race/income groups indicates that 40% of the MIW girls took supplements as compared to approximately

4% in the LIW group and 11% in both the black income groups. However, only one-half of those six girls in the MIW group took supplements containing folate. Generally, those supplements contained 400 µg of folate. The supplements were evidently taken irregularly, since only one of the girls took folate-containing supplements on both days included in the dietary evaluation; therefore, the folate intake was increased by 200 µg per day when means of the two days evaluated were computed.

C. BIOCHEMICAL RESULTS

Mean serum and red cell folate levels across the eight race and/or income categories (table 4) were all within normal limits for adolescents. These findings are in agreement with those of others (1,5), including the "Ten-State Nutrition Survey" done in the late sixties (3). Means were not statistically different between groups.

Daniels et al. (1) reported significantly higher (P 0.001) plasma folate concentrations in middle-upper income adolescent girls compared to low-income. However, these authors pointed out that 40% of the middle-upper income girls took non-prescription vitamin/mineral supplements, which were assumed to contain some folate in most cases.

A comparison between percentages of girls with adequate serum and red cell folate levels (table 5) shows that 93.5% of all subjects were considered to have sufficient blood levels, with 89% of the whites and 97% of the blacks having levels of serum folate above 5.9 ng/ml and red cell folate above 159 ng/ml. Only 6.5% of all subjects (n=61) were marginal or deficient in both serum and red cell folate. In both the LIW and MIW groups, 11.1% were marginal or deficient in serum and

red cell folate, compared to 0% and 6.3% in the respective black groupings of income levels. Because serum folate measurements are indicative of recent intake, and red cell levels are more descriptive of long-term stores, subjects that exhibit low levels in both parameters can be suspect of poor folate nutriture which might be chronic.

D. RELATIONSHIPS AMONG DIETARY AND BIOCHEMICAL DATA

Pearson correlations made between all dietary and biochemical measures of folate nutriture for both race and income groups (tables 6-9) indicate a positive correlation ($P<0.0001$) between serum and red cell folate levels, indicating that those subjects with adequate red cell values (good stores) also have adequate serum levels (which are a reflection of dietary folate intakes).

An important relationship exists between dietary folate intake and kilocalories consumed across three of the four groups on which correlations were performed (tables 6 through 8). No difference in nutrient density was found between the four groups, therefore indicating a trend toward more folate taken in when more calories are consumed. Elsborg et al. (2) found a correlation between folate and kilocalories also, but had no data on the nutrient density of the diets. In both the black and low-income groups, hemoglobin was positively correlated with folate intakes, however, the relationship between these two parameters has not been satisfactorily explained in the literature (62,63). The correlation is assumed due to folacin's role in protein synthesis.

E. CONCLUSIONS

The following conclusions can be stated upon evaluation of the results of this study:

1. Eighty-two percent of the girls consumed less than 2/3 of the RDA, with mean intakes for all groups below 200 μg folate/day (50% of RDA).

2. Mean serum and red cell folate levels were, for all groups, within normal limits.

3. Thirty percent of the subjects had marginal or deficient serum folate levels indicating unsatisfactory recent folate intake or utilization. Eight percent of all subjects had deficient folate stores as indicated by deficient red cell folate levels. Six and one-half percent of the subjects were considered to be at a definite nutritional risk since they had both low serum and red cell levels of folate, indicating recent poor intake and stores.

4. The correlation between dietary folate and kilocalories is evident in white, black and low-income groups, and is assumed to be a realistic one indicating an inherent choosing of nutritionally sound foods in these groups.

CHAPTER VI

SUMMARY

The study of nutrition as it relates to the individual's health should entail prevention of deficiency states through adequate nutrition as its major component. To accomplish this, reliable tools and techniques, as well as plausible guidelines for the assessment of nutritional status must be available.

In the present study, folic acid nutriture in adolescent females was found to be adequate in the majority of subjects when assessed from biochemical analyses of serum and red blood cell folate levels. Assessment of two 24-hour dietary recalls was, however, not in full agreement with the biochemical results, as over 80% of these girls consumed less than 2/3 of the RDA for folate. Other reports in the literature have shown a similar discontinuity in these two parameters used to assess adolescent folate nutriture.

Reiteration that the RDA is only a guideline and is not inflexible to the interpretation of adequate consumption seems appropriate. Also, we must remember that 24-hour diet recalls only serve to depict recent intake or dietary habits. Therefore, consideration of the 24-hour recall and its interpretation in a nutritional assessment will provide perhaps non-typical and speculative information. The dietary portion of a nutritional assessment should still be utilized, provided refinements in its approach and interpretation are forthcoming.

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LITERATURE CITED

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