FOLATE STATUS AND DIETARY FOLATE INTAKE OF WOMEN
DURING ORAL CONTRACEPTIVE USE AND PREGNANCY

by

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ABSTRACT

During pregnancy, the expansion of fetal and maternal tissues increases the demand for folic acid and may result in reduced levels of serum and red cell folate in the mother. If dietary intake of folate is also poor, maternal folate stores may be further depleted. Recent research has investigated the possibility of a similar alteration in folate status among women using oral contraceptives. Although there have been several reports in the literature demonstrating reduced serum folate concentrations in women taking the pill, other studies have contradicted these findings. Furthermore, it has not been established to what extent dietary folate intake determines the serum or red cell folate levels of women using oral contraceptives.

A cross-sectional survey was designed to determine the relationship between dietary folate intake and serum and erythrocyte folate concentrations in women who are pregnant or using oral contraceptives, as compared with a group of control women. In addition, this study was intended to examine any differences or similarities in folate status among the three groups.

A sample of 48 women of similar age, parity, and socio-economic status was selected from the Vancouver area. These subjects were classified into three groups: a control group of 18 women, who had neither been pregnant nor using oral contraceptives for at least six months prior to being in the study; an oral contraceptive group of 22 women, all of whom had been taking combination-type oral contraceptives for at least four months prior to being in the study; and a pregnant group of 8 women, who were in their third or fourth month of pregnancy at the time of commencing their participation in the study. All subjects were in good health and were not taking folic acid supplements. Any woman was excluded from the study if she had a condition (other than
pregnancy) that might alter folate metabolism, or if she was using a drug (other than oral contraceptives) known to interfere with folate metabolism.

For the oral contraceptive users and control women, fasting blood samples were taken at two different phases of the menstrual cycle—day 5 and day 20—for two consecutive cycles. In the group of pregnant women, three fasting blood samples were taken, one during each trimester of pregnancy.

Each subject also kept two three-day diet records—one for the three days preceding the first blood sample, and the other for the three days preceding the last blood sample. The mean daily dietary folate intake was calculated from these records using food composition tables. The accuracy of these calculated estimates for folate was tested by assaying food samples collected by 15 of the subjects. Folate was measured microbiologically with *Lactobacillus casei*.

The results indicate that serum folate concentrations were higher in the control group than in the oral contraceptive group. This difference was statistically significant (*p*<0.05) for the serum folate values at day 5 of the menstrual cycle, but not at day 20 of the cycle. There was no significant difference in the red cell folate levels between these two groups of subjects although, again, the control women had higher levels than did the oral contraceptive users. Within each of the two groups, serum and red cell folate concentrations did not vary significantly with the time of the menstrual cycle.

In the group of pregnant women studied, both serum folate and red cell folate levels were found to increase over the course of pregnancy. However, the rise in serum folate was not statistically significant, and the increase in red cell folate was significant between the first and second trimesters (*p*<0.05) but not between the second and third trimester. It is not clear why the folate status of the pregnant subjects improved during the course of pregnancy.
There was no difference in the levels of dietary folate intake among the three groups of subjects. The degree of correlation between the serum folate levels and dietary folate intake was consistently higher in the control women than in the women using oral contraceptives. The difference in correlation coefficients between the two groups was statistically significant (p < 0.01) only at day 5 of the cycle. In the group of pregnant women the correlation between serum folate and dietary folate was higher in the first trimester than in the third trimester, but the difference in correlation coefficients was not statistically significant. There was no significant correlation between red cell folate levels and dietary folate intake in any of the three groups of subjects.

These results indicate that oral contraceptive use reduces serum folate levels, and that this decline in serum folate is independent of dietary folate intake. Thus, there appears to be some direct effect of synthetic sex hormones in reducing serum folate levels. Whether this is an effect at the level of intestinal absorption or tissue utilization remains unclear. If women using oral contraceptives are not able to fully absorb or utilize their dietary folate, it may be that their recommended daily allowance for folate should be increased.
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CHAPTER I

INTRODUCTION

It is known that during pregnancy there is an increased demand for folic acid, which may result in reduced levels of serum and red cell folate. Moreover, the depletion of maternal folate stores may be further aggravated if the pregnant woman's dietary intake of folate is low. There is also evidence in the literature of a similar reduction in serum and red cell folate concentrations among women using oral contraceptives. The role of dietary folate intake in determining serum or red cell folate levels in these women has, however, not been established.

There has been extensive research into the effect of pregnancy on folate status. Low serum and erythrocyte folate values are most frequently encountered during the last trimester of pregnancy when expansion of maternal tissues and fetal growth rate are the greatest. Therefore this decline in folate status has been attributed primarily to fetal demand and increased erythropoiesis. However, other factors more directly associated with folate metabolism have also been implicated: increased rate of folate clearance from the plasma (Landon and Hytten, 1972), decreased renal reabsorption of folates (Hytten and Leitch, 1971; Fleming, 1972), poor folate stores prior to pregnancy (Cole et al., 1974; Fleming et al., 1974a), and low intake of dietary folate (Hibbard and Hibbard, 1972; Cole et al., 1974; Fleming et al., 1974a).

Oral contraceptives are said to simulate some of the physiological changes associated with early pregnancy. Because of this, recent research
has focused on investigating whether changes in folate status and metabolism analogous to those in pregnancy occur among women taking oral contraceptives. Although several studies have revealed a tendency towards decreased serum and red cell folate levels in oral contraceptive users (Shojania et al., 1971; Roetz and Nevinny-Stickel, 1973; Smith et al., 1975), other researchers have not found these values to be significantly lower than those of control women not using the pill (Stephens et al., 1972; Paine et al., 1975; Prasad et al., 1975).

Investigation into the possible mechanisms behind these altered folate levels in the blood has implicated several factors. There appears to be a decreased absorption of polyglutamate forms of folate in some oral contraceptive users (Streiff, 1970; Necheles and Snyder, 1970), but this does not seem to be true for all women (Shojania and Hornady, 1973) and may not be sufficient to account for the reduction in folate levels. Other factors have also emerged which could be of comparable importance: an increased rate of folate clearance from the plasma (Stephens et al., 1972; Shojania et al., 1975), an increased urinary excretion of folates (Shojania et al., 1975), and the presence of a folic acid binding protein in the serum and leukocytes of oral contraceptive users which could reduce folate availability (da Costa and Rothenberg, 1974; Waxman and Screiber, 1974).

However, there has been a lack of research, in both pregnant women and women using oral contraceptives, concerning the extent to which dietary folate intake determines their serum and red cell folate values. It may be that an inadequate level of dietary folate is contributing to the development of low folate concentrations in the blood of some of these women.

Low dietary intake of folate has been implicated as a factor in the development of folate deficiency (Herbert, 1962). In economically undeveloped
areas of the world where nutrition is below the recommended standards, the incidence of folate deficiency and megaloblastic anemia is greatly increased. This is especially true among women who have the additional stress of one pregnancy after another with little chance for the body to reestablish its folate stores between pregnancies (Hibbard and Hibbard, 1972; Cole et al., 1974). We would not expect this to be the case in better-nourished societies.

This is confirmed by comparative statistics which show that the incidence of megaloblastic anemia due to insufficient dietary folate is rare in industrially developed countries where nutrition is supposedly adequate (Weir, 1973). However, evidence presented by the recent Nutrition Canada Survey demonstrates that there are very large segments of the Canadian population at risk with respect to folic acid; 67% of adult men and women in the general population are at risk, increasing to 97% of Eskimo men. Contrary to what one might expect, a smaller proportion of the pregnant women studied—only 4% to 6%—were found to be at risk. Nutrition Canada however, based these percentages almost solely on serum folate measurements. The authors themselves suggest: "It is not possible to assess the clinical significance and public health consequences of these findings without further hematological studies and an evaluation of dietary folate intakes."

The primary purpose of the present study was to examine the relationship between serum and erythrocyte folate levels, and dietary folate intake in women who are pregnant or using oral contraceptives, as well as in a group of control women. The study was also designed to investigate differences and similarities in folate status among the three groups of subjects.

For oral contraceptive users and controls, folate status was determined by measuring serum folate, red cell folate, and dietary folate intake at two different stages in the menstrual cycle. Statistical tests were performed to evaluate any variations in these parameters within and between the groups of women at the two phases of the cycle.

A group of pregnant women was also selected and their folate status was assessed; serum and red cell levels of folate and dietary folate intake were determined during each of the three trimesters. These parameters of folate status were also compared statistically with the same parameters in controls and women using oral contraceptives.

The results of this study should help to clarify the extent to which serum and red cell folate concentrations are a function of dietary levels of folate, and what effect oral contraceptive use or pregnancy has on this relationship.
CHAPTER II

REVIEW OF THE LITERATURE

Absorption and Metabolism of Folic Acid

Current knowledge concerning the absorption of dietary folic acid and its subsequent transformation to the metabolically active forms is incomplete. A review of this area is requisite for evaluating the literature concerned with folate metabolism during pregnancy and oral contraceptive therapy.

Physiological indices of folate status. Folic acid, in its reduced forms, is a vitamin of major importance as a cofactor in all one-carbon transfer systems, the most significant being the biosynthesis of purine and pyrimidine nucleotides. Therefore, the role of folic acid in DNA synthesis and cellular growth is vital, and folates are utilized extensively in tissues where there is a great turnover or multiplication of cells. This is evidenced by the increased requirement for the vitamin during growth and pregnancy, as well as by the observation that a folate deficiency will be most readily reflected in those tissues undergoing a continuous, rapid replication of cells. The tissues primarily affected are the bone marrow and blood, the intestinal mucosa and, in women, the uterine and cervical cells. Although any of these may show morphological changes due to a lack of folate, such changes are most pronounced in the marrow and peripheral blood (Blakley, 1969). Alterations in cell morphology, however, are a late sign of folate deficiency.

The most common measures of folate status are levels of the vitamin in serum and whole blood. Plasma folic acid levels reflect recent nutritional
status with respect to folate intake, whereas the amount of folate in red blood cells more specifically indicates folate stores in the body (Chanarin, 1969).

Serum folate is believed to be derived from liver and other tissue stores by a displacement mechanism (Rosenberg and Godwin, 1971; Gerson and Cohen, 1972). Absorbed folic acid enters these tissues and thus acts to displace endogenous 5-methyltetrahydrofolate to the plasma. If dietary intake of folic acid is reduced this phenomenon is diminished, resulting in a rapid decrease in serum folate levels.

A reduction of the folate in red blood cells requires a longer period of deprivation since erythrocyte folate is tightly bound and not released until the cell itself is destroyed at the end of its life cycle (Hoffbrand, 1971). Therefore measurement of red cell folate is indicative of long-term folate status.

Whereas erythrocyte folate levels are used as a measure of folate stores, it is liver supplies of folate which actually form the largest reservoir of the vitamin in the body (O’Broin et al., 1975). A person’s folate stores are theoretically sufficient to provide folate for several months if dietary intake is reduced to an inadequate level (Herbert, 1962).

**Absorption of folic acid.** The adequacy of folate nutrition depends not only on the availability of adequate folate-rich foods, but also on the individual’s ability to absorb sufficient folate from these dietary sources.

Some disagreement still surrounds the mechanism of folate absorption, both with regard to the mode of transport across the intestinal mucosa and the form in which dietary folates are absorbed.

1. **Site of absorption.**

Absorption of folates has been shown to take place along the entire
length of the small intestine although the greatest proportion is absorbed from the proximal jejunum (Bernstein et al., 1970). In general, the rate of absorption is inversely related to the length of the $\gamma$-glutamyl side chain on the folate molecule (Gerson and Cohen, 1972). Only deconjugated folates—monoglutamate and some diglutamates—will eventually reach the portal circulation. Hydrolysis of the glutamic acid residues occurs by the action of intestinal folate conjugase ($\gamma$-carboxypeptidase, referred to as pteroylpolyglutamyl hydrolase) present in the lysosomal fraction of intestinal mucosal cells (Rosenberg and Godwin, 1971). Most evidence indicates that this is the site of hydrolysis although it is not clear how the larger molecules of folate reach the inside of the mucosal cells. There is perhaps some deconjugation in the lumen itself by the action of conjugase present in sloughed-off mucosal cells, but this remains hypothetical (Gerson and Cohen, 1972).

2. Mode of transport across the mucosa.

The mode of transport of folates across the intestinal wall (i.e., whether active or passive) is still unresolved. The earliest research with rats yielded conflicting results regarding transport and it was not until more refined techniques for studying absorption were developed that it became possible to examine the process in more detail.

Hepner et al. in 1968, using an intestinal perfusion technique on humans, concluded that crystalline pteroylglutamic acid (PGA) was absorbed by a saturable transport mechanism. However, their findings have not been substantiated by others. Gerson et al. in 1971, using a similar method but employing $^3$HPGA, showed that glucose enhanced the absorption of folic acid, probably by a solvent-drag effect secondary to sodium and water flow. This implicated a passive process and corroborated the work of Smith et al.
(1970) using everted gut sacs from rats. Gerson points out, however, that although this is strong evidence for a passive transport of folic acid, it does not exclude the possibility of a parallel system involving either active transport or facilitated diffusion.

Reduction and methylation of absorbed folates. The majority of folate derivatives in the body are formylated or methylated, and reduced, these changes being prerequisite for the coenzyme function. The length of the glutamyl side chain appears to also be a factor in coenzyme activity, although the effect of chain length in this regard is not yet completely understood (Krumdieck et al., 1975). There has been some dispute concerning the site of conversion of absorbed folates to their metabolically active forms (primarily 5-methyltetrahydrofolate).

While some researchers have suggested that reduction and methylation occur in the gut wall during absorption (Chanarin and Perry, 1969; Perry and Chanarin, 1970; Whitehead et al., 1972), other work in this area contradicts these findings. Studies by Baugh and associates (1971, 1975) with intestinal loops in dogs showed no evidence of conversion of folates to reduced or methylated forms during passage through the mucosal cells. Rather, there has been some research indicating that these changes are accomplished in the liver. Whitehead and Cooper (1967) demonstrated that orally administered PGA appears unaltered in the portal circulation, and only after passage through the liver does the folate emerge as 5-methyltetrahydrofolate. In the process, hepatic folate is evidently exchanged for the more recently absorbed folates entering from the portal circulation (Melikian et al., 1971). This has been confirmed in studies using $^{14}$CPGA (Butterworth et al., 1969) and $^{3}$HPGA (Pratt and Cooper, 1971).

Whitehead et al. (1972) have suggested that reduced dietary folates are absorbed differently from folic acid (PGA), which could account for
the discrepant results in the above studies. Whereas PGA may not be methylated until it reaches the liver, reduced folates appear to be methylated in the intestine.

Thus, both the hydrolysis of the polyglutamate side chain and the methylation of dietary folates (which are primarily reduced) occur prior to the entry of folates into the hepatic portal system, and probably take place in the intestinal mucosa.

A deficiency of folate may be caused by any condition which interferes with the above processes. Inadequate intake of folate could lead to a depletion of liver supplies; a disturbance of intestinal function could impair absorption; disorders of the liver could prevent formation of the functional derivatives of folate; the use of certain drugs can impair absorption of utilization of folates; and certain conditions such as hemolysis can place an inordinate demand on body stores of the vitamin. If such situations remain untreated they can result in the development of a megaloblastic anemia.

**Folate Deficiency and Megaloblastic Anemia**

**Definition of folate deficiency and megaloblastic anemia.** It is relevant at this point to distinguish between a folic acid deficiency and an outright megaloblastic anemia which is an expression of extreme folate deficiency.

Herbert's classic work (1962) has outlined the sequence of changes which take place when the body is deprived of sufficient folic acid (i.e., limited to an intake of 5.0 μg folate per day or less). Herbert summarized his findings as follows:
Dietary Folic Acid Deprivation in Man: Biochemical and Hematologic Sequence of Events

<table>
<thead>
<tr>
<th>Sequential Changes</th>
<th>Time of Occurrence (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low serum folate (&lt;3 ng./mL.)</td>
<td>3</td>
</tr>
<tr>
<td>Hypersegmentation of Neutrophils</td>
<td>7</td>
</tr>
<tr>
<td>High urinary Ficlu excretion</td>
<td>14</td>
</tr>
<tr>
<td>Low red blood cell folate (&lt;20 ng./mL.)</td>
<td>17</td>
</tr>
<tr>
<td>Macroovalocytosis</td>
<td>18</td>
</tr>
<tr>
<td>Megaloblastic marrow</td>
<td>19</td>
</tr>
<tr>
<td>Anemia</td>
<td>20</td>
</tr>
</tbody>
</table>

The formation of megaloblasts in the bone marrow and macrocytes in the peripheral blood reflects a state of imbalanced growth due to impaired synthesis of DNA precursors. The result is that, although cells continue to synthesize RNA and protein, there is a delay at certain points in mitosis which causes the formation of giant cells (Chanarin, 1969; Hoffbrand, 1971).

There are also changes in the production of white blood cells. One type of leukocyte, the neutrophil, will show an increased number of lobes in its nucleus. There are normally two to five; this will increase to six or more.

A megaloblastic anemia, by definition, is characterized by the presence of megaloblasts in the marrow. However, a folate deficiency may be defined by the presentation of any change from a low serum folate to the appearance of macrocytes in the peripheral blood. Most often it is the first four signs outlined by Herbert which have been used to establish the existence of a folate deficiency. And it is the existence of marginal levels of these parameters which have concerned most researchers investigating folate status.
**Etiology of folate deficiency.** There are numerous factors which may act singly, but which more often occur together in some combination to cause a folic acid deficiency that may, if untreated, lead to a megaloblastic anemia. The relative significance of these factors has not been established, so they are discussed here in an arbitrary order.

1. **Impaired absorption.**

A defect in absorption of folic acid is most commonly due to some disorder of, or damage to, the jejunal mucosa as in sprue, steatorrhea, coeliac disease, or following a jejunal resection (Bernstein et al., 1970). Malabsorption has also been implicated as a mechanism by which other conditions may act to alter folate status; these include pregnancy, oral contraceptive use, anticonvulsant therapy, and alcoholism.

2. **Alcoholism.**

The occurrence of folic acid deficiency in alcoholics is common and is probably the result of poor dietary habits (Halsted et al., 1967, 1971; Stebbins et al., 1973). However, absorption may also be impaired due to damage to the intestinal mucosa, a direct effect of the alcohol itself (Stebbins et al., 1973; Weir, 1973).

3. **Liver disease.**

If cirrhosis occurs in connection with alcoholism, or if there is some other damage to liver function, this may hamper the ability of the liver to store or exchange folates (Brown et al., 1973). Such a condition can also result in increased urinary loss of folates (Weir, 1973).

4. **Increased requirement for folic acid.**

Any condition in which there is a marked increase in cell multiplication, as in pregnancy, lactation, growth, malignancy, or hyperthyroidism will increase the need for folic acid above the normal Recommended Daily
Allowance (Hoffbrand, 1971). This may also occur under conditions where the utilization of folate is blocked as in homocystinuria.

5. Increased loss of folic acid.

There may be an abnormally rapid catabolism of folic acid in situations where hemolysis or hemorrhage occur, as well as when a hemolytic anemia exists (sickle cell anemia, thalassemia, or congenital spherocytosis) (Weir, 1973). In addition, a person undergoing hemodialysis may lose a significant amount of folic acid which, in conjunction with the anorexia and vomiting frequently accompanying dialysis, could precipitate a folate deficiency (Hoffbrand, 1971).

6. Inflammatory disease and infection.

Such conditions as arthritis, tuberculosis, and psoriasis have been known to contribute to a folate deficiency. This is probably due to a combination of poor appetite with an increased requirement for folate induced by increased leukocyte turnover (Hoffbrand, 1971).

7. Drug interferences.

There are many drugs which interfere with folate metabolism, usually by a disruption of availability or utilization of folate at the cellular level (Stebbins et al., 1973). Folate antagonists, most notably methotrexate and aminopterin, used in treatment of cancer, have a great affinity for dihydrofolate reductase and act by displacing folic acid from this enzyme. Pyrimethamine, an antimalarial drug, is also an inhibitor of dihydrofolate reductase but is far less potent than methotrexate. Anticonvulsants, the most common being diphenylhydantoin, act by some mechanism which has not been established. There may be a competitive inhibition of folate enzymes, an impairment of polyglutamate absorption by conjugase inhibition, or a displacement of folate from its transport proteins. Oral contraceptive
agents also interfere with the metabolism of folic acid, although the process by which this happens remains unclear. The effect of oral contraceptives on folate status will be discussed below in greater detail.

8. Poor dietary intake of folates.

A low level of dietary folate may occur in conjunction with any of the above conditions and thus contribute to the development of a folate deficiency. The significance of inadequate dietary folate is often overlooked but it may be of importance in infancy, old age, poor socio-economic conditions, or any instance where anorexia occurs. Diet has also been implicated as a factor in determining the folate status of pregnant women (Hibbard and Hibbard, 1972; Cole et al., 1974), but its significance to the folate status of women using oral contraceptives remains to be clarified. The present research was concerned specifically with examining pregnancy, oral contraceptive use, and dietary folate intake as factors in the etiology of folate deficiency.

The Effect of Pregnancy on Folate Status

Pregnancy induces some striking physiological alterations in the female's body in the course of adapting to the needs of the developing fetus. Much of the nutrient intake of the woman, as well as a large portion of her body stores of nutrients, are directed toward the placenta and fetus. In addition, maternal energy and nutrient supplies must be conserved in anticipation of the demands of labor, delivery, and subsequent nursing.

Anemias of pregnancy: iron and folate interrelationships. The requirement for both iron and folic acid is greatly increased in pregnancy and it is pertinent to look at these together. They have a common role in hematopoiesis, which is accelerated during pregnancy, and may be interrelated in other ways which have not been fully elucidated. An iron
deficiency is generally associated with a reduced serum folate level (Velez et al., 1966; Toskes et al., 1974), and Toskes et al. suggest that an iron deficiency may actually predispose a person to folic acid deficiency.

It is known that iron deficiency and folic acid deficiency do frequently occur together, and the risk of developing both of these is especially increased during pregnancy (Fritchard, 1970). The alterations in both iron and folate metabolism that take place in pregnancy may contribute to the development of anemia in some women.

In discussing anemias of pregnancy it is critical to distinguish between what is called the "physiological anemia of pregnancy" and those pathological conditions which are of more serious concern.

During pregnancy there is a remarkable increase in plasma volume which tapers off during the last six weeks of gestation. However, the maximum increase in red blood cells does not occur until the woman is near term. Consequently, most of pregnancy is spent in a state of hemo-dilution, where the plasma volume is expanding without a comparable, simultaneous rise in red cell volume. And, since the size of the cells and their hemoglobin content normally show no change, the net effect is that the hemoglobin concentration and hematocrit fall (Hytten and Leitch, 1971).

Some believe that this physiological alteration is a true state of iron deficiency anemia and cite the evidence that iron given in therapeutic doses will prevent the fall in hemoglobin concentration (Fritchard, 1970). Others reason that these changes do not represent a pathological state because the increased oxygen requirements of pregnancy are met even with the lowered hemoglobin levels. Therefore, the lowered hemoglobin does not in itself indicate impaired erythropoiesis but may simply be a consequence
of the proportionately greater increase in plasma volume over red blood cell mass (Hytten and Leitch, 1971; Hall, 1974).

In addition to the increase in plasma volume during pregnancy, there is also an unrelenting drain on maternal iron stores by the fetus. This demand is always met even at the expense of the mother. Thus, if by the time the greatest increase in red cell production is occurring in late pregnancy, iron stores have already been depleted, the desirable increase in red cell volume may be limited and a true anemia may result.

However, an anemia is not ordinarily brought about simply by fetal demand. And the case has been made that a healthy, well-nourished woman does not require iron supplementation in pregnancy (Izak et al., 1973; Desforges, 1973). Usually it is the presence of some additional stress acting to deplete maternal iron stores (such as hemorrhage, infection, or hemolytic anemia), or the existence of poor iron stores prior to conception which forces the woman into a state of negative iron balance (Desforges, 1973). This may, in turn, have a bearing on the woman's folate status.

Although the association between iron balance and folate status remains obscure, two pieces of evidence have emerged which suggest that there is some relationship between them. First there is the well-documented fact that a decrease in serum folate occurs in conditions associated with iron deficiency (Velez et al., 1966; Toskes et al., 1974). Secondly, and conversely, it has been shown that pregnant women suffering from a folate-responsive megaloblastic anemia have a higher incidence of iron deficiency than those without such an anemia (Hoffbrand, 1971).

The basis for these changes is uncertain although the explanation may be quite simple. In iron deficiency there is a decrease in the
survival of peripheral red blood cells and a concomitant increase in heme catabolism. According to Toskes et al. (1974), this in itself may be sufficient to account for the decreased serum folate levels and the increased folate requirement. Paradoxically, once an iron deficiency anemia is established, treatment with an iron supplement may cause a further decrease in serum folate because of the additional demand on folate stores in the initial spurt of new erythrocyte formation (Hoffbrand, 1971).

Perhaps the major point to be aware of in clinical practice is that the morphological changes in the blood associated with iron deficiency may mask evidence of a coexisting folate deficiency (Toskes et al., 1974). Therefore, if a pregnant woman develops an iron deficiency, it may be a wise precaution to also determine her serum and red cell folate levels.

**Folic acid deficiency of pregnancy.** Reduced serum and red cell folate concentrations are a common accompaniment of pregnancy. There is little question that lowered maternal folate levels must be primarily attributed to fetal demand. This is substantiated by the very high concentration of folate found in cord blood at delivery when compared to maternal folate concentration (Landon and Hey, 1974; Baker et al., 1975).

However, other factors relating more specifically to folate metabolism have also been implicated in the etiology of folate deficiency in pregnancy.

1. **Decreased renal reabsorption of folates.**

Urinary loss of many nutrients is increased in pregnancy (Hyttten and Leitch, 1971). The loss of folates by this route could cause a depletion of the dialyzable fraction of serum folate and thus account somewhat for the lowered serum folate characteristic of pregnancy.
(Fleming, 1972). However, there is wide variability in the amount of
urinary folate excreted and urinary loss is probably not sufficient to
cause a folate deficiency (Landon and Hytten, 1971).

2. Increased rate of folate clearance from the plasma.

The increased rate of plasma folate clearance is a definite
change observed during pregnancy. However, it is influenced by other
factors which are also altered by pregnancy (absorption, tissue uptake,
and excretion of folate, as well as volume of extracellular fluid) and
cannot be taken as an accurate determinant of folate status in pregnancy
(Landon and Hytten, 1972).

3. Folic acid binding protein in the blood.

A folic acid binding protein (FABP) has been found in the serum
and leukocytes of women who are pregnant or using oral contraceptives
(de Costa and Rothenberg, 1974). The significance of this FABP is not
known, but it may act to bind folates so they cannot be utilized (Waxman
and Schreiber, 1974).

4. Poor folate stores prior to pregnancy.

5. Poor intake of dietary folate.

With pregnancy's added strain on endogenous folate stores, exogenous
folate intake assumes greater importance. Most studies of nutritional
folate status have made only perfunctory examination of the dietary prac­
tices of the subjects. This variable may be of more significance in
determining folate status than is often supposed. Food availability,
economic advantage, and ethnic custom are of particular importance in
this regard.

Research among pregnant women, primarily in Australia (Cole et al.,
1974; Fleming et al., 1974a) and South Africa (Colman et al, 1974), has
shown striking differences in folate status among women from different ethnic and socio-economic groups. Cooking practices and traditional food preferences were implicated as factors in determining folate status. Large amounts of lightly cooked green vegetables as eaten by the Chinese in Malaysia (Hibbard and Hibbard, 1972) or the beans and lentils which form a staple in the diet of Mediterranean immigrants to Australia (Cole et al., 1974) appear to exert a protective effect against folate deficiency.

Assessment of folate status in pregnancy. Estimates of the incidence of folate deficiency in pregnancy vary within a wide range. This can be mainly attributed to the use of different criteria for judging folate status. The methods used to assess folate status reflect different aspects of folic acid metabolism. Therefore it is not surprising that the results of different tests should lead to different conclusions. It is important to be familiar with these criteria in order to accurately interpret what they tell us about folic acid metabolism in pregnancy.

Hansen (1968) discusses the relative merits of the different methods.

The Figluexcretion test measures the amount of formiminoglutamic acid (Figlu) excreted in the urine after a histidine load. If there is insufficient folate circulating to pick up the formimino group, then Figlu accumulates and is excreted in larger amounts than normal. Whereas this test is a reliable and sensitive measure of folic acid deficiency in most instances, the results seem to be quite unreliable in pregnancy. This is probably due to the increased amounts of histidine excreted during pregnancy, which could create a falsely high level of Figlu excretion.

Plasma clearance of folates is another commonly used test of deficiency, and it is also altered in pregnancy. There is some question
however, as to whether or not an increased rate of clearance necessarily indicates a deficiency in pregnancy. It may simply be another of the physiological adaptations associated with normal pregnancy.

The neutrophil lobe average, when it is increased, is indicative of a severe degree of folate depletion (Hibbard and Hibbard, 1971). However it also seems to increase in a normal pregnancy when other signs of folate deficiency are absent, so its usefulness as a measure of deficiency has been questioned.

Serum folate reflects the balance between dietary intake and plasma turnover of folates (Gerson and Cohen, 1972). A steady decrease in serum folate is commonly observed in the course of pregnancy. This should return to normal non-pregnant levels by six weeks postpartum (Hanson, 1968). A low serum folate may be due to the increased plasma clearance of folates and not necessarily diagnostic of a deficiency.

A decrease in red cell folate, however, is of more value in determining the existence of a folate deficiency in pregnancy since it is definitely associated with a depletion of folic acid stores. However, low red cell folate is a relatively late sign of deficiency, and it may not be wise to wait for this change to appear before diagnosing a deficiency.

There is a very high incidence of low serum folate and accelerated plasma clearance of folate among pregnant women (Benjamin et al., 1966; Hansen, 1968; Pritchard, 1970; Rae and Robb, 1970; Roetz and Hampel, 1972; Cole et al., 1974). It is still debatable whether these should be regarded as indicative of a folate deficiency or if they are normal changes associated with pregnancy, like the decrease in hemoglobin concentration.

Red cell folate levels seem to be the most reliable indicator of a significant deficiency, and also the factor least disturbed by a coexisting iron deficiency (Roberts et al., 1971). But to delay diagnosis
of folate deficiency until a low red cell folate appears may present a risk to both mother and child.

Maternal and fetal complications associated with folate deficiency in pregnancy. A folate deficiency in pregnancy has been associated with abruptio placentae, toxemia, and premature labor in the mother (Hibbard, 1964; Streiff and Little, 1967), as well as low birth weight, fetal malformations, and impaired central nervous system development in the newborn (Stone, 1968; Gross et al., 1974). The majority of these studies however, are retrospective and therefore not truly representative of the incidence with which such complications occur. Moreover, the presence of a folate deficiency severe enough to cause these abnormalities may indicate the existence of some other underlying factor which could be just as strongly implicated (Hall, 1972; Hall, 1972b). Although it remains to be proven that maternal folate deficiency, per se, is responsible for these complications, still the risk of incurring them should be avoided if at all possible.

Supplementation with folic acid in pregnancy. Folate deficiency and its extreme form, megaloblastic anemia, are almost always the result of more than one factor contributing to a folate imbalance. In pregnancy, it is the woman faced with some additional complication who is most susceptible to such a deficiency. She may be considered to be at risk in this regard if she has a multiple pregnancy or if her pregnancy is accompanied by any of the following conditions: hemolytic anemia, thalassemia, epilepsy being treated with anticonvulsants, alcoholism, infection, or poor diet. In these situations a folic acid supplement may be warranted to preclude the possibility of any maternal or fetal complications (Fleming et al., 1974b). This practice would not be challenged by most obstetricians.

Rather, it is the question of routine folic acid supplementation for all pregnant women which is controversial. The position can be argued
that for the majority of women who are healthy and who meet the Recommended Daily Allowance for dietary folate a folic acid supplement is unnecessary. Some clinicians, however, advocate the administration of such supplements to all pregnant women. There is perhaps some wisdom in this attitude, which Hansen (1968) expresses, "... a normal serum folate level probably is the best guarantee that the different dividing cells receive an adequate amount of folic acid, which must be assumed to be of special significance for normal growth in pregnancy."

The decision to prescribe a folic acid supplement to a pregnant patient is left to the individual obstetrician and opinions concerning the practice of supplementation vary widely.

Considering the additional burden placed on a woman's folate stores by pregnancy, it would be prudent to examine the future mother's folate status prior to conception. There is evidence that oral contraceptives may adversely influence folate status. The clinical significance of low serum folate levels and other signs of altered folate status should not be underrated. Following the discontinuation of hormonal contraception a woman could easily become pregnant. If, at this point, there is insufficient saturation of her folate stores, a folate deficiency could develop in the sensitive period of pregnancy.

The Effects of Oral Contraception on Folate Status

Oral contraceptives have been said to induce a state in the body analogous to pregnancy. The fixed level of estrogen provided by the pill, along with the absence of ovulation, acts to simulate the hormonal conditions of early pregnancy. These two situations are therefore comparable in some respects, and one would expect some similar alterations in physiology to exist.
There is evidence that oral contraceptives have an effect on folate status which is similar in many respects to that found in pregnancy. Whether this effect is the result of an etiology common to both conditions is unclear.

Megaloblastic anemia associated with oral contraceptive use. There has been extensive work recently published documenting the possible adverse effects of hormonal contraception on folate status.

A survey of the literature reveals numerous incidences of megaloblastic anemia occurring in association with oral contraceptive use (Necheles and Snyder, 1970; Streiff, 1970; Buhac and Finn, 1971; Ryser et al., 1971; Toghill and Smith, 1971; Flury and Angehrn, 1972; Salter, 1972; Johnson et al., 1973; Shojania and Hornady, 1973; Lewis, 1974; Meguid and Loebl, 1974). These, however, are all based on individual case studies and do not represent controlled experimental situations. In reviewing these reports, it is of significance that those which included a test for malabsorption did, in fact, find evidence for an underlying intestinal disorder that could have affected folate absorption (Streiff, 1970; Ryser et al., 1971; Toghill and Smith, 1971; Johnson et al., 1973). Moreover, in most of the other studies, it was stated that an occult malabsorption syndrome of some kind was not, and should not, be excluded as a possible contributing factor (Necheles and Snyder, 1970; Flury and Angehrn, 1972; Shojania and Hornady, 1973; Lewis, 1974; Meguid and Loebl, 1974). In all cases, discontinuation of oral contraceptive therapy and initiation of folic acid supplementation were sufficient to cure the anemia.

Thus it seems that a woman using oral contraceptives must also have some other condition affecting folate metabolism before megaloblastic changes will develop. Conversely, the effect of such a condition might
not become apparent except for the added stress of treatment with synthetic hormonal contraceptives. Therefore, if there already exists some unmanifested form of malabsorption such as a tendency towards coeliac disease, the further insult of oral contraceptives may be sufficient to precipitate an anemia.

Oral contraceptive use and folate deficiency. Megaloblastic anemia occurring in conjunction with oral contraceptive use is a rare event relative to the large number of women at risk. A more common observation among women using oral contraceptives is the tendency towards reduced serum folate concentrations. However, this has been a highly variable finding. It appears that certain women may have an enhanced susceptibility to lowered folate levels when using oral contraceptives, whereas others suffer no such adverse effects. Even a careful perusal of the research published in this area makes it difficult to draw conclusions applicable to all individuals.

Review of the studies finding a reduced serum folate in oral contraceptive users. Of those studies which have established some positive correlation between low serum folate levels and oral contraceptive use, the most significant are those by Shojaania and associates in 1971, and Roetz and Nevinny-Stickel in 1973. The latter researchers showed that serum folate levels decline over time after the initiation of oral contraceptive therapy. Looking at the converse situation, Shojaania et al. found that the serum folate concentration improved within three months after discontinuing oral contraception. The conclusion of these workers was that there is an impairment of folate metabolism directly related to the use of hormonal contraceptives. However, they also make the qualification that the effect on folate metabolism—whatever the mechanism may
be---is very mild, and that it takes a large number of subjects with a long history of oral contraceptive use to demonstrate any effect.

The major criticism of the above studies is that they never specified whether or not serum folate determinations were made on fasting blood samples. This can make a great difference in folate levels since eating a meal with folate-rich foods will elevate the serum folate for several hours afterwards.

The mechanism by which oral contraceptives might act to decrease serum folate has been a matter of some speculation. There have been a few studies implicating a direct effect of synthetic orally-administered hormones on absorption of folates from the intestine (Snyder and Necheles, 1969; Streiff, 1970). Streiff's findings have formed the primary basis for the theory that oral contraceptives act to inhibit the activity of intestinal conjugase.

Streiff studied nine oral contraceptive users and nine control women; all had normal serum folate levels and none were anemic. He observed the changes in serum folate levels after these women were given an oral dose of either monoglutamic folate or polyglutamic folate. The rise in serum folate was comparable in the two groups when the monoglutamic form was given, but after administration of polyglutamic folate the rise in serum folate in the oral contraceptive group was about 50% of that in the control group. From this he concluded that, since the polyglutamic forms of folate must be deconjugated in the small intestine before they can be absorbed, there might be some direct interference of the hormones with conjugase activity. His hypothesis was also based on an in vitro demonstration of the inhibitory effect of mestranol (a synthetic estrogen) on conjugase activity (Streiff and Greene, 1970).
Streiff was however, reluctant to implicate oral contraceptives as the exclusive cause of this malabsorption. He suggested that if folate absorption is already hampered by some other condition, or if dietary intake of folates is inadequate, a further inhibition of absorption (such as that imposed by oral contraceptives) could be enough to precipitate an overt deficiency or anemia.

Review of the studies finding no difference in serum folate between oral contraceptive users and control women. There have been numerous studies which contradict the above results but which do not, significantly, dismiss the possibility that some alteration of folate status may be incurred by oral contraceptive use. The research of most interest in this connection has been that of Stephens and associates in 1972 and, more recently, Paine et al. (1975).

Paine et al. surveyed a very large sample of women (N=526) and found no significant difference in serum folate levels between oral contraceptive users and controls. They obtained the same result when blood samples were analyzed by either the *L. casei* microbiological method or the new radioassay for folate. The strength of their findings lies in the size of the sample studied. However, they did not state whether they used fasting blood samples and this may have skewed their results.

The work of Stephens et al. appears to have been more carefully controlled and logically planned than any of the other studies. These authors looked at both folate status and folate absorption in a series of experiments. First they examined serum folate levels in fasting blood from a group of oral contraceptive users and a group of control women. They found no statistically significant difference in the serum folate values between the two groups, and no significant variation in serum folate levels at different stages of the menstrual cycle.
This was followed by absorption studies on the same women to test for differences in their ability to absorb different forms of folate. Although they were able to confirm Streiff's finding of reduced polyglutamate absorption in the oral contraceptive users, further investigation showed that, after saturation of the subjects with folic acid, the differences between the groups disappeared. Stephens explained their results by suggesting that oral contraceptives may cause an increase in the rate of clearance of folates from the plasma. This accelerated clearance is apparent when the subject's tissues are not fully saturated with folates. But when tissues are pre-saturated prior to the absorption test, the rate of clearance becomes the same for both groups of women. This finding may be interpreted to mean that there is no true malabsorption of folates in women taking oral contraceptives.

Rapid plasma clearance of folates in pill users may be caused by a mechanism similar to the one which results in increased clearance during pregnancy. The implication here is that there is some hormonal effect common to both situations.

The last part of the study by Stephens et al. involved an in vitro examination of the effect of sex hormones on conjugase activity. Although previous work by Streiff and Green (1970) had indicated that mestranol, a synthetic estrogen, inhibited conjugase activity in vitro, Stephens et al. found no inhibition of intestinal conjugase by any of the three synthetic hormones they tested (estradiol, progesterone and estrone). In fact, there has been recent evidence to suggest that estrogen has an enhancing effect on conjugase activity (Krumdieck et al., 1975). This was, however, shown in rat uterus and is not necessarily applicable to human intestinal conjugase. A direct effect of steroid hormones on conjugase activity remains to be proven.
Further research attempting to clarify these results. In a paper published in 1973 Shojania and Hornady attempted to reconcile the question of malabsorption induced by oral contraceptives. They compared folate absorption in women on the pill who had a low serum folate and in women who were also using the pill but who had normal serum folate levels.

They found no consistent malabsorption of folates in oral contraceptive users, but when they made the above distinction between two populations of women a pattern did emerge. One population consisted of those who had a normal fasting serum folate and showed normal polyglutamate absorption, whereas the population that had low fasting serum folate levels showed reduced absorption of polyglutamates.

In 1975, Shojania et al. extended their investigation to include a study of plasma clearance and urinary excretion of folates. In the group of women they studied, plasma clearance of an injected dose of pteroylglutamic acid was shown to be much faster among the oral contraceptive users than in controls. One possible explanation given for this difference was that tissues are less saturated with folates in oral contraceptive users, thus enhancing uptake from the plasma.

In this same study it was also discovered that there was a direct correlation between urinary folate excretion and serum or red cell folate levels in both oral contraceptive users and control women. However, the pill users excreted more folate for a given level of serum folate or red cell folate than did their control counterparts. The authors felt that this increased excretion of folates among women taking hormonal contraceptives may partially account for their frequently lower blood folate levels.

Related factors of unknown significance.

1. Folic acid binding protein (FABP).

Da Costa and Rothenberg (1974) have reported the existence of a specific folic acid binding protein (FABP) in the blood of both women who
are pregnant and women using oral contraceptives. A factor was isolated from the serum and leukocytes of these women which effectively bound unreduced folates and dihydrofolates. Significantly, this folate binder was absent from the blood of control women, suggesting that its production may be hormonally induced.

It may be that this is not a new factor in the blood but simply an already existing FABP which undergoes an increase in concentration. Such a shift in the relative concentration of plasma FAEP's has been shown to occur in pregnancy (Markkanen et al., 1973). Whichever is the case, the exact function of such a binding protein and its position in the etiology of reduced folate status in pill users have yet to be resolved.

Da Costa and Rothenberg (1974) could not find a correlation between the presence of the binder and the serum folate concentration. Their findings imply that this factor may act to sequester folates from the metabolically active pool of coenzymes and thereby contribute to the altered folate status of both pregnant women and women using the pill.

In a related study, Waxman and Schreiber (1974) looked at the effect of FABP on $^3$H - labelled folates. They found that those folates bound to FABP were less available for uptake by tissues than folates not attached to the binding factor. Uptake was shown to be inversely related to the amount of FABP in the serum. These authors suggest that this FABP may be responsible for cellular uptake and distribution, rather than serum transport, of folates.

Shojania et al. (1975) have postulated that it is the change in plasma folate binders that is responsible for the increased urinary excretion of folates they observed among oral contraceptive users.
2. Megaloblastic changes in the cervical epithelium.

There is another observed effect of oral contraceptive use on folate utilization, the significance of which is still unclear, but which warrants inclusion here. This is the finding by several workers (Klaus, 1971; Whitehead, et al., 1973; Lindenbaum et al., 1975) that oral contraceptive use is associated with distinctly megaloblastic changes in the cervical cells which can be observed from Papanicolaou smears.

It is disturbing to note that these morphological changes in the cervical epithelium are in no way correlated with other evidence of a systemic folate deficiency (such as reduced serum or red cell folate, macrocytosis, or hypersegmented neutrophils). In fact, the serum folate levels were found to be similar in women with either normal or abnormal smears (Lindenbaum et al., 1975).

However, folic acid therapy was shown to significantly revert the cervical morphology towards normal within three weeks, and a discontinuation of the folic acid supplement resulted in a recurrence of the abnormalities (Whitehead et al., 1973; Lindenbaum et al., 1975). This strongly implicates some direct effect of oral contraceptives in altering folate metabolism at the level of the target organ.

Sex steroids are known to stimulate DNA synthesis and cell proliferation in their target tissues (O'Malley and Means, 1974). Therefore, one would expect an increased utilization of folate coenzymes in these tissues and a resultant depletion of folate stores in the localized target area. The subsequent impairment of DNA synthesis may be evidenced by the presence of megaloblastic changes. The full significance of these cervical changes cannot be elaborated on without further elucidation of the dynamics behind their development.
Summary of the mechanisms by which oral contraceptives may act
to alter folate status. In summary, the possible mechanisms of action of
oral contraceptives in altering folate status may be enumerated as follows:

1. Decreased absorption of polyglutamate forms of folate by
direct inhibition of intestinal conjugase activity.

2. Increased rate of plasma clearance of folates.

3. Increased urinary excretion of folates.

4. Presence of a FABP which binds folates so they are not
available in their metabolically active forms.

5. Interference with folate utilization at the cellular level
in sex steroid target tissues.

While it is not as yet possible to evaluate the relative impor-
tance of the factors implicated above, it is nevertheless evident that
they must all be considered significant clues to the physiological action
of oral contraceptives on folate metabolism.

It is significant that in almost all the studies of folic acid
status in pregnant women and oral contraceptive users the influence of
dietary intake of folates has been totally neglected. This seems a
serious omission since dietary folate is known to directly influence
serum levels of the vitamin and must be accounted for before conclusions
can be made about the influence of other factors on serum folate levels.

However, it is not surprising that this problem has been avoided
since the difficulties involved in assessing dietary folate intake are
numerous. Consideration of this problem involves introducing the addi-
tional variables of food folate analysis and dietary evaluation. These
variables increase the complexity of any investigation into folate status
and require careful examination before attempting the design of further
studies in this area.
The Assessment of Dietary Folate Status

Nutritional adequacy with regard to folates will depend on food availability, dietary habits, and physiological absorptive capacity. The assessment of folate status must therefore include both a dietary estimation of folate intake and an examination for factors which might interfere with its absorption.

This discussion will center on the difficulties surrounding evaluation of dietary folate intake and will encompass two areas of concern: (1) Problems inherent in the assay of food folates which one should be aware of in assessing available data on the folate content of foods. (2) Problems in estimating dietary folate intake.

Problems inherent in the assay of food folates. Derivatives of folic acid are found in a wide variety of foods, most commonly as reduced polyglutamates. Folic acid (pteroylmonoglutamic acid), itself, generally constitutes only a small portion (about 5%-10%) of dietary folates and is apparently present only because of the oxidation of reduced folates during processing, storage and cooking (Hurdle, 1973). It is the multiplicity of forms of folate in food and the differing availability of these forms to various test organisms that makes the evaluation of food folates a difficult task (O’Broin et al., 1975).

Those folate derivatives which are detected by microbiological assay without prior enzymatic hydrolysis with conjugase are called "free"; whereas those which are microbiologically active only after such hydrolysis are "conjugated" (i.e., the polyglutamates). "Total" folate is a measure of both the free and conjugated forms together. Different test microorganisms will be able to utilize different forms of folate and therefore, their growth-response curves will vary for the same
food sample. Growth of *Lactobacillus casei*, the most commonly used organism, is supported by most folates with up to three glutamate residues (Hoppner et al., 1972).

The determination of dietary folic acid presents additional problems because the significance of "free" and "total" folates as determined by microbiological assay is poorly understood in the context of biological availability in mammals. We cannot equate "total" folate in food with "available" folate because the *in vivo* action of conjugase is not completely understood and cannot be assumed to be the same as that *in vitro*. For this reason, some researchers have suggested that assaying food folates without conjugase treatment (i.e., "free" folate) gives a more accurate indication of available folate in the diet (Herbert, 1963). The measurement of truly available folate probably lies somewhere between the values for "free" and "total."

In addition to the basic problem of defining available folate, there are other factors which may influence laboratory determinations of folate activity:

1. Use of ascorbic acid as an antioxidant.

Many folate composition tables were compiled before the protective effect of ascorbate against oxidation of reduced folates was recognized. Use of ascorbic acid makes more folates available to the test organism and can give values up to forty times higher (Hurdle, 1973).

2. Inhibitors and binders of folate present in foods.

Variability in folate values may also be partially accounted for by the presence of certain known and unknown inhibitors of conjugase (as in yeast) and binders of folate (as in milk) and the extent of their activity (Tamura and Stokstad, 1973).
3. The effect of cooking.

Processing and cooking, especially in large quantities of water, will destroy heat-labile folates (Herbert and Bertino, 1967), and can result in losses of 90% or more of folate activity (Herbert, 1963; Hurdle, 1973).

4. Sensitivity to pH.

Folates are available for absorption in a very narrow pH range (Butterworth, 1968). Buffering is essential to the assay of folates as they will be destroyed by extremes of pH, especially acidity (O’Broin et al., 1975).

5. The effect of storage.

Folates may be oxidized during storage and this can account for a significant loss of activity (Tamura and Stokstad, 1973).

Problems in estimating dietary folate intake. Dietary assessment is a difficult task, largely because it is so dependent on subjective evaluations. Unless dietary habits can be observed without the knowledge of the subject, some personal bias will enter in; and unless intake of food items is measured precisely, an estimation of dietary intake is likely to be distorted. Consequently, the limitations of the available methodology for assessing nutritional status must be taken into account when evaluating dietary studies (see Fidanza, 1974).

The determination of dietary folate intake is further complicated by factors peculiar to folic acid. The accuracy of a measurement of dietary folate intake will be most influenced by:

1. The interest, intelligence, and education of the subjects.
2. The choice of method for determining intake (e.g., 24 hour recall, diet record, or diet history).
3. The reliability of food collections.
4. The choice of food composition tables.

The relative merit of these tables depends on the precautions which were taken to insure accurate measurements of food folate (as outlined in the previous section).

A good model for the application of this knowledge can be found in the study by Moscovitch and Cooper (1973). Their examination of the folate content of the diets of pregnant women involved the use of both a dietary record and direct assay of food samples. A similar methodology was adapted to the design of the present research.

Conclusion

We have seen that reports regarding the folate status of women who are pregnant or using oral contraceptives are contradictory. It has been difficult to evaluate and compare the research in the literature, mainly because the methodology has varied, often considerably, from one study to another. Obviously, the value of any further research in this area will depend upon choosing those parameters which may be significant variables and insuring adequate investigation of these variables.

The major factors which have emerged as important variables in the literature reviewed here are:

1. Choice of population studied.
2. Presence of malabsorption in subjects.
4. Fasting or post-absorptive blood samples.
5. Dietary intake of folates.

Certainly the least adequately investigated factor, in studies of both pregnant women and oral contraceptive users, has been the role
that dietary intake of folates has in determining their blood folate levels. While the examination of this role will be one of the principal aims of the present study, all of the above variables are significant and each was considered in the methodological design of this study.
CHAPTER III

MATERIALS AND METHODS

For the purpose of studying the folate status of young women as affected by oral contraceptives or pregnancy a sample of 48 women was selected from the Vancouver area. These subjects were classified into three groups: a control group of 18 women, mean age 22.6 (range: 18-29 years), who had neither been pregnant nor using oral contraceptives for at least six months prior to being in the study; an oral contraceptive group of 22 women, mean age 22.5 (range: 19-28 years), all of whom had been using combination-type oral contraceptives for at least four months prior to being in the study; and a pregnant group of 8 women, mean age 26.5 (range: 22-33 years), who were in their third or fourth month of pregnancy at the time of commencing their participation on the study. Approval for this research was received from the Health Sciences Screening Committee of the University of British Columbia and informed consent was obtained in writing from each participant (Appendix A).

All subjects in the control group and oral contraceptive group were women enrolled as students at the University of British Columbia in Vancouver, British Columbia. They were informed of the study in one of several ways: through personal contact, by notices posted on university bulletin boards, by announcements given to several classes, or through the Birth Control Clinic at the Student Health Services on campus.
Pregnant women were contacted with the cooperation of several private obstetricians in the City of Vancouver who had agreed to distribute the preliminary questionnaire to interested patients. The obstetricians were restricted to choosing only women in early pregnancy who were in good health and who had not been prescribed a folic acid supplement.

Any interested woman was given a letter explaining the purpose of the research and what would be expected of her (Appendix B), as well as a preliminary questionnaire which was designed to gather necessary information regarding her health and medical history (Appendix C). The volunteers were screened by means of this questionnaire and were excluded or selected as subjects on the basis of the following criteria:

**Age:** The minimum acceptable age was 18, the maximum, 35.

**Medical history:** Evidence of any chronic inflammatory disease or infection, liver disease, kidney dysfunction, epilepsy, diabetes, tuberculosis, malaria, or an anemia within the past year were grounds for elimination from the study.

**Drug use:** If any woman was receiving regular treatment with antibiotics, anticonvulsants, antimalarial drugs, or other drugs (besides oral contraceptives) known to interfere with folic acid metabolism, she was not included in the study. If a woman was undergoing temporary treatment with antibiotics she was used as a subject only after antibiotic therapy had been discontinued.

**Blood donation:** If a woman had recently donated blood she was asked to wait at least one month before being included in the study.

**Dietary information:** Any severe dietary limitation, including caloric restriction to less than 1200 kilocalories a day, was cause for exclusion from the study.
Use of supplements: Subjects were carefully questioned regarding their use of nutritional supplements. No one taking a vitamin supplement that included folic acid was allowed to participate in the study. Iron supplementation was permitted since a very large proportion of the women were using such a supplement.

Use of oral contraceptives: A detailed history of past and present oral contraceptive use was obtained from all subjects. This included the specification of types (brands) of oral contraceptives taken and the length of time in months that each one was used. (See Appendix D for a breakdown of the number of subjects in the oral contraceptive group who were using each type of pill and the length of time each subject had been taking the pill.)

Obstetrical History: A history of past pregnancies, including those which had ended in miscarriage or therapeutic abortion, was obtained from each woman. If a woman had been pregnant within the last six months she was not admitted as a subject to either the oral contraceptive or control group. All subjects in these two groups were nulliparous, except for three women who had one child each, and one woman with two children.

For each woman in the pregnant group the approximate date of conception and day of delivery were established. Of the pregnant women, 4 had no previous children, 3 had one child each, and one had 3 children.

Collection of Blood

For the oral contraceptive users and control women fasting blood samples were taken at two different phases of the menstrual cycle, for two consecutive cycles. Figuring day 1 in control women as the first day of the menstrual flow, venous blood samples were drawn on day 5, when sex steroid levels in the blood were low, and day 20, when hormone levels were relatively
high. In women using oral contraceptives, day 5 was taken as the last day before beginning a new cycle of pills since most of the synthetic hormones from the previous cycle had been excreted by this time, and day 20 was counted from there. (This sampling procedure was adopted from Stephens et al., 1972.) Depending on the intervention of weekends and holidays, days 3 to 6 of the cycle were considered "day 5" and days 18 to 21 were considered "day 20."

In the group of pregnant women, fasting venous blood samples were taken three times during the course of pregnancy. The first sample was drawn as soon as possible after the woman had returned her questionnaire; this was sometime during the third or fourth month of pregnancy. The following two samples were taken at approximately 8 to 10 week intervals. In this way it was possible to have a blood sample from each trimester of pregnancy. (See Appendix E for the exact weeks of sampling for each subject.)

Blood was collected in the morning following an overnight fast. The samples were drawn by venipuncture and collected into Vacutainer Tubes.* Approximately 10 to 15 mls. of blood was collected for each sample, the amount depending on which analyses were to be done on the sample. The schedule for sampling was as outlined in Table 1. Whenever hemolysis occurred, a note was made of it and the degree of hemolysis estimated on a relative scale (severe, moderate, slight, very slight). If hemolysis was severe, a fresh blood sample was drawn on the following day.

Treatment of blood samples. The blood collected into the tube containing no additive was allowed to stand at room temperature for at least one hour in order to form a firm clot. This tube was then centrifuged at 2000 rpm for 15 minutes and the serum removed with a Pasteur pipette.

---

*Vacutainer tubes from Becton, Dickinson and Co., Canada, Ltd.
Aliquots of the serum were placed in small plastic tubes* as follows: 1.5 mls. for serum folate assay (for all samples), and an additional 1.0 ml. for iron assay (for all samples from the pregnant women, but only for the first and last samples from the oral contraceptive and control groups). All tubes of sera were kept frozen at -20°C. until the day of assay.

Table 1

Procedure for Collecting Blood Samples

<table>
<thead>
<tr>
<th>Additive in Vacutainer tube</th>
<th>Milliliters of blood collected</th>
<th>Pregnant Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control and Oral Contraceptive Groups</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycle I</td>
<td>Cycle II</td>
</tr>
<tr>
<td></td>
<td>day 5</td>
<td>day 20</td>
</tr>
<tr>
<td>1st sample</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>2nd sample</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>3rd sample</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4th sample</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>No additive (for serum)</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>0.5 ml. 3.8% Na citrate</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.07 ml. 15% EDTA</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

A microhematocrit was performed on the blood collected into the tube containing sodium citrate as soon as possible after the blood had been drawn. The citrated whole blood was kept frozen at -20°C. until the day of assay. This blood was used only for the determination of red cell folate levels.

*Falcon polystyrene tubes.
Whole blood treated with EDTA was run through the Model S Coulter Counter and was used to make blood smears, which were subsequently stained* for microscopic examination.

**Collection of dietary data.** Each subject was asked to keep two three-day diet records, one for the three days before the first blood sample was taken and the other for the three days prior to the last blood sample. This procedure was the same for all three groups of subjects.

Women were instructed in how to record food items accurately, and were asked not to change their usual eating habits. The subjects were not informed of the specific nutrients under examination; this was done in order to avoid some of the bias in their food intake during the time they were keeping the diet records.

**Collection of food at the time of consumption for analysis of folate content.** To evaluate the accuracy of estimations of food folate made from the available food composition tables, the folate levels in meal samples were determined for comparison with calculated values. Fifteen women volunteered to collect duplicate samples of all the food they ate for one 24-hour period. This food was then used for a direct assay of the free and total folate content. The women also kept a written record of the food they collected so that an estimation could be made of the folate values using food tables.

In order to protect the labile forms of folate from oxidation the food was collected into a plastic bucket containing 500 mls. of phosphate buffer (pH 6.10) and 150 mg. of ascorbic acid per 100 mls. buffer. During the course of collection the food samples were kept refrigerated and on the

*Camco Quick Stain, buffered differential Wright's Stain.*
day following collection they were homogenized. The homogenate was stored in 10 ml. aliquots at -20°C, until the day of assay. All of the samples were assayed for folate within one month of the time they were collected.

**Analyses**

*Analysis of blood samples.* Hemoglobin, hematocrit, and blood cell counts were determined with the Model S Coulter Counter. Blood films were given only cursory examination unless there was reason to suspect the existence of morphological abnormalities; in such a case the slide was more thoroughly examined for evidence of macrocytosis.

Serum folate was assayed using the *Lactobacillus casei* microbiological method of Baker et al. (1959), using ascorbic acid to prevent folate oxidation. Red cell folate was determined using the *L. casei* method as modified by Hoffbrand (1966) and Spray (1969), with the exception that the blood was not treated with ascorbic acid prior to freezing. This was deemed unnecessary as there was no significant loss of folate activity in citrated blood when the ascorbate treatment was omitted.* Both serum and whole blood were added to phosphate buffer containing 150 mg. of ascorbic acid per 100 mls. buffer on the day of assay, immediately after being thawed. Folate determinations were done in triplicate for each sample.

For control purposes, pooled sera with serum folate values in the low (1.5-2.0 ng/ml), mid-normal (5.0-6.0 ng/ml) and high-normal (11.0-12.0 ng/ml) ranges were kept frozen in aliquots and determined each time a folate assay was performed. Normal values for serum folate were in the range of 3.0 to 16.0 ng/ml, and for red cell folate were in the range of 190-700 ng/ml.

*Dr. R.F. Pratt, Hematology Department, St. Paul's Hospital, Vancouver, British Columbia, personal communication.*
A standard curve was determined with each assay using Folic Acid "Baker Grade."* Folic Acid Casei Medium** and Lactobacilli Broth AOAC** were used in lieu of preparing the assay medium and maintenance culture broth ourselves. Serum iron and total iron binding capacity were determined using the Hycel Kit for "Serum Iron and Iron Binding Capacity Tests."*** For women from the oral contraceptive and control groups, the iron assays were performed on pooled serum from the first and last blood samples. For pregnant women the iron assays were done on each of the three blood samples.

Evaluation of diet records. The daily intake of free and total folate was calculated from the diet records using the tables published by Hoppner et al. (1972), and Hurdle (1968). Portion sizes and amounts were estimated using the tables of Bowes and Church as revised by Church and Church (1970).

Analysis of food samples. The free and total folate of the collected food samples was determined using the L. casei microbiological method adapted for food analysis by Herbert (1963), with the exception that the concentration of ascorbic acid used was 150 mg. per 100 mls. of buffer. Total folate was assayed using chicken pancreas conjugase.**** For control purposes, the pooled sera for low, mid-normal, and high-normal serum folate levels were determined with each assay.

Statistical Analysis of the Data

The raw data were analyzed statistically by computer at the Computing Centre of the University of British Columbia. The SPSS computer program

**"Difco" Certified, from Difco Laboratories, Detroit, Michigan.
***Hycel #HY294, from Hycel, Inc., Houston, Texas.
****Difco Chicken Pancreas, from Difco Laboratories, Detroit, Michigan.
package (Kita and Morley, 1973) was employed to draw up a program for the desired analyses.

The Student's t-test with $p = 0.05$ was used to test for the statistical significance of differences among the three groups of subjects regarding each of the hematological and dietary variables. The statistical significance was also determined for differences in these variables at different phases of the menstrual cycle and in the different trimesters of pregnancy.

Tests of correlation among the variables within and between the groups of subjects were done using the Pearson Test for Correlation. Those correlations of most interest were the ones between serum and red cell folate levels and dietary folate intake. The differences between the groups of subjects in the correlation coefficients were tested for significance using Fisher's $z$-transformation.

Comparison of the assayed values of the food samples with the calculated ("expected") values for these samples was initially done using linear regression. The Pearson Product Moment Correlation Coefficient was calculated and its significance determined. This procedure was carried out on all 15 food samples and again on 13 of these samples after excluding two values which were considerably higher than the others (due to inclusion of liver in these two food samples). Because these two outlying values appeared to be skewing the results, it was decided to also perform a test of correlation using Spearman's Rank Order Test. This is a non-parametric test of correlation for situations where normal distribution is not a valid assumption.
CHAPTER IV

RESULTS

Serum Folate, Red Cell Folate, and Other Hematological Parameters

The effect of oral contraceptives. The results in Table 2 show that women taking oral contraceptives had significantly lower levels of serum folate than did controls at day 5 of the menstrual cycle (p < 0.05), although the difference between the two groups failed to reach a statistically significant level at day 20 of the cycle. Within each of the two groups of subjects there was no significant difference in serum folate levels measured at the different phases of the menstrual cycle.

Table 2

Serum Folate Concentrations Compared at Two Phases of the Menstrual Cycle in Control Women and Women Using Oral Contraceptives

<table>
<thead>
<tr>
<th></th>
<th>Serum Folate (ng/ml)</th>
<th></th>
<th></th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Day 5</td>
<td>Day 20</td>
<td>P*</td>
</tr>
<tr>
<td>Controls (N = 18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.54 ± 3.58</td>
<td>8.14 ± 4.97</td>
<td>&gt; 0.05</td>
<td>7.84 ± 4.04</td>
</tr>
<tr>
<td>O.C. Users (N = 22)</td>
<td>5.34 ± 2.20</td>
<td>5.93 ± 2.46</td>
<td>&gt; 0.05</td>
<td>5.63 ± 2.20</td>
</tr>
<tr>
<td>P*†</td>
<td>&lt; 0.05</td>
<td>&gt; 0.05</td>
<td></td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

*Two-tailed, using Student's t-test.
†Separate variance estimate (F ratio significant, p < 0.05).
Table 3 shows that there was no significant difference in red cell folate concentrations between the controls and pill users. Neither was there any statistically significant difference in red cell folate levels at the two different phases of the menstrual cycle within either of the groups.

Table 3
Red Cell Folate Concentrations Compared at Two Phases of the Menstrual Cycle in Control Women and Women Using Oral Contraceptives

<table>
<thead>
<tr>
<th></th>
<th>Red Cell Folate (ng/ml)</th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 20</td>
</tr>
<tr>
<td>Controls (N = 18)</td>
<td>210.1 ± 73.6</td>
<td>199.7 ± 74.0</td>
</tr>
<tr>
<td>O.C. Users (N = 22)</td>
<td>184.2 ± 74.1</td>
<td>184.0 ± 72.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two-tailed, using Student's t-test.

†Pooled variance estimate (F ratio not significant, p > 0.05).

In controls there was a statistically significant correlation between serum folate levels and red cell folate levels (p < 0.05) at both day 5 (r = 0.6171) and day 20 (r = 0.5181) of the menstrual cycle. However, in the oral contraceptive group this correlation failed to reach levels of statistical significance at either phase of the cycle.

The comparisons for hemoglobin, M.C.V., serum iron, and total iron binding capacity between the control women and women taking oral contraceptives are found in Table 4. No difference was found in hemoglobin concentration between the two groups, but M.C.V. was significantly higher in the oral contraceptive users at day 20 of the cycle (p < 0.05). Therefore, although
the amount of hemoglobin did not vary with oral contraceptive use, the volume of the red blood cells was somewhat greater among pill users than in control subjects. Although serum iron concentrations were higher among the women using oral contraceptives than in controls, the difference was not quite significant statistically \((p = 0.057)\). However, subjects taking oral contraceptives demonstrated significantly higher values for total iron binding capacity than did their control counterparts \((p < 0.05)\). Within each of the two groups there was no variation in hemoglobin, M.C.V., serum iron, or total iron binding capacity with the time of the menstrual cycle.

There was a significant difference \((p < 0.01)\) in white cell count between the two groups, the oral contraceptive users showing the higher values (Appendix I). In addition, leukocyte count in subjects using oral contraceptives was significantly higher on day 20 than on day 5 of the cycle \((p < 0.01)\). A similar variation in leukocyte count during the cycle was not found among control subjects.

Additional data for the other hematological parameters tested (red cell count, hematocrit, M.C.H., M.C.H.C.) are found in Appendix I. Only those findings of statistical significance have been discussed here.

The effect of type of oral contraceptive used and duration of oral contraceptive use on serum folate levels. Since the results showed that serum folate concentrations were lower in oral contraceptive users than in controls, it was decided to examine more closely the effect of different hormonal preparations and their duration of use on the serum folate levels.

The Pearson test of correlation was performed between serum folate values and the number of months of oral contraceptive use. No significant relationship was found between the duration of oral contraceptive therapy and serum folate concentration \((r = -0.0549, p > 0.10)\).
Table 4
Differences in Hemoglobin, MCV, Serum Iron, and Total Iron Binding Capacity Between Control Women and Women Using Oral Contraceptives

<table>
<thead>
<tr>
<th></th>
<th>Hemoglobin (g/100 ml) Mean ± S.D.</th>
<th>MCV (µ³) Mean ± S.D.</th>
<th>Serum Iron (µg/dl) Mean ± S.D.</th>
<th>T.I.B.C. (µg/dl) Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 20</td>
<td>Overall Mean</td>
<td>Day 5</td>
</tr>
<tr>
<td>Controls (N = 18)</td>
<td>13.0 ± 0.7</td>
<td>13.2 ± 0.5</td>
<td>13.1 ± 0.6</td>
<td>86.1 ± 2.6</td>
</tr>
<tr>
<td>O.C. Users (N = 22)</td>
<td>13.4 ± 0.8</td>
<td>13.3 ± 0.6</td>
<td>13.3 ± 0.6</td>
<td>87.5 ± 3.8</td>
</tr>
<tr>
<td>P *</td>
<td>&gt; 0.10²</td>
<td>&gt; 0.10²</td>
<td>&gt; 0.10²</td>
<td>&gt; 0.10²</td>
</tr>
</tbody>
</table>

*Two-tailed, using Student's t-test.

1 Separate variance estimate (F ratio significant, p < 0.05)

2 Pooled variance estimate (F ratio not significant, p > 0.05)
Subjects using oral contraceptives were separated into groups depending on the type of hormonal preparation they were taking. The mean serum folate concentrations at two different phases of the menstrual cycle were calculated for each of these groups of oral contraceptive users and are shown in Table 5.

The difference in serum folate levels between the women using Ortho-Novum 1/50 and those using Ovral was not statistically significant at either phase of the cycle. There was also no significant variation in the serum folate measured at different times in the menstrual cycle among women taking Ortho-Novum 1/50. In those using Ovral, however, the mean serum folate concentration at day 20 of the cycle was statistically higher than at day 5 (p < 0.05).

The effect of pregnancy. The results in Table 6 show that as pregnancy progresses the serum folate and red cell folate levels increase. However, the rise in serum folate is not statistically significant, and the increase in red cell folate is significant between trimesters 1 and 2, and trimesters 1 and 3, but not between trimesters 2 and 3. Therefore the greatest increase in red cell folate in this group of women occurred before the third trimester.

The correlation between serum folate and red cell folate was positive throughout pregnancy, but became statistically significant only in the third trimester (r = 0.754, p < 0.05).

The variation in hemoglobin, MCV and iron values during pregnancy are shown in Table 7. Hemoglobin values were significantly higher in the third trimester as compared with the second trimester. Mean corpuscular volume (MCV) generally increased with the duration of pregnancy, although it was only significantly higher in trimester 2 as compared with trimester 1.
<table>
<thead>
<tr>
<th>Oral Contraceptive Agent</th>
<th>Subject</th>
<th>Serum Folate (ng/ml)</th>
<th>Day 5</th>
<th>Day 20</th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortho-Novum 1/50 (Norethindrone, 1.0 mg) (Mestranol, 0.05 mg)</td>
<td>A.T.</td>
<td>9.15</td>
<td>9.53</td>
<td>9.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.M.</td>
<td>1.90</td>
<td>3.96</td>
<td>2.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>9.23</td>
<td>8.85</td>
<td>9.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N.B.</td>
<td>7.23</td>
<td>7.08</td>
<td>7.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.H.</td>
<td>4.20</td>
<td>2.85</td>
<td>3.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M.D.</td>
<td>5.63</td>
<td>6.80</td>
<td>6.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K.H.</td>
<td>6.03</td>
<td>4.18</td>
<td>5.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.M.</td>
<td>10.95</td>
<td>12.25</td>
<td>11.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S.M.</td>
<td>5.30</td>
<td>7.50</td>
<td>6.40</td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>6.62</td>
<td>7.00</td>
<td>6.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovral (Norgestrel, 0.50 mg) (Ethinyl estradiol, 0.05 mg)</td>
<td>L.H.</td>
<td>4.35</td>
<td>5.98</td>
<td>5.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>5.65</td>
<td>5.40</td>
<td>5.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.O.</td>
<td>3.80</td>
<td>5.25</td>
<td>4.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.S.</td>
<td>4.78</td>
<td>5.08</td>
<td>4.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A.C.</td>
<td>4.53</td>
<td>4.90</td>
<td>4.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L.P.</td>
<td>4.07</td>
<td>6.80</td>
<td>5.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W.B.</td>
<td>4.85</td>
<td>4.05</td>
<td>4.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F.N.</td>
<td>3.70</td>
<td>5.85</td>
<td>4.78</td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>4.47</td>
<td>5.41</td>
<td>4.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norlestrin (Norethindrone acetate, 2.5 mg) (Ethinylestradiol, 0.05 mg)</td>
<td>V.B.</td>
<td>4.69</td>
<td>5.70</td>
<td>5.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.M.</td>
<td>3.68</td>
<td>3.90</td>
<td>3.79</td>
<td></td>
</tr>
<tr>
<td>Ortho-Novum 1/80 (Norethindrone, 1.0 mg) (Mestranol, 0.08 mg)</td>
<td>H.R.</td>
<td>5.65</td>
<td>10.00</td>
<td>7.83</td>
<td></td>
</tr>
<tr>
<td>Ortho-Novum 2 mg. (Norethindrone, 2.0 mg) (Mestranol, 0.10 mg)</td>
<td>M.S.</td>
<td>2.79</td>
<td>3.14</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>Demulen (Ethynodiol diacetate, 1.0 mg) (Ethinyl estradiol, 0.05 mg)</td>
<td>B.M.</td>
<td>3.25</td>
<td>3.60</td>
<td>3.43</td>
<td></td>
</tr>
</tbody>
</table>
Table 6

Differences in Serum Folate and Red Cell Folate Concentrations Measured at Each Trimester of Pregnancy (N = 8)

<table>
<thead>
<tr>
<th>Trimester</th>
<th>Serum Folate (ng/ml)</th>
<th>Red Cell Folate (ng/ml)</th>
<th>Pearson Correlation Coefficient between Serum and Red Cell Folate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.76 ± 2.66</td>
<td>167.6 ± 44.8</td>
<td>0.5972*</td>
</tr>
<tr>
<td>2</td>
<td>5.11 ± 1.881</td>
<td>268.9 ± 82.43</td>
<td>0.0646*</td>
</tr>
<tr>
<td>3</td>
<td>8.71 ± 7.211,2</td>
<td>327.9 ± 168.72,3</td>
<td>0.7544**</td>
</tr>
</tbody>
</table>

1 Not significantly different from folate level in 1st trimester (p>0.05).
2 Not significantly different from folate level in 2nd trimester (p>0.05).
3 Significantly different from folate level in 1st trimester (p<0.05).

*p > 0.05
**p < 0.05
Table 7

Differences in Hemoglobin, MCV, Serum Iron, and Total Iron Binding Capacity Measured at Each Trimester of Pregnancy (N = 8)

<table>
<thead>
<tr>
<th>Trimester</th>
<th>Hemoglobin (g/100ml) Mean ± S.D.</th>
<th>MCV (µm) Mean ± S.D.</th>
<th>Serum Iron (µg/dl) Mean ± S.D.</th>
<th>T.I.B.C. (µg/dl) Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.8 ± 1.0</td>
<td>87.5 ± 3.3</td>
<td>97.6 ± 29.4</td>
<td>335.7 ± 61.9</td>
</tr>
<tr>
<td>2</td>
<td>11.5 ± 0.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>89.0 ± 2.8&lt;sup&gt;3&lt;/sup&gt;</td>
<td>101.9 ± 24.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>408.7 ± 35.6&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>12.0 ± 0.8&lt;sup&gt;1,4&lt;/sup&gt;</td>
<td>88.3 ± 3.3&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>104.0 ± 48.2&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>463.4 ± 38.5&lt;sup&gt;3,4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Not significantly different from value in 1<sup>st</sup> trimester (p>0.05).

<sup>2</sup> Not significantly different from value in 2<sup>nd</sup> trimester (p>0.05).

<sup>3</sup> Significantly different from value in 1<sup>st</sup> trimester (p<0.05).

<sup>4</sup> Significantly different from value in 2<sup>nd</sup> trimester (p<0.05).
These findings indicate that, whereas the volume of red blood cells showed the greatest increase before the third trimester, the hemoglobin concentration did not rise significantly until the last trimester.

There was a statistically significant increase in total iron binding capacity throughout the course of pregnancy; serum iron increased during pregnancy, but not to a statistically significant degree. The result was a net increase in the unsaturated iron binding capacity as pregnancy progressed.

The raw data, t-values, and levels of significance for all the hematological variables compared during pregnancy are found in Appendix J. Only those findings that were statistically significant have been included in the results section.

Comparison of pregnant subjects with controls and oral contraceptive users. Table 8 outlines the results of t-test comparisons for serum folate, red cell folate, serum iron, and total iron binding capacity among the three groups of subjects. In the first trimester of pregnancy there were no significant differences in any of these parameters when compared with controls or oral contraceptive users. The pregnant women, at this point in pregnancy, appeared comparable to non-pregnant women with regard to the parameters tested.

During the second trimester, pregnant subjects had serum folate concentrations which were not significantly different from those of oral contraceptive users, but which were statistically lower \((p<0.05)\) than those of control women. However, by the last trimester, serum folate values had increased in the pregnant women and were no longer different from the control levels.

During both the second and third trimesters pregnant women demonstrated higher red cell folate concentrations than either the controls
Table 8

Differences in Serum Folate, Red Cell Folate, Serum Iron, and Total Iron Binding Capacity Between Controls or Oral Contraceptive Users and Pregnant Women for Each Trimester of Pregnancy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls Mean ± S.D.</th>
<th>O.C. Users Mean ± S.D.</th>
<th>Pregnant Women Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st Trimester</td>
</tr>
<tr>
<td>Serum Folate</td>
<td>7.84 ± 4.04</td>
<td>5.64 ± 2.20</td>
<td>5.76 ± 2.66**2†</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Cell Folate</td>
<td>204.9 ± 71.8</td>
<td>184.1 ± 71.7</td>
<td>167.6 ± 44.8**2†</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Iron</td>
<td>84.0 ± 22.8</td>
<td>100.7 ± 29.6</td>
<td>85.4 ± 44.0**2†</td>
</tr>
<tr>
<td>(μg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.I.B.C.</td>
<td>327.6 ± 73.5</td>
<td>382.5 ± 65.7</td>
<td>335.7 ± 61.9**2†</td>
</tr>
<tr>
<td>(μg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1*Not significantly different from control value (p>0.05).
2Not significantly different from O.C. users value (p>0.05).
3Significantly different from control value (p<0.05).
4Significantly different from O.C. users value (p<0.05).

*Separate variance estimate (F ratio significant, p<0.05), compared with controls.
**Pooled variance estimate (F ratio not significant, p>0.05), compared with controls.
†Separate variance estimate (F ratio significant, p<0.05), compared with O.C. users.
‡Pooled variance estimate (F ratio not significant, p>0.05), compared with O.C. users.
or oral contraceptive users. These differences were only significant between the pregnant women and pill users, however. The difference between pregnant and control values was not statistically significant.

Serum iron levels were higher among the pregnant subjects in the last two trimesters than in controls, but the difference was not statistically significant. Total iron binding capacity in both the second and third trimesters was significantly higher than in controls; this variable was also significantly higher in pregnant women than in oral contraceptive users, although only during the third trimester.

When comparing pregnant women with controls, the only consistently significant finding was that the total iron binding capacity increased and became significantly higher than control levels as pregnancy progressed. The most impressive relationship observed when comparing pregnant women with oral contraceptive users was the continuous rise in red cell folate during the course of pregnancy to levels which were significantly higher than those in the pill users.

**Dietary Folate Intake**

**Comparison of dietary folate intake among controls, oral contraceptive users, and pregnant subjects.** As demonstrated in Table 9, no statistically significant difference was found in dietary intake of folate among the three groups of subjects. A one-way analysis of variance revealed that there was no significant difference in the sample variances for free folate or total folate intake in the three groups.

Two tests for homogeneity of variance were also performed (Cochran's test and the Bartlett-Box test) and it was determined that the variances in folate intake among the groups of subjects were homogeneous. Therefore, a pooled variance estimate was used to perform a t-test contrasting the
Table 9

One-Way Analysis of Variance Comparing Dietary Folate Intake Among Control Women, Women Using Oral Contraceptives, and Pregnant Women

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Mean ± S.D.</th>
<th>Analysis of Variance</th>
<th>Contrast Coefficient Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F-ratio</td>
<td>P*</td>
</tr>
<tr>
<td>Free Folate Intake</td>
<td>Controls (N = 18)</td>
<td>171.7 ± 81.0 (^1)</td>
<td>0.436</td>
<td>0.639</td>
</tr>
<tr>
<td></td>
<td>O.C. Users (N = 21)</td>
<td>150.5 ± 60.3 (^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnant (N = 8)</td>
<td>165.6 ± 80.9 (^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free Folate Intake</td>
<td>Controls (N = 18)</td>
<td>300.8 ± 97.1</td>
<td>0.870</td>
<td>0.429</td>
</tr>
<tr>
<td>((\mu)g/day)</td>
<td>O.C. Users (N = 21)</td>
<td>272.1 ± 79.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnant (N = 8)</td>
<td>315.8 ± 99.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two-tailed.

**Pooled variance estimate.

\(^1\)Canadian Recommended Daily Allowance: 200 \(\mu\)g free folate.

\(^2\)Canadian Recommended Daily Allowance: 250 \(\mu\)g free folate.
dietary folate intake of the controls and oral contraceptive users with
the intake of the pregnant group (Contrast Coefficient Matrix). There was
no significant difference among the three groups of subjects with respect
to their dietary intake of either free folate or total folate. It is of
interest to note that in all groups of subjects the mean daily folate intake
was below the Canadian Recommended Daily Allowance of 200 micrograms free
folate (250 micrograms free folate for pregnant women).

Relationship between dietary folate intake and serum and red cell
tolate levels. For each group of subjects the Pearson Correlation Coef­
ficients were determined comparing serum folate with free and total folate
intake and red cell folate with free and total folate intake.

The significance of the difference between the correlation coef­
ficients of controls and oral contraceptive users was figured using Fisher’s
z-transformation; the results of this test are shown in Table 10. We
found a consistently higher correlation between the serum folate levels
and dietary folate intake in the controls than in the oral contraceptive
users. This was the case whether serum values were correlated with free
folate intake or total folate intake. However, the difference in correla­
tion coefficients between these two groups was only statistically signi­
ficant at day 5 of the menstrual cycle.

In general, it can be seen that serum folate concentration appears
to be directly and significantly related to dietary folate intake among
control women, whereas a comparable relationship does not exist in women
using oral contraceptives.

The correlation between red cell folate levels and dietary folate
intake was not of statistical significance in either the controls or the
pill users.
Table 10

Pearson Correlation Coefficients Comparing Serum or Red Cell Folate Levels with Dietary Folate Intake in Control Women and Women using Oral Contraceptives

<table>
<thead>
<tr>
<th>Variable Pair</th>
<th>Day of Cycle</th>
<th>Pearson Correlation Coefficient Controls (N = 18)</th>
<th>O.C. Users (N = 21)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Folate and Free Folate Intake</td>
<td>5</td>
<td>0.7208</td>
<td>-0.0229</td>
<td>.0072</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.6727</td>
<td>0.2155</td>
<td>.0872</td>
</tr>
<tr>
<td>Serum Folate and Total Folate Intake</td>
<td>5</td>
<td>0.6189</td>
<td>-0.0832</td>
<td>.0198</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.5850</td>
<td>0.1741</td>
<td>.1528</td>
</tr>
<tr>
<td>Red Cell Folate and Free Folate Intake</td>
<td>5</td>
<td>0.2302</td>
<td>-0.1907</td>
<td>.2150</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.0719</td>
<td>-0.2157</td>
<td>.4010</td>
</tr>
<tr>
<td>Red Cell Folate and Total Folate Intake</td>
<td>5</td>
<td>0.1122</td>
<td>-0.1384</td>
<td>.4654</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.0667</td>
<td>-0.1406</td>
<td>.5486</td>
</tr>
</tbody>
</table>

*Two-tailed test using Fisher’s z-transformation.

Table 11 shows the results of the same tests for correlation and significance applied to the pregnant women. There was a difference in correlation between serum folate and dietary folate between the first and third trimesters of pregnancy. These differences were not statistically significant, but nevertheless there was a higher correlation in the first trimester. Again, there was no comparable relationship found between red cell folate levels and dietary folate intake in the pregnant women.

In all groups of subjects there was a consistently stronger correlation between free folate intake and serum or red cell folate than there was between total folate intake and serum or red cell folate.
Table 11
Differences between First and Third Trimesters of Pregnancy in Pearson Correlation Coefficients Comparing Serum or Red Cell Folate Levels and Dietary Folate Intake

<table>
<thead>
<tr>
<th>Variable Pair</th>
<th>Pearson Correlation Coefficient</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Trimester</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; Trimester</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Folate and Free Folate Intake</td>
<td>0.4563</td>
<td>-0.1300</td>
<td>0.3222</td>
<td></td>
</tr>
<tr>
<td>Serum Folate and Total Folate Intake</td>
<td>0.4429</td>
<td>-0.0599</td>
<td>0.3954</td>
<td></td>
</tr>
<tr>
<td>Red Cell Folate and Free Folate Intake</td>
<td>0.3024</td>
<td>0.2563</td>
<td>0.9362</td>
<td></td>
</tr>
<tr>
<td>Red Cell Folate and Total Folate Intake</td>
<td>0.1769</td>
<td>0.2477</td>
<td>0.9044</td>
<td></td>
</tr>
</tbody>
</table>

*Two-tailed test using Fisher's z-transformation.

Correlation between the calculated and assayed values of folate for food samples collected at the time of consumption. The calculated and assayed values for free and total folate for each food sample are listed in Appendix K.

The results of the Spearman's Rank Order Test between the calculated and assayed folate values are found in Table 12. The correlation coefficients between the calculated and assayed values were statistically significant for both free folate and total folate. These correlations remained significant when the two outlying values were omitted. Our findings indicate that the values for folate calculated from the folate composition tables of Hoppner et al. (1972) and Hurdle (1968) are comparable to the values for folate obtained by direct assay of the food.

The ratio of free folate to total folate in foods has been variously reported from 1:2 to 1:4. Therefore, this ratio was calculated for both
the assayed values and the values obtained from food tables in this study. It was found that the ratio of free folate to total folate was 1:1.77 for both the assayed and calculated folate values.

Table 12

Spearman's Rank Order Test of Correlation Between Calculated and Assayed Values of Folate for Food Samples Collected at the Time of Consumption

<table>
<thead>
<tr>
<th>Variable Pair</th>
<th>Spearman's Correlation Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 15</td>
</tr>
<tr>
<td>Free Folate</td>
<td>0.6714&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Calculated Value vs. Assayed Value</td>
<td></td>
</tr>
<tr>
<td>Total Folate</td>
<td>0.7571&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Calculated Value vs. Assayed Value</td>
<td></td>
</tr>
</tbody>
</table>

*Values for food samples containing liver omitted.

<sup>1</sup>_<sub>p</sub><0.01

<sup>2</sup>_<sub>p</sub><0.05
CHAPTER V

DISCUSSION

When comparing controls with oral contraceptive users it was found that, although the level of dietary folate intake was similar in the two groups, the women taking oral contraceptives demonstrated a significantly (p<0.05) lower mean level of serum folate than did the controls. This relationship was further evidenced by the statistically lower correlation between dietary folate intake and serum folate levels in the oral contraceptive users.

From these results it appears that oral contraceptive use reduces serum folate levels, and that this decline in serum folate is independent of dietary folate intake. Therefore, it cannot be concluded that low dietary folate intake is the cause of decreased serum folate levels in women using oral contraceptives. Rather, there appears to be some direct effect of the synthetic sex hormones in reducing serum folate concentrations.

The finding that serum folate concentrations were lower among the women using oral contraceptives is supported by the work of Shojania et al. (1971) and Roetz and Nevinny-Stickel (1973), both of which were longitudinal studies, as well as Smith et al. (1975). It is contradicted, however, by the research of Stephens et al. (1972), Paine et al. (1975) and Prasad et al. (1975), all of whom found no statistically significant difference in serum folate levels between controls and oral contraceptive users. The reason for the discrepancy in results in this area remains obscure. It could be due to not only differences in methodology, but also a lack of
control for factors other than oral contraceptives which are known to influence folate status.

A criticism of the study by Stephens et al. (1972) is that there was little information given regarding the sample of women studied or their dietary folate intake. Paine et al. (1975) also failed to examine the dietary practices of their subjects. Although our findings indicate that serum folate was not directly correlated with dietary folate intake in oral contraceptive users, nevertheless, dietary folate will directly influence the serum folate levels of control women. For this reason folate intake must be considered an important variable.

In the study by Paine et al. (1975) as well as that of Prasad et al. (1975) the authors did not specify whether their determinations for serum folate were made on fasting blood samples, and this may have skewed their results. Folate-rich foods are known to elevate the level of serum folate for several hours post-prandial.

Prasad et al. did, however, make a contribution towards clarifying the ambiguous findings in this area. They made a distinction among their subjects regarding socio-economic status, and found that those women in the lower socio-economic group had significantly lower serum and red cell folate concentrations than those from the higher socio-economic group. This finding implicates socio-economic status as a factor which may influence folate status.

It may be that what is found to be true for one sample population cannot be extrapolated to another population of women. Factors such as the socio-economic status, dietary habits, ethnic custom, age, and parity of the subjects, as well as the seasonal availability of folate-rich foods, have been suggested as determinants of folate status. These should be controlled for as far as possible in the choice of a target population.
Control and oral contraceptive subjects in the present study were
drawn from a relatively homogeneous sample population (university students)
within a narrow range of age (18 to 29 years) and parity (all were nulli-
parous except for three subjects who had one child each and one subject who
had two children). While this careful control of subject selection lends
strength to our findings, conclusions drawn from these results may only be
applicable to women from a similar population. This study was also some-
what limited in that it was a cross-sectional survey. It would be of value
to design a similar study on a longitudinal basis using subjects as their
own controls, and following their folate status over a more extended period
of time, both prior to and during oral contraceptive therapy.

The mechanism by which serum folate levels are reduced among women
using oral contraceptives is unclear. Whether the effect of these exogenous
hormones is at the level of folate absorption in the intestine or uptake by
the cells remains a matter of speculation.

If folate absorption is disturbed in some way by oral contraceptives,
one would expect a lower serum folate level among pill users for the same
intake of dietary folate. This was confirmed by our results. However, if
it is the polyglutamate forms of folate which are poorly absorbed, as sug-
gested by Streiff (1970), then one might expect a greater decrease in serum
folate relative to total folate intake than to free folate intake. This was
not observed in the present study; rather, it was the correlation between
serum folate and free folate intake which was most reduced among the oral
contraceptive users. These results may be interpreted as evidence supporting
the hypothesis of an interference of oral contraceptives at the level of
folate absorption, but not specifically a hormonal inhibition of conjugase
activity. Research corroborating this hypothesis has not been reported
in the literature.
If the alteration in folate metabolism is at the level of tissue uptake, it may be connected with the presence of the folic acid binding protein (FABP) found in women who are pregnant or using oral contraceptives (da Costa and Rothenberg, 1974). This binding protein appears to be responsible for cellular uptake rather than serum transport of folates (Waxman and Schreiber, 1974). Therefore, Shojania et al. (1975) have suggested that it may account for the increased rate of plasma folate clearance found in oral contraceptive users. The induction of this FABP by both oral contraceptive therapy and pregnancy implies some hormonal influence common to both situations. Clarification of the nature of this factor, the mechanism of its production, and its physiological function may contribute to our understanding of the process by which sex steroids act to alter folate status.

Serum folate levels were not found to vary with the normal fluctuation of circulating hormone levels in the menstrual cycle, in either the controls or oral contraceptive users. This is in agreement with the findings of Stephens et al. (1972), the only other researchers to examine serum folate changes in the cycle.

However, the difference in serum folate between the controls and oral contraceptive users was statistically significant at day 5 of the cycle and failed to reach statistical significance at day 20. The fact that this difference is most pronounced when endogenous secretion or exogenous intake of sex steroids was the lowest is difficult to explain. If there is an effect of the hormones in reducing serum folate levels, one would expect it to be most evident when these hormones are present in the greatest relative amounts. Perhaps menstruation itself exerts some as yet undefined effect on serum folate levels, although this remains speculative.
Among the oral contraceptive subjects in this study, the duration of oral contraceptive therapy (range: 4 to 120 months) was found to not be significantly correlated with serum folate concentrations. This confirms the results of Paine et al. (1975) who also found no relationship of this kind in a larger group of subjects (280 oral contraceptive users).

Our findings also indicate that women using different hormonal preparations did not have statistically significant differences in serum folate. The subjects taking Ortho-Novum 1/50, however, did show higher serum folate levels than those using Ovral; the difference in serum folate between these two groups was very close to statistical significance. These oral contraceptive preparations differ with regard to both the type of progestogen and synthetic estrogen and the relative concentrations of these hormones. Since the groups of women studied here were so small, it would be advisable to further investigate the folate status of larger groups of women using various hormonal contraceptives.

Results of the present study show that there was no adverse effect of oral contraceptive use on red cell folate concentrations. This is in contrast to the work of Prasad et al. (1975) and Smith et al. (1975). Prasad et al. examined the folate status of women from a high socio-economic group and found oral contraceptive users to have red cell folate levels that were significantly lower than controls; there was no comparable difference among women from a low socio-economic group. The authors offered no explanation for these results. Smith et al. studied a homogeneous sample of oral contraceptive users with controls matched for age, parity, and body weight; the pill users were found to have significantly lower red cell folate values. They also gave no reason for these findings.
Since both of these studies involved subjects within a wider age range than in the present study, it may be that they were looking at women who had undergone a much longer period of oral contraceptive therapy. In the present study only three subjects had been taking oral contraceptives for more than three years (Appendix D). One could speculate that a reduction in red cell folate levels occurs only after long-term oral contraceptive use, i.e., of five or more years duration.

Serum iron and total iron binding capacity were significantly higher among the women using oral contraceptives in our study than in the controls. This has been found by other researchers as well (Doar, 1973; Roetz and Nevinny-Stickel, 1973; Smith et al., 1975), and may be explained by the reduced blood loss during menstruation that is commonly associated with oral contraceptive therapy. It should be pointed out, however, that some of the subjects in this study were taking iron supplements, and it may not be valid to compare these women with others not using an iron supplement.

The elevated white cell count among oral contraceptive users in this study has been previously associated with steroid therapy (Beck, 1973). It cannot be taken as an indicator of pathological disturbance since leukocytosis is a common result of stress or drug use, and the leukocyte counts in our oral contraceptive users were not above the normal range.

Serum folate levels in the pregnant subjects studied were not statistically different from those of the controls. In addition, there was no change in the levels of serum folate or dietary folate intake over the course of pregnancy. It may be inferred that, although intake of dietary folate was below the RDA in these women, it was sufficient to maintain normal serum folate levels during pregnancy. While the tendency towards reduced serum
folate concentrations is commonly associated with pregnancy, it is also a highly variable finding and values for serum folate in pregnant women fall within a wide range (Hansen, 1968).

The sharp rise in red cell folate among our pregnant subjects as pregnancy progressed is more difficult to explain. The fact that the pregnant women showed an increase over time in correlation between serum folate and red cell folate may partially account for their improved erythrocyte folate levels. One may speculate that there is a compensatory physiological adaptation in pregnancy that allows for greater availability and storage of folates. The most likely hypothesis is that the high concentration of FABP found in pregnant women enables them to store greater amounts of folate by increasing cellular uptake (Waxman and Schreiber, 1974). This assumes, of course, that there are adequate levels of circulating folate, which in turn depends on adequate folate intake and the absence of conditions that would further increase the demand for folate. Our pregnant subjects were in good health, as judged by their obstetricians, and all were from middle-class households. These factors may have also contributed to their superior folate status during pregnancy.

It may also be postulated that, since there is evidence that a deficiency of iron may increase the folate requirement (Velez et al., 1966; Taskes et al., 1974), conversely, a supply of iron adequate to meet the increased needs of pregnancy might exert a sparing effect on folate. Perhaps the use of an iron supplement during pregnancy prevents the ineffective erythropoiesis and excessive heme catabolism associated with iron deficiency anemia, and would thereby relieve some of the increased demand for folate in pregnancy. However, the absence of a positive correlation between serum iron or total iron binding capacity and serum folate or red cell folate in the pregnant
subjects does not substantiate this hypothesis. The relationship between iron balance and folate status should be more thoroughly examined. It may be that there is a more complex synergy between these nutrients than has been supposed. In particular, it would be of value to determine the consequences of iron supplementation on folate status. If there is a sparing effect of iron on folate, this may mean that iron supplementation in pregnancy would preclude the use of a folate supplement in women with otherwise normal folate requirements.

If it is assumed that a low serum folate level is indicative of marginal folate status and a low red cell folate level is the most reliable index of a true folate deficiency, then the pregnant women in this study would not be considered at risk with respect to folate. The evidence in the Nutrition Canada National Survey that a smaller percentage of pregnant women were at risk with respect to folic acid may be epidemiological support for this finding.

The increase, as pregnancy progressed, in hemoglobin concentration, serum iron, and MCV is common among women who are taking iron supplements in pregnancy (Pritchard, 1970). Total iron binding capacity was also found to increase, and this is a normal adaptation to the increased need for iron in the pregnant woman (Hytten and Thomson, 1970). In addition, the rise in leukocyte count over the course of pregnancy is a usual occurrence (Hytten and Thomson, 1970). Therefore, these were all changes to be expected.

The values for food folate as determined from diet records and calculated from food composition tables were shown to be comparable to the folate values obtained by direct assay of the food. The degree of correlation between the calculated and assayed values using the Spearman's Rank Order Test was in close agreement with that obtained by Moscovitch and Cooper (1973).
in a similar study. This finding lends confidence to the accuracy of our estimations of dietary folate intake calculated from food tables. It also supports the assumption that diet records were a reliable method of measuring folate intake in the subjects who participated in this study.

Nevertheless, there is still strength in the argument that estimation of folate intake as free and total folate is not indicative of the actual biological availability of dietary folates. Among all three groups of subjects tested, free folate intake was consistently more highly correlated with serum and red cell folate levels than was total folate intake. This substantiates the hypothesis of Herbert in 1963 that free folate may be the more accurate measure of available folate. More sophisticated studies involving the assessment of biological availability of food folates appear necessary.

The early work of Herbert (1962) indicated that the serum folate level is very sensitive to changes in the folic acid intake. Hansen (1968) also suggested that serum folate reflects the balance between the daily intake and plasma turnover of folic acid. Although the dietary intake of folate was below the Canadian Recommended Daily Allowance in all our groups of subjects, this level of consumption was apparently adequate for the maintenance of normal serum folate levels among the control women and the pregnant women studied. Among the subjects using oral contraceptives, however, the same level of dietary folate appears to be inadequate to sustain their serum folate at concentrations comparable to those in controls.

Although the women on oral contraceptives did not demonstrate deficient concentrations of serum folate, these findings may well imply that they are at risk with respect to folate. Should their dietary folate intake be even more restricted, or should there be some additional demand on their folate stores, their chances of developing a folate deficiency would be
further increased. Women under oral contraceptive therapy would be prudent to take in the full RDA of 200 μg free folate daily. This practice may be sufficient to insure maintenance of normal serum folate levels in oral contraceptive users who are in good health. It should be pointed out, however, that there is no evidence that the quantity of dietary folate required to maintain a certain serum folate level in women taking oral contraceptives is greater than in women not using the pill.

The question of folic acid supplementation remains controversial, but deserves clarification since it is of great practical concern to both pregnant women and the large population of women taking oral contraceptives. Further research designed to investigate the current Canadian Recommended Daily Allowance for folate seems advisable. It should be determined whether or not this allowance is sufficient to support normal serum folate concentrations in women during oral contraceptive therapy and pregnancy. In addition, it should be specified under what conditions this allowance is not adequate so that appropriate revisions may be recommended.
BIBLIOGRAPHY


APPENDIX A.

DATE __________________________

NAME _______________________________________________________

ADDRESS _______________________________________________________

PHONE __________________________

CONSENT FORM

I have read the accompanying statement and questionnaire concerning the nutritional study of women who are pregnant, or oral contraceptive users, and agree to participate in the described study.

I was made aware of the fact that:

1. the study does not represent a health risk

2. all information will be held in confidence

3. I have the right to withdraw from the study at any time.

______________________________
Signature
APPENDIX B.

Division of Human Nutrition
School of Home Economics
University of British Columbia
June, 1974

We are undertaking a project this year to study some of the effects of oral contraceptives and pregnancy on nutrition. This research will help to clarify the special nutritional requirements of women who are using the pill or who are pregnant. We would like to know if you are interested in participating in this study. Three groups of women are needed:

(1) **Oral Contraceptive Users**: If you are now taking birth control pills and have been using them for at least three months you could be in this group. We would need to be able to contact you four times within two consecutive months (these two months may be anytime between July and January).

(2) **Pregnant Women**: If you are not more than three months pregnant you would be able to participate as a member of this group. We would need to see you three times during your pregnancy, in the 3rd, 5th and 8th months.

(3) **Control Group**: This will be a group of women (age 18-35) who are neither pregnant nor currently taking birth control pills. It doesn't matter if you have taken the pill in the past but we would prefer that it had been at least 6 months since you last used the pill. We would need to contact you four times within two consecutive months (sometime between July and January).

What will you need to do?

If you are interested in participating first fill out the Preliminary Questionnaire attached to this letter (this is our one and only piece of red tape). I will then contact you personally.

Each woman who agrees to take part in the study will then keep a dietary record for 7 days (you will be instructed in how to do this) and will be asked to give a series of blood samples. Women who are in the control group or the oral contraceptive group will give four blood samples over a period of two months; the samples will be taken at times to be determined according to each woman's individual menstrual cycle. Pregnant women will be asked to give three blood samples, one each in their 3rd, 5th and 8th months. The amount of blood taken will not be sufficient to cause any significant depletion.

We are unable to tell you specifically what we are looking for until after you have completed your participation. This is done for control purposes, to avoid prejudicing your food intake; we don't want you to change your usual eating habits because that would bias the results of the study. We will however let you know the outcome of our research when it is completed.

Your attention and consideration is appreciated. We can't do it without you.

If you have any questions please feel free to call me.

Sincerely,

Jean Pietarinen
APPENDIX C.

PRELIMINARY QUESTIONNAIRE

This questionnaire is designed to obtain information about you which we will need in assessing your blood values and dietary record. We also wish to determine if anyone should be excluded from the study for medical reasons.

All information will be held in confidence.
Please answer all questions as fully as you can.

* * * * * * *

Date ____________

Name ____________________________

Address ____________________________

Phone at home ____________________________

When is the best time to call you? ____________________________

General Medical

Age _______ Height _______ Weight _______

In the past five years have you had any
disease (specify) ____________________________
illness (specify) ____________________________
serious accident (specify) ____________________________

Do you now have any chronic illness or condition? ____________________________

If so, are you being treated for it? ____________________________

Specify any drugs you take in treatment: ____________________________

In the last 6 months have you taken any prescription drugs (for example, antibiotics) other than birth control pills? ______

If so, specify what ____________________________

When last taken ____________________________

Have you had any previously diagnosed anemia? ______

If so, specify how it was treated ____________________________

________________________________________

Have you had any recent transfusions? ____________________________

When was the last time you donated blood? ____________________________
Dietary Information

Are you now on any kind of special diet (including weight-reducing diet)?

Specify

Are there any foods which you must restrict or avoid for medical reasons?

Are there any foods to which you are allergic?

Do you take any nutritional supplements (for example, vitamin pills or iron)?

If so, specify what

How much?

How often?

Are any of these supplements prescribed by a doctor?

Gynecological/Obstetrical

Are your menstrual periods regular?

How long is it usually between the start of one period to the start of the next one?

When was the first day of your last menstrual flow?

Do you use birth control pills now?

If so, what kind Since when?

Have you used birth control pills in the past?

If so, what kind(s) When?

Are you now pregnant? How many months?

Have you ever been pregnant before?

Please list each pregnancy by year, noting live births, miscarriages and abortions

* * * * * * * * *

Please return completed questionnaire to me in the enclosed envelope. Thank you.
### APPENDIX D. Brand of Oral Contraceptive Used and Duration of Use Among Subjects Taking Oral Contraceptives

<table>
<thead>
<tr>
<th>Subject</th>
<th>Oral Contraceptive Agent</th>
<th>Duration of Use (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.S.</td>
<td>Ortho-Novum 2 mg.</td>
<td>4</td>
</tr>
<tr>
<td>A.T.</td>
<td>Ortho-Novum 1/50</td>
<td>4</td>
</tr>
<tr>
<td>L.H.</td>
<td>Ovral</td>
<td>9</td>
</tr>
<tr>
<td>V.B.</td>
<td>Norlestrin</td>
<td>9</td>
</tr>
<tr>
<td>C.S.</td>
<td>Ovral</td>
<td>10</td>
</tr>
<tr>
<td>P.M.</td>
<td>Ovral</td>
<td>12</td>
</tr>
<tr>
<td>A.C.</td>
<td>Ovral</td>
<td>15</td>
</tr>
<tr>
<td>S.M.</td>
<td>Ortho-Novum 1/50</td>
<td>15</td>
</tr>
<tr>
<td>W.B.</td>
<td>Ovral</td>
<td>16</td>
</tr>
<tr>
<td>C.M.</td>
<td>Norlestrin</td>
<td>17</td>
</tr>
<tr>
<td>K.H.</td>
<td>Ortho-Novum 1/50</td>
<td>18</td>
</tr>
<tr>
<td>C.H.</td>
<td>Ortho-Novum 1/50</td>
<td>18</td>
</tr>
<tr>
<td>L.P.</td>
<td>Ovral</td>
<td>19</td>
</tr>
<tr>
<td>J.P.</td>
<td>Ortho-Novum 1/50</td>
<td>22</td>
</tr>
<tr>
<td>B.M.</td>
<td>Demulen</td>
<td>23</td>
</tr>
<tr>
<td>M.D.</td>
<td>Ortho-Novum 1/50</td>
<td>36</td>
</tr>
<tr>
<td>J.T.</td>
<td>Ovral</td>
<td>36</td>
</tr>
<tr>
<td>J.M.</td>
<td>Ortho-Novum 1/50</td>
<td>36</td>
</tr>
<tr>
<td>N.B.</td>
<td>Ortho-Novum 1/50</td>
<td>36</td>
</tr>
<tr>
<td>C.O.</td>
<td>Ovral</td>
<td>60</td>
</tr>
<tr>
<td>H.R.</td>
<td>Ortho-Novum 1/80</td>
<td>96</td>
</tr>
<tr>
<td>C.M.</td>
<td>Norlestrin</td>
<td>120</td>
</tr>
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</table>
### APPENDIX E. Schedule for Collecting Blood Samples from Pregnant Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>1st sample</th>
<th>2nd sample</th>
<th>3rd sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.S.</td>
<td>13</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>F.K.</td>
<td>15</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>G.B.</td>
<td>16</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>S.M.</td>
<td>18</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>V.T.</td>
<td>12</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>L.D.</td>
<td>17</td>
<td>28</td>
<td>36</td>
</tr>
<tr>
<td>J.M.</td>
<td>15</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>A.S.</td>
<td>20</td>
<td>31</td>
<td>40</td>
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</table>
APPENDIX F. Mean Daily Dietary Intake of Folate Calculated from Two 3-Day Diet Records for Each Subject

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>First 3-Day Record*</th>
<th>Second 3-Day Record**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free Folate Intake</td>
<td>Total Folate Intake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μg/day)</td>
<td>(μg/day)</td>
</tr>
<tr>
<td>Oral Contraceptive Users (N = 21)</td>
<td>A.T.</td>
<td>100.13</td>
<td>223.78</td>
</tr>
<tr>
<td></td>
<td>L.H.</td>
<td>237.58</td>
<td>471.79</td>
</tr>
<tr>
<td></td>
<td>H.R.</td>
<td>148.63</td>
<td>267.95</td>
</tr>
<tr>
<td></td>
<td>J.M.</td>
<td>95.02</td>
<td>228.35</td>
</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>132.28</td>
<td>250.07</td>
</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>101.61</td>
<td>192.98</td>
</tr>
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<td></td>
<td>N.B.</td>
<td>191.33</td>
<td>321.00</td>
</tr>
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<td></td>
<td>C.H.</td>
<td>225.40</td>
<td>383.88</td>
</tr>
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<td></td>
<td>C.O.</td>
<td>101.63</td>
<td>169.52</td>
</tr>
<tr>
<td></td>
<td>V.B.</td>
<td>70.32</td>
<td>167.50</td>
</tr>
<tr>
<td></td>
<td>B.M.</td>
<td>161.66</td>
<td>280.87</td>
</tr>
<tr>
<td></td>
<td>M.S.</td>
<td>149.52</td>
<td>282.77</td>
</tr>
<tr>
<td></td>
<td>C.S.</td>
<td>199.22</td>
<td>329.34</td>
</tr>
<tr>
<td></td>
<td>A.C.</td>
<td>258.07</td>
<td>378.85</td>
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<tr>
<td></td>
<td>L.P.</td>
<td>73.72</td>
<td>141.73</td>
</tr>
<tr>
<td></td>
<td>C.M.</td>
<td>184.33</td>
<td>314.47</td>
</tr>
<tr>
<td></td>
<td>W.B.</td>
<td>75.67</td>
<td>181.83</td>
</tr>
<tr>
<td></td>
<td>K.H.</td>
<td>230.45</td>
<td>378.75</td>
</tr>
<tr>
<td></td>
<td>C.M.</td>
<td>180.69</td>
<td>269.04</td>
</tr>
<tr>
<td></td>
<td>P.M.</td>
<td>98.01</td>
<td>214.45</td>
</tr>
<tr>
<td></td>
<td>S.M.</td>
<td>96.23</td>
<td>232.52</td>
</tr>
<tr>
<td>Mean+S.D.</td>
<td></td>
<td>148.2± 59.8</td>
<td>270.5± 85.2</td>
</tr>
</tbody>
</table>

(continued)

*Three days prior to day 5 of the menstrual cycle, or 1st trimester.

**Three days prior to day 20 of the menstrual cycle, or 3rd trimester.
<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>First 3-Day Record*</th>
<th>Second 3-Day Record**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free Folate Intake (µg/day)</td>
<td>Total Folate Intake (µg/day)</td>
</tr>
<tr>
<td>Controls</td>
<td>E.S.</td>
<td>430.13</td>
<td>490.62</td>
</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>219.90</td>
<td>339.68</td>
</tr>
<tr>
<td></td>
<td>B.J.</td>
<td>67.83</td>
<td>160.25</td>
</tr>
<tr>
<td></td>
<td>G.B.</td>
<td>55.75</td>
<td>164.70</td>
</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>235.18</td>
<td>426.67</td>
</tr>
<tr>
<td></td>
<td>K.M.</td>
<td>149.74</td>
<td>326.69</td>
</tr>
<tr>
<td></td>
<td>C.J.</td>
<td>157.96</td>
<td>351.85</td>
</tr>
<tr>
<td></td>
<td>S.V.</td>
<td>370.79</td>
<td>508.37</td>
</tr>
<tr>
<td></td>
<td>E.L.</td>
<td>138.83</td>
<td>236.43</td>
</tr>
<tr>
<td></td>
<td>P.F.</td>
<td>317.90</td>
<td>480.57</td>
</tr>
<tr>
<td></td>
<td>J.H.</td>
<td>100.37</td>
<td>223.37</td>
</tr>
<tr>
<td></td>
<td>B.L.</td>
<td>80.96</td>
<td>185.93</td>
</tr>
<tr>
<td></td>
<td>V.M.</td>
<td>40.22</td>
<td>110.67</td>
</tr>
<tr>
<td></td>
<td>L.M.</td>
<td>233.10</td>
<td>353.13</td>
</tr>
<tr>
<td></td>
<td>M.P.</td>
<td>151.35</td>
<td>283.87</td>
</tr>
<tr>
<td></td>
<td>S.R.</td>
<td>167.94</td>
<td>270.93</td>
</tr>
<tr>
<td></td>
<td>J.S.</td>
<td>92.35</td>
<td>198.95</td>
</tr>
<tr>
<td></td>
<td>S.W.</td>
<td>156.20</td>
<td>278.94</td>
</tr>
<tr>
<td>Mean+S.D.</td>
<td></td>
<td>175.9±108.8</td>
<td>299.5±119.9</td>
</tr>
</tbody>
</table>

| Pregnant Women | J.S.    | 106.55 | 288.83 | 93.12 | 261.03 |
|                | P.K.    | 237.69 | 381.51 | 261.05 | 465.79 |
|                | G.B.    | 398.19 | 529.35 | 175.22 | 378.83 |
|                | S.M.    | 93.15  | 220.82 | 96.00  | 231.25 |
|                | V.T.    | 54.12  | 187.85 | 127.60 | 283.34 |
|                | L.D.    | 177.59 | 331.12 | 205.20 | 350.17 |
|                | J.M.    | 284.42 | 439.42 | 156.87 | 332.29 |
|                | A.S.    | 74.49  | 135.07 | 108.43 | 230.48 |
| Mean+S.D.  |         | 178.3±120.6 | 314.3±133.2 | 152.9±59.0 | 317.3±81.1 |
## APPENDIX G. Serum Folate Concentrations of Each Subject

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Serum Folate (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Oral Contraceptive Users (N = 22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.T.</td>
<td>9.15</td>
<td>9.53</td>
</tr>
<tr>
<td>L.H.</td>
<td>4.35</td>
<td>5.98</td>
</tr>
<tr>
<td>H.R.</td>
<td>5.65</td>
<td>10.00</td>
</tr>
<tr>
<td>J.M.</td>
<td>1.90</td>
<td>3.96</td>
</tr>
<tr>
<td>J.T.</td>
<td>5.65</td>
<td>5.40</td>
</tr>
<tr>
<td>J.P.</td>
<td>9.23</td>
<td>8.85</td>
</tr>
<tr>
<td>N.B.</td>
<td>7.23</td>
<td>7.08</td>
</tr>
<tr>
<td>C.H.</td>
<td>4.20</td>
<td>2.85</td>
</tr>
<tr>
<td>C.O.</td>
<td>3.80</td>
<td>5.25</td>
</tr>
<tr>
<td>V.B.</td>
<td>4.69</td>
<td>5.70</td>
</tr>
<tr>
<td>M.D.</td>
<td>5.63</td>
<td>6.80</td>
</tr>
<tr>
<td>B.M.</td>
<td>3.25</td>
<td>3.60</td>
</tr>
<tr>
<td>M.S.</td>
<td>2.79</td>
<td>3.14</td>
</tr>
<tr>
<td>C.S.</td>
<td>4.78</td>
<td>5.08</td>
</tr>
<tr>
<td>A.C.</td>
<td>4.53</td>
<td>4.90</td>
</tr>
<tr>
<td>L.P.</td>
<td>4.07</td>
<td>6.80</td>
</tr>
<tr>
<td>C.M.</td>
<td>3.68</td>
<td>3.90</td>
</tr>
<tr>
<td>W.B.</td>
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<td>4.05</td>
</tr>
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<td>K.H.</td>
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<td>4.18</td>
</tr>
<tr>
<td>C.M.</td>
<td>10.95</td>
<td>12.25</td>
</tr>
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<td>3.70</td>
<td>5.85</td>
</tr>
<tr>
<td>S.M.</td>
<td>5.30</td>
<td>7.50</td>
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<tr>
<td>Mean±S.D.</td>
<td>5.34±2.20</td>
<td>5.93±2.46</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Serum Folate (ng/ml)</th>
<th>Day 5</th>
<th>Day 20</th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>E.S.</td>
<td>13.45</td>
<td>10.33</td>
<td>11.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>5.28</td>
<td>5.03</td>
<td>5.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B.J.</td>
<td>4.70</td>
<td>4.63</td>
<td>4.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G.B.</td>
<td>5.45</td>
<td>7.73</td>
<td>6.59</td>
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</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>9.70</td>
<td>12.80</td>
<td>11.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K.M.</td>
<td>4.55</td>
<td>5.15</td>
<td>4.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.J.</td>
<td>5.93</td>
<td>6.23</td>
<td>6.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S.V.</td>
<td>14.20</td>
<td>11.28</td>
<td>12.74</td>
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</tr>
<tr>
<td></td>
<td>E.L.</td>
<td>14.90</td>
<td>18.88</td>
<td>16.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P.F.</td>
<td>10.25</td>
<td>9.15</td>
<td>9.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.H.</td>
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<td>5.63</td>
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<td>B.L.</td>
<td>5.00</td>
<td>4.33</td>
<td>4.67</td>
<td></td>
</tr>
<tr>
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<td>V.M.</td>
<td>5.55</td>
<td>3.90</td>
<td>4.73</td>
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</tr>
<tr>
<td></td>
<td>L.M.</td>
<td>3.20</td>
<td>2.53</td>
<td>2.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M.P.</td>
<td>6.58</td>
<td>7.10</td>
<td>6.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S.R.</td>
<td>9.63</td>
<td>20.30</td>
<td>14.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.S.</td>
<td>5.68</td>
<td>6.75</td>
<td>6.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S.W.</td>
<td>5.73</td>
<td>4.88</td>
<td>5.31</td>
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</tr>
<tr>
<td>Mean+S.D.</td>
<td></td>
<td>7.54 ± 3.58</td>
<td>8.14 ± 4.97</td>
<td>7.84 ± 4.04</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pregnant Women (N = 8)</th>
<th>1st Trimester</th>
<th>2nd Trimester</th>
<th>3rd Trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.S.</td>
<td>3.25</td>
<td>3.60</td>
<td>7.45</td>
</tr>
<tr>
<td>P.K.</td>
<td>11.00</td>
<td>6.70</td>
<td>5.90</td>
</tr>
<tr>
<td>G.B.</td>
<td>7.30</td>
<td>4.25</td>
<td>5.45</td>
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<td>S.M.</td>
<td>3.75</td>
<td>7.70</td>
<td>11.70</td>
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<td>V.T.</td>
<td>7.30</td>
<td>7.60</td>
<td>5.45</td>
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<td>L.D.</td>
<td>4.45</td>
<td>3.35</td>
<td>3.80</td>
</tr>
<tr>
<td>J.M.</td>
<td>5.55</td>
<td>3.85</td>
<td>25.50</td>
</tr>
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<td>3.50</td>
<td>3.80</td>
<td>4.40</td>
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<tr>
<td>Mean+S.D.</td>
<td>5.76 ± 2.66</td>
<td>5.11 ± 1.88</td>
<td>8.71 ± 7.21</td>
</tr>
</tbody>
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APPENDIX H. Red Cell Folate Concentrations of Each Subject

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Red Cell Folate (ng/ml)</th>
<th>Day 5</th>
<th>Day 20</th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Contraceptive</td>
<td>A.T.</td>
<td>315.9</td>
<td>242.3</td>
<td></td>
<td>279.1</td>
</tr>
<tr>
<td>Users</td>
<td>L.H.</td>
<td>158.2</td>
<td>157.4</td>
<td></td>
<td>157.8</td>
</tr>
<tr>
<td></td>
<td>H.R.</td>
<td>201.1</td>
<td>197.6</td>
<td></td>
<td>199.3</td>
</tr>
<tr>
<td></td>
<td>J.M.</td>
<td>86.2</td>
<td>87.5</td>
<td></td>
<td>86.8</td>
</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>182.3</td>
<td>189.7</td>
<td></td>
<td>186.0</td>
</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>187.6</td>
<td>148.4</td>
<td></td>
<td>168.0</td>
</tr>
<tr>
<td></td>
<td>N.B.</td>
<td>199.8</td>
<td>165.6</td>
<td></td>
<td>182.7</td>
</tr>
<tr>
<td></td>
<td>C.H.</td>
<td>136.4</td>
<td>137.8</td>
<td></td>
<td>137.1</td>
</tr>
<tr>
<td></td>
<td>C.O.</td>
<td>131.2</td>
<td>190.8</td>
<td></td>
<td>161.0</td>
</tr>
<tr>
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<td>V.B.</td>
<td>145.7</td>
<td>151.8</td>
<td></td>
<td>148.8</td>
</tr>
<tr>
<td></td>
<td>M.D.</td>
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<td>141.3</td>
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<td>155.7</td>
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<td>B.M.</td>
<td>131.6</td>
<td>144.4</td>
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<td>138.0</td>
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<tr>
<td></td>
<td>M.S.</td>
<td>137.9</td>
<td>160.0</td>
<td></td>
<td>149.0</td>
</tr>
<tr>
<td></td>
<td>C.S.</td>
<td>121.1</td>
<td>138.8</td>
<td></td>
<td>129.9</td>
</tr>
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<td>A.C.</td>
<td>137.2</td>
<td>146.0</td>
<td></td>
<td>141.6</td>
</tr>
<tr>
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<td>L.P.</td>
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<td>268.8</td>
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<td>261.7</td>
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<tr>
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<td>C.M.</td>
<td>222.9</td>
<td>234.2</td>
<td></td>
<td>228.5</td>
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<tr>
<td></td>
<td>W.B.</td>
<td>161.7</td>
<td>183.7</td>
<td></td>
<td>172.7</td>
</tr>
<tr>
<td></td>
<td>K.H.</td>
<td>193.0</td>
<td>226.1</td>
<td></td>
<td>209.6</td>
</tr>
<tr>
<td></td>
<td>C.M.</td>
<td>198.0</td>
<td>173.4</td>
<td></td>
<td>185.7</td>
</tr>
<tr>
<td></td>
<td>P.M.</td>
<td>130.9</td>
<td>137.6</td>
<td></td>
<td>134.3</td>
</tr>
<tr>
<td></td>
<td>S.M.</td>
<td>429.0</td>
<td>445.1</td>
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<td>437.0</td>
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</tbody>
</table>

Mean ± S.D. 184.2 ± 74.1 184.0 ± 72.2 184.1 ± 71.7

(continued)
<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Red Cell Folate (ng/ml)</th>
<th></th>
<th></th>
<th>Overall Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
<td>Day 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (N = 18)</td>
<td>E.S.</td>
<td>256.3</td>
<td>195.2</td>
<td>225.7</td>
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</tr>
<tr>
<td></td>
<td>J.T.</td>
<td>201.7</td>
<td>143.1</td>
<td>172.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B.J.</td>
<td>141.1</td>
<td>119.0</td>
<td>130.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G.B.</td>
<td>283.1</td>
<td>313.8</td>
<td>298.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J.P.</td>
<td>174.5</td>
<td>188.3</td>
<td>181.4</td>
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</tr>
<tr>
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<td>K.M.</td>
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<td>133.6</td>
<td>129.3</td>
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</tr>
<tr>
<td></td>
<td>C.J.</td>
<td>174.7</td>
<td>140.0</td>
<td>157.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S.V.</td>
<td>279.6</td>
<td>280.7</td>
<td>280.2</td>
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<tr>
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<td>E.L.</td>
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<td>253.7</td>
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<td>P.F.</td>
<td>206.4</td>
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<td>203.8</td>
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<tr>
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<td>J.H.</td>
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<td>171.6</td>
<td>172.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B.L.</td>
<td>178.3</td>
<td>180.3</td>
<td>179.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V.M.</td>
<td>166.0</td>
<td>155.4</td>
<td>160.7</td>
<td></td>
</tr>
<tr>
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<td>L.M.</td>
<td>84.4</td>
<td>87.0</td>
<td>85.7</td>
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</tr>
<tr>
<td></td>
<td>M.P.</td>
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<td>296.0</td>
<td>316.3</td>
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</tr>
<tr>
<td></td>
<td>S.R.</td>
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<td>294.0</td>
<td>281.1</td>
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</tr>
<tr>
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<td>J.M.</td>
<td>130.3</td>
<td>126.3</td>
<td>128.3</td>
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</tr>
<tr>
<td></td>
<td>S.W.</td>
<td>267.5</td>
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<td>291.2</td>
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</tr>
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<td>Mean±S.D.</td>
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<td>210.1 ± 73.6</td>
<td>199.7 ± 74.0</td>
<td>204.9 ± 71.8</td>
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</table>

<table>
<thead>
<tr>
<th>Pregnant Women (N = 8)</th>
<th>1st Trimester</th>
<th>2nd Trimester</th>
<th>3rd Trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.S.</td>
<td>116.6</td>
<td>96.5</td>
<td>73.3</td>
</tr>
<tr>
<td>F.K.</td>
<td>235.2</td>
<td>198.5</td>
<td>276.8</td>
</tr>
<tr>
<td>G.B.</td>
<td>180.4</td>
<td>277.7</td>
<td>364.1</td>
</tr>
<tr>
<td>S.M.</td>
<td>103.6</td>
<td>298.0</td>
<td>307.7</td>
</tr>
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<td>V.T.</td>
<td>156.3</td>
<td>310.3</td>
<td>323.9</td>
</tr>
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<td>350.2</td>
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<td>300.4</td>
<td>248.1</td>
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<td>Mean±S.D.</td>
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<td>268.9 ± 82.4</td>
<td>327.9 ± 168.7</td>
</tr>
<tr>
<td>Variable</td>
<td>Day of Cycle</td>
<td>Control Group</td>
<td>Oral Contraceptive Group</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
<td>------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Folate (ng/ml)</td>
<td>5</td>
<td>7.54 ± 3.58</td>
<td>5.34 ± 2.20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.14 ± 4.97</td>
<td>5.93 ± 2.46</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Red Cell Folate (ng/ml)</td>
<td>5</td>
<td>210.1 ± 73.6</td>
<td>184.2 ± 74.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>199.7 ± 74.0</td>
<td>184.0 ± 72.2</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Hemoglobin (g/100ml)</td>
<td>5</td>
<td>13.0 ± 0.7</td>
<td>13.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>13.2 ± 0.5</td>
<td>13.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>5</td>
<td>38.3 ± 2.3</td>
<td>39.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>38.6 ± 1.8</td>
<td>38.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>White Cell Count (x 10^3)</td>
<td>5</td>
<td>4.6 ± 0.8</td>
<td>6.1 ± 1.4</td>
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<tr>
<td></td>
<td>20</td>
<td>4.9 ± 1.1</td>
<td>7.1 ± 1.9</td>
</tr>
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<td>P</td>
<td>N.S.</td>
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<td>Red Cell Count (x 10^6)</td>
<td>5</td>
<td>4.44 ± 0.23</td>
<td>4.50 ± 0.28</td>
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<tr>
<td></td>
<td>20</td>
<td>4.48 ± 0.19</td>
<td>4.43 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>M.C.V. (μm^3)</td>
<td>5</td>
<td>86.1 ± 2.6</td>
<td>87.5 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>86.0 ± 2.2</td>
<td>88.0 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>M.C.H. (μg)</td>
<td>5</td>
<td>29.3 ± 0.9</td>
<td>29.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>29.4 ± 1.0</td>
<td>30.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
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### APPENDIX I. (continued)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day of Cycle</th>
<th>Control Group</th>
<th>Oral Contraceptive Group</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td></td>
</tr>
<tr>
<td>M.C.H.C. ( % )</td>
<td>5</td>
<td>33.7 ± 0.7</td>
<td>33.4 ± 0.7</td>
<td>N.S.</td>
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<tr>
<td></td>
<td>20</td>
<td>33.5 ± 0.7</td>
<td>33.6 ± 0.6</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Serum Iron (μg/dl)</td>
<td></td>
<td>84.0 ± 22.8</td>
<td>100.7 ± 29.6</td>
<td>N.S.</td>
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<tr>
<td>Total Iron Binding Capacity (μg/dl)</td>
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<td>327.6 ± 73.5</td>
<td>382.5 ± 65.7</td>
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<td>Unsaturated Iron Binding Capacity (μg/dl)</td>
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<td>243.6 ± 76.8</td>
<td>281.9 ± 78.2</td>
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<td>Free Folate Intake (μg/day)</td>
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<td>175.9 ± 108.8</td>
<td>148.2 ± 59.8</td>
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<td>20</td>
<td>167.5 ± 81.4</td>
<td>152.7 ± 83.9</td>
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</tr>
<tr>
<td></td>
<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Total Folate Intake (μg/day)</td>
<td>5</td>
<td>299.5 ± 119.9</td>
<td>270.5 ± 85.2</td>
<td>N.S.</td>
</tr>
<tr>
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<td>20</td>
<td>302.0 ± 104.4</td>
<td>273.7 ± 102.0</td>
<td>N.S.</td>
</tr>
<tr>
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<td>P</td>
<td>N.S.</td>
<td>N.S.</td>
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N.S. = Not Significant, P > 0.05
APPENDIX J. Differences in Hematological and Dietary Parameters Measured During Each Trimester of Pregnancy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± S.D.</th>
<th>1st Trimester</th>
<th>2nd Trimester</th>
<th>3rd Trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Folate (ng/ml)</td>
<td></td>
<td>5.76 ± 2.66</td>
<td>5.11 ± 1.88</td>
<td>8.71 ± 7.21</td>
</tr>
<tr>
<td>Red Cell Folate (ng/ml)</td>
<td></td>
<td>167.6 ± 44.8</td>
<td>268.9 ± 82.4</td>
<td>327.9 ± 168.7</td>
</tr>
<tr>
<td>Hemoglobin (g/100ml)</td>
<td></td>
<td>11.8 ± 1.0</td>
<td>11.5 ± 0.8</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td></td>
<td>34.3 ± 2.9</td>
<td>33.9 ± 2.0</td>
<td>34.7 ± 2.6</td>
</tr>
<tr>
<td>White Cell Count (x 10^3)</td>
<td></td>
<td>7.9 ± 2.0</td>
<td>8.9 ± 2.0</td>
<td>9.0 ± 1.9</td>
</tr>
<tr>
<td>Red Cell Count (x 10^6)</td>
<td></td>
<td>3.93 ± 0.40</td>
<td>3.78 ± 0.31</td>
<td>3.93 ± 0.34</td>
</tr>
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<td>M.C.V. (μ3)</td>
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<td>87.5 ± 3.3</td>
<td>89.0 ± 2.8</td>
<td>88.3 ± 3.3</td>
</tr>
<tr>
<td>M.C.H. (μg)</td>
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<td>30.1 ± 1.0</td>
<td>30.3 ± 0.9</td>
<td>30.5 ± 1.5</td>
</tr>
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<td>M.C.H.C. (%)</td>
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<td>33.8 ± 0.6</td>
<td>33.6 ± 0.6</td>
<td>33.8 ± 0.7</td>
</tr>
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<td>Serum Iron (μg/dl)</td>
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<td>97.6 ± 29.4</td>
<td>101.9 ± 24.5</td>
<td>104.0 ± 48.2</td>
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<td>Total Iron Binding Capacity (μg/dl)</td>
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<td>335.7 ± 61.9</td>
<td>408.9 ± 35.6</td>
<td>463.4 ± 38.5</td>
</tr>
<tr>
<td>Unsaturated Iron Binding Capacity (μg/dl)</td>
<td></td>
<td>225.9 ± 69.5</td>
<td>309.3 ± 40.8</td>
<td>362.3 ± 63.5</td>
</tr>
<tr>
<td>Free Folate Intake (μg/day)</td>
<td></td>
<td>178.3 ± 120.6</td>
<td>-</td>
<td>152.9 ± 59.0</td>
</tr>
<tr>
<td>Total Folate Intake (μg/day)</td>
<td></td>
<td>314.3 ± 133.2</td>
<td>-</td>
<td>317.3 ± 81.1</td>
</tr>
</tbody>
</table>

1Significantly (P < 0.05) different from value in 1st trimester; 22nd trimester.
3Not significantly (P > 0.05) different from 1st trimester; 42nd trimester.
APPENDIX K. Calculated and Assayed Values for Folate for Duplicate Food Samples Collected at the Time of Consumption

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Values Calculated from Food Tables</th>
<th>Assayed Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free Folate (μg)</td>
<td>Total Folate (μg)</td>
</tr>
<tr>
<td>1</td>
<td>123.20</td>
<td>198.40</td>
</tr>
<tr>
<td>2</td>
<td>101.25</td>
<td>208.90</td>
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<tr>
<td>3</td>
<td>66.05</td>
<td>131.10</td>
</tr>
<tr>
<td>4</td>
<td>35.20</td>
<td>111.50</td>
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<tr>
<td>5</td>
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<td>284.70</td>
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<tr>
<td>6</td>
<td>60.27</td>
<td>150.80</td>
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<td>105.45</td>
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<tr>
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<td>155.35</td>
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<tr>
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<tr>
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<td>606.50</td>
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<tr>
<td>15</td>
<td>720.48</td>
<td>832.00</td>
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</tbody>
</table>