CHAPTER I

INTRODUCTION

Folic acid is one of the vitamin. Its molecular structure is composed of:

1. pteridine nucleus (pyrimidine and pyrazines rings)
2. para-aminobenzoic acid
3. glutamic acid

Folic acid is also known as a pteroylglutamic acid (PGA). The empirical formula is $C_{19}H_{19}N_7O_6$ (see figure 1).

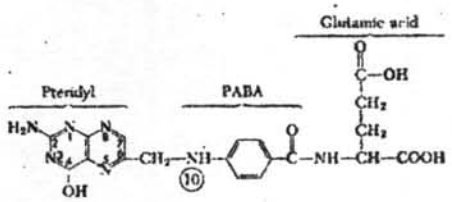
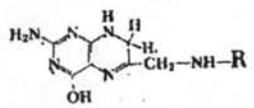


FIGURE 1. PTEROYLGLUTAMIC ACID (PGA)

Folic acid is a yellow, spear-shaped, crystalline compound which has a molecular weight of 441. It is soluble in diluted solution of alkali-carbonates and hydroxides as well as in diluted solutions of hot

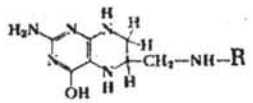
sulfuric and hydrochloric acid. On the other hand, it is insoluble in organic solvents such as alcohol, ether and benzene. The compound is more stable in an alkaline medium than in an acid and it is generally stable in the dark. The exposure of folic acid to ultraviolet light results in a rapid cleavage of the molecule at the 9th and 10th positions which gives a pteridine and a free aromatic amine (Stockstad *et al.*, 1947).

After reduction, folic acid will become biochemically active. This can be brought about enzymetically or chemically by the addition of two molecules of hydrogen to give 7,8-dihydro folic acid and subsequently, two more molecules of hydrogen to give 5,6,7,8 tetrahydrofolic acid. Their structural formulae are shown in figure 2 and 3, respectively.



7,8-Dihydrofolic acid

FIGURE 2. 7,8-dihydrofolic acid.



5,6,7,8-Tetrahydrofolic acid (FH_4)

FIGURE 3. 5,6,7,8 tetrahydrofolic acid.

From a clinical standpoint, the most important activity of folic acid is in the orderly production of new red blood cells in the bone marrow. A dietary folic deficiency causes a macrocytic anemia which resembles pernicious anemia without the involvement of the nervous system. Glossitis, gastrointestinal lessions, diarrhea and intestinal malabsorption may accompany the macrocytic anemia. A similar syndrome may be produced by the administration of a specific folic acid antagonist, aminopterin.

Folic acid is found widely in nature such as in dark green leafy vegetables, asparagus, lima beans, nut, whole-grain cereals, lentils, liver and kidney. A good source of folate in the monoglutamate form is oranges and orange juice.

The human daily requirement for folic acid is not exactly known (Krause, 1969). The U.S. Food and Drug Administration has set a daily lower limit of 0.1 mg of folic acid in vitamin preparations which are sold without prescriptions. The daily allowance recommended by the Food and Nutrition Broad varies from 0.05 mg for infants to 0.8 mg for pregnant women (Burton, 1976).

Folic acid in serum and milk is bound to protein which are called folic acid binding protein (FABP). There are two forms of FABP, one of which is an unsaturated form (unsaturated folate-binding capacity or UFBC). The other is a saturated form (saturated folate-binding capacity or SFBC). The sum of these two proteins is the total folate-binding capacity (TFBC).

FABP is composed of two ^{3}H-PGA binding protein peaks in Sephadex G-200 gel filtration (Waxman and Schreiber, 1973b). Peak I is a macromolecular protein peak (molecular weight greater than 200 000). Peak II is a smaller protein peak (molecular weight 50 000). Both of them are separated by ethanol fraction, resulting in the precipitation of peak I but not peak II. The treatment of 8M urea results in the loss of peak I in sera and human milk and to a lesser extent, peak II of FABP. Peak II is as beta-globulin and is recovered in the transferrin band region in polyacrylamide gel electrophoresis (Waxman and Schreiber, 1973b). Markkanen and Peltola (1970, 1971) found that serum folic acid activity (FAA) was in transferrin, alpha-2-macroglobulin and albumin fraction. Alpha-2-macroglobulin bound the most of the serum FAA (Markkanen *et al*., 1972). Retief and Huskisson (1969, 1970) found that most of the serum folate binder had molecular weight ranged from 70 000 to 120 000. Anti-bodies against transferrin do not block ^{3}H-PGA binding to FABP. Peak II has similar properties to beta-macroglobulin, but it is immunologically distinct from the beta-lactoglobulin and has a slightly larger molecular weight. Peak II is always in folate deficient serum and the patients with uremia whereas peak I is found in human milk and lymphocyte membranes (Waxman, 1975).

FABP has been purified over 500 folds from human milk by using PGA-affinity column chromatography (Waxman and Schreiber, 1974b). Only peak II of the FABP was attached to the affinity column whereas the large FABP, peak I, passed through. Peak II was further purified by passing through DEAE cellulose when it was eluted with hypo-osmolar

buffer with a specific activity of 750 ng/mg of protein. Therefore, the FABP of human milk probably represents two proteins which bind folate by hydrogen bonds. Although it has not yet been proved, peak I may represent a complex of peak II attached to the cell membrane, since it was extracted from cell membranes and sedimented at 200 000 g suggesting that it was particulate.

The FABP which is found in normal sera and in serum folate deficiency (Waxman and Schreiber, 1973a), leukocyte lysates from patients with chronic myelogenous leukemia (Rothenberg and da Costa, 1971), patients with uremia (Hines *et al*., 1973), leukocyte lysates of pregnant women and women taking oral contraceptives (da Costa and Rothenberg, 1974b) and in milk (Metz *et al*., 1968; Waxman and Schreiber, 1973a) have been shown to share some similar characteristics (Waxman and Schreiber, 1973c). These characteristics have also been found in cow's milk (Ghitis, 1967), hog kidney (Kamen and Caston, 1974) and the brush border membranes of rat's small intestinal epithelial cells (Leslie and Rowe, 1972), The binding of ^{3}H-PGA to FABP is a saturated process with a rapid association and a slow dissociation rate. It is a pH dependent (maximum at pH 7.4). The FABP is destroyed by boiling for 30 minutes, but not by heating at 56^{o}C for 30 minutes. The binding of ^{3}H-PGA to FABP can be dissociated when the pH of citrate and acetate is less than 3 or was extracted with detergents (sodium dodecyl sulfate, SDS). FABP binds oxidized folyl mono and polyglutamates in preference to reduced folate, although a poor inhibitor of ^{3}H-PGA in binding to FABP, is the more effective inhibitor than formyl tetrahydrofolate.

Methotrexate is also bound by FABP but to a lesser extent than folic acid or dihydrofoloic acid. The binding of ^{3}H-PGA to FABP is not inhibited by dilantin or ethanol (Waxman and Schreiber, 1973b).

There are many methods to determine the FABP content in the samples. For example:

1. DEAE-cellulose chromatography (Metz *et al*., 1968),

2. DEAE cellulose chromatography and filtration in Sephadex gel G-150 (Ford *et al*, 1969),

3. Chromatography of the radioactivity on a DEAE Sephadex column (Zettner and Duly , 1974),

4. Filtration with Sephadex G-25 chromatography and recycled with Sephadex G-200 filtration (Markkanen and Peltola, 1970-1971),

5. Affinity chromatography (Salter *et al*., 1972; Waxman and Schreiber, 1975),

6. Sephadex G-200 chromatography (Markkanen *et al*., 1974),

7. A coated charcoal radioisotope dilution assay (Metz *et al*, 1967; Ghitis *et al*, 1969; Waxman and Schreiber, 1973a, 1973b; da Costa and Rothenberg, 1974; Eichner *et al*., 1975; Colman and Herbert, 1976; Gorst *et al*., 1976; Paine *et al*., 1976).

Ghitis *et al*., (1966-1969) concluded that folic acid in cow's milk was bound to large molecular protein and there were two different kinds of folate binding proteins. The first type of binding appeared to be highly specific as it was not broken by heating. The fact that

unbound milk folate are unable to rebind may indicate that milk has no unsaturated binding capacity for its own folates. The second type of binding property did not seem to be specific. The amount of ^{3}H-PGA bound to milk was the same whether the incubation time was 5 minutes or 60 minutes, at 0°C or 37°C. Ford *et al*., (1969) also showed that the FABP in cow's milk was destroyed by boiling for 10 minutes. The pH ranged from 5.5 to 8.0 did not influence the binding capacity of milk to bind PGA. Ghitis *et al*., (1967) found that cow's milk has the capacity to bind approximately 50 ng/ml. Metz *et al*., (1968) reported that the amount of ^{3}H-PGA bound ranged from 46 to 62 ng/ml of cow's milk. It was similar to that of human milk.

Zettner and Duly (1974) showed that 1.0 ml of sera can be bind less than 1.0 ng/of exogenous ^{3}H-PGA which was a rather small quantity as compared to the average concentration of endogenous folate. And the concentration of binders may vary considerably from person to person. Eichner *et al*, (1975) showed that the FABP in normal men was lower than that in normal women and the mean value of FABP in normal for both groups was 8.6%. Waxman and Schreiber (1973a) reported that the mean value of FABP was 3.6% of 0.5 ng ^{3}H-PGA added or 45 pg/ml. while the serum folate was more than 10 ng/ml. This figure was similar to the results reported by Gorst *et al*., 1976; Colman and Herbert, 1976 (76 and 61 pg/ml, respectively). da Costa and Rothenberg (1974b) found that the FABP in normal serum bound 1 ± 1% of 0.1 ng ^{3}H-PGA (ranged from 0 to 20%). Retief *et al*., (1976) also found that the mean value of FABP in normal serum was 10 ng/ml and serum folate was greater than 3 ng/ml.

The value of FABP in pregnant women was higher than that in non-pregnant women. The reason for this was possibly due to the hormonal adaptation in the folate binder synthesis. The value of FABP in pregnant women was 20 ± 3% (da Costa and Rothenberg, 1974b). Colman and Herbert (1976) found that the mean value of FABP was highest (207 ± 83 pg/ml) in the second trimester of pregnant women, while in the first and third trimester were 133 ± 40 and 155 ± 52 pg/ml, respectively. Retief *et al*., (1976) also reported the mean value of FABP was 230 pg/ml in pregnant women.

de Costa and Rothenberg (1974b) reported that the mean FABP level in serum of women taking oral contraceptives was 22 ± 8%. Eichner *et al*., (1975) also found higher serum FABP in women taking oral contraceptives (18.3%) than that in non-drug taking serum (15.2%). But the different mean values were not statistically significant.

Waxman and Schreiber (1973a) found that the mean value of FABP in folate deficient serum was greater than in normal, but may fall to the normal value by treating with folic acid. They also found that the mean value of FABP in folate deficiency was 26.7% of 0.5 ng ^{3}H-PGA added or 333 pg/ml, while serum folate was less than 5 ng/ml. It was closer to the figure of 300 pg/ml reported by Retief *et al*., (1976). Gorst *et al*., (1976) showed that the levels of serum FABP in patients with folate deficiency was higher than that of the normal subjects. The mean value of FABP in these patients was 139 pg/ml, while the mean serum folate was 1.2 ng/ml.

Eichner *et al*, (1975) showed that there was a relationship between the serum folate level and serum FABP level in patients with uremia. The mean value of FABP in patients with uremia (28%) was higher than that in normal (8.6%). These mean values were significantly different. Colman and Herbert (1976) reported that the mean value of FABP in patients with uremia was 119 ± 18 pg/ml. Paine *et al*., (1976) found that the levels of serum FABP in patients with uremia ranged from 6% to 64%. The mean values of serum FABP in this disease (26%) and control (9.9%) showed significant difference.

Colman and Herbert (1976) reported that the mean value of FABP in patients with cirrhosis of liver was 131 ± 32 pg/ml. Waxman and Schreiber (1973a) showed that the levels of serum FABP in patients with cirrhosis of liver was lower than the normal subjects.

Although, nobody knows the real function of folate binder but Rothenberg and da Costa (1971) suggested that FABP may have some relationship to DNA synthesis, therefore, this folate cofactor is essential for the *de novo* synthesis of thymidylate from deoxyuridylate. Because the value of FABP in milk is higher than that in serum, Waxman and Schreiber (1973b) suggested that FABP may be membrane derived protein which is important in regulating cellular uptake, distribution and storage of various folate coenzymes. At the saturable process, the cellular uptake of the various folate is consistently with a carrier mediated mechanism (Hepner *et al*., 1968; Lichtenstein *et al*., 1969; Das and Hoffbrand, 1970; Corcino *et al*., 1971 and Izak *et al*., 1972).

Waxman and Schreiber (1974) showed that the folate deficient HeLa cell could absorb ^{3}H-PGA better than in normal cell (27.9% and 5.5%, respectively). This bound to different kinds of FABP were greater in the unbound state. It was found that the more amount of FABP present in serum, the less HeLa cell will take ^{3}H-PGA. It was also proved that FABP in serum prevents the cellular uptake of the folate it binds (Waxman, 1975).

The purpose of the present studies is to determine the level of FABP in sera of blood donors; patients with various diseases i.e., malaria, pyrexia of unknown origin (PUO), opisthorchiasis, anemia and liver disease (e.g. hepatitis, cirrhosis, jaundice, amoebic liver abscess (ALA), Carcinoma (CA. liver), etc.) by the method of the coated charcoal radioisotope dilution assay of Waxman and Schreiber (1973a).