The Biochemistry of Folic Acid and Vitamin B$_{12}$

F. MICHAEL LUBRAN, M.D., Ph.D.
Harbor General Hospital
Torrance, CA 90509

Folic acid and vitamin B$_{12}$ are the precursors of coenzymes involved in many important biochemical reactions. Most of our knowledge of the biochemistry of these vitamins has been obtained from the study of bacterial metabolism: there have been fewer studies of animal or human tissues. The role of these vitamins in man has been determined mainly by the investigation of patients with folate or B$_{12}$ deficiency. There is still much that is not known about their behavior in man; nor is it certain that all the biochemical reactions in which they participate have been discovered. Reactions involving folic acid and B$_{12}$ in microorganisms and even animals do not necessarily occur in man. In particular, megaloblastic anemia as seen in the human does not occur in animals deficient in folate or B$_{12}$. The major biochemical reactions of folic acid and vitamin B$_{12}$ will be described here with emphasis on those known or believed to be important in human metabolism. The literature on the subject is vast. Recent reviews are cited in the references.

Folic Acid

Folic acid is pteroylglutamic acid (figure 1). It consists of a pteridine portion linked through p-aminobenzoic acid to L-glutamic acid. It is found in plants (its name is derived from the Latin folium, a leaf), animal tissues and human red cells as a polyglutamate, typically containing three to seven glutamic acid residues linked by gamma-peptide bonds. Human small intestinal juice contains the enzyme folic conjugase which is necessary for the hydrolysis and absorption of the polyglutamates. Absorption is an active process that occurs mainly in the duodenum and jejunum.

Folic acid itself has no biological activity. It is the precursor of a group of coenzymes derived from tetrahydrofolic acid in which the N atoms at 5 and 8 and the C atoms at 6 and 7 of the pyrazine ring have been reduced. The tetrahydrofolate derivatives contain groups attached to N-5, N-10 or both atoms by a bridge (table 1). The coenzyme is attached to its specific enzyme through the pyrimidine ring.

Folic acid is reduced to dihydrofolic acid and then to tetrahydrofolic acid by the enzyme dihydrofolic acid reductase, which requires pyridine nucleotides (NADH$_2$ or NADPH$_2$). Dihydrofolic acid, which is the natural substrate, is reduced more readily than folic acid by this enzyme. Folic acid reductases, which convert folic acid to dihydrofolic acid only, have been described in some bacteria. Their role in man is uncertain. Dihydrofolic acid reductase is widely distributed in bacterial and animal cells. It is strongly inhibited by folic acid antagonists such as aminopterin.
BIOCHEMISTRY OF FOLIC ACID AND VITAMIN B₁₂

Table I. The Tetrahydrofolate Coenzymes

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Formula</th>
<th>Attached to N</th>
<th>Oxidation level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>-CH₃</td>
<td>5</td>
<td>Methanol</td>
</tr>
<tr>
<td>Methylene</td>
<td>-CH₂⁻</td>
<td>5 and 10</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Methenyl</td>
<td>-CH⁻</td>
<td>5 and 10</td>
<td>Formate</td>
</tr>
<tr>
<td>Formyl</td>
<td>-CHO</td>
<td>5 or 10</td>
<td>Formate</td>
</tr>
<tr>
<td>Formimino</td>
<td>-CH=NH</td>
<td>5</td>
<td>Formate</td>
</tr>
</tbody>
</table>

Folate coenzymes include tetrahydrofolate and substituted tetrahydrofolates

Functions of Folic Acid Coenzymes

The major function of the folate coenzymes is the transfer of one-carbon groups in a variety of synthetic reactions, which depend upon the state of oxidation of the group transferred. The lowest level of oxidation is that of methanol (methyl group); next is formaldehyde (methylene group); highest is formate (methenyl, formyl, formimino groups). Coenzymes containing these groups are readily interconvertible by specific enzymes, except for 5-methyltetrahydrofolate. The methylene level is reduced to methyl by 5,10-methylenetetrahydrofolate reductase, a flavo-protein requiring FADH₂ (reduced flavin adenine dinucleotide); the reverse reaction does not occur in man. Regeneration of tetrahydrofolate from methyltetrahydrofolate is B₁₂ dependent and occurs during the synthesis of methionine. This process is described in the section on B₁₂. The NADP-dependent enzyme, 5,10-methylenetetrahydrofolate dehydrogenase, brings about the interconversion of coenzymes containing groups at the formaldehyde and formate levels of oxidation. Methenyl and formyl groups are interconverted by a cyclohydrolase; methenyl and formimino derivatives by a cyclodeaminase.

The single carbon groups transferred by the folate coenzymes are derived from the following compounds: serine (β-C) mainly, formate and histidine to a small extent only. Single carbon atoms derived from these groups are found in: serine (β-C), formate, formaldehyde, purines (C-2 and C-8), thymine (5-methyl C) and methionine (5-methyl C). The reactions involved are described below. All are believed to take place in human metabolism.

1) L-serine-glycine interconversion

Serine hydroxymethyltransferase, tetrahydrofolate and pyridoxal-5-phosphate reversibly convert L-serine to glycine. The enzyme is present in human red cells.

\[
\text{CH}_3\text{OH} - \text{CH} - \text{COOH} + \text{H}_2\text{PteGlu} \rightarrow \text{CH}_2\text{COOH} + 5\text{,10-CH}_2\text{H}_2\text{PteGlu}
\]

2) Formiminoglutaric acid (FIGlu) conversion

FIGlu is an intermediate in the enzymatic degradation of histidine in animals. It is converted to glutamic acid by FIGlu formiminatortransferase and tetrahydrofolate.

\[
\text{COOH} - \text{CH} - \text{CH}_2\text{COOH} + \text{H}_2\text{PteGlu} \rightarrow \text{COOH} - \text{CH} - \text{CH}_2\text{CH}_2\text{COOH}
\]

The formiminotetrahydrofolate produced in this reaction is converted by cyclodeaminase into 5,10-methylenetetrahydrofolate; this in turn is converted by cyclohydrolase into 10-formyltetrahydrofolate. In patients with
folic acid deficiency, the catabolism of FIGlu is decreased and its excretion in the urine is increased. Excretion is more marked in the presence of a histidine load. Vitamin B₁₂ deficiency may also give rise to increased urinary excretion of FIGlu, but the increase is not as marked as in folic acid deficiency.

3) Utilization of formate

Tetrahydrofolate formylase (formate-activating enzyme), ATP and tetrahydrofolate give rise, reversibly, to 10-formyltetrahydrofolate; this coenzyme transfers the formyl group to appropriate substrates (for example, in the synthesis of purines).

\[
\text{H}_{4}\text{PteGlu} + \text{HCDOOH} + \text{ATP} \rightarrow \text{10-CHO-H}_{4}\text{PteGlu} + \text{ADP} + \text{Pi}
\]

Only 10-formyltetrahydrofolate participates in formate transfer in purine synthesis. The energy of hydrolysis of 5-formyltetrahydrofolate is too low for this transfer to occur. This enzyme can be converted into the 10-formyl form by ATP and magnesium ions.

4) Formylation of glutamate

This takes place through the action of glutamate transformylase and 5-formyltetrahydrofolate; 10-formyltetrahydrofolate is inactive in this reaction.

\[
\text{Glu} + \text{5-CHO-H}_{4}\text{PteGlu} \rightarrow \text{formylGlutamate} + \text{H}_{4}\text{PteGlu}
\]

The reverse reaction is important in formate metabolism, since formylglutamate is an intermediate in the conversion of the α-C atom of glycine into active formate. Urinary formate excretion rises in folic acid deficiency. Excretion is increased by oral tryptophan but not histidine.

---

**Syntheses Involving Folic Acid Coenzymes**

Folic acid coenzymes are involved in the synthesis of purines, pyrimidines (and thus, indirectly, in the synthesis of DNA) and methionine. The last synthesis also involves vitamin B₁₂ and will be described later.

1) **Purine synthesis**

Folic acid is concerned in the introduction of carbon atoms into positions 8 and 2 in the purine ring; different coenzymes are involved. C-8 is introduced by the formation of formylglycinamide ribonucleotide (FGAR) from glycinamide ribonucleotide (GAR) by 5,10-methenyltetrahydrofolate and GAR transformylase (figure 2). Tetrahydrofolate is formed. The formyl group of FGAR later condenses with the amide N to form an imidazole ring.

C-2 is introduced by formylation of 5-amino-4-imidazole-carboxamide ribonucleotide (AICAR) with 10-formyltetrahydrofolate and AICAR transformylase to give 4-formamido-5-imidazolecarboxamide ribonucleotide (FAICAR) and tetrahydrofolate (figure 3). AICAR undergoes ring closure to form inosinic acid, which is subsequently converted to adenylic, xanthyllic and guanylic acids by appropriate enzyme systems. Folate inhibitors (aminopterin, methotrexate) do not inhibit the folate dependent steps of purine synthesis.

2) **Pyrimidine synthesis**

Folic acid is not concerned in the synthesis of the pyrimidine ring, but in the introduction of the methyl group of thy-
BIOCHEMISTRY OF FOLIC ACID AND VITAMIN B\textsubscript{12}

Figure 3. Introduction of carbon atom 2 into purine ring.

Figure 4. Introduction of methyl group into a pyrimidine.

mine (figure 4) and, in phage infected E. coli, the hydroxymethyl group of 5-hydroxymethylcytosine. The folate coenzyme is 5,10-methylenetetrahydrofolate. In the synthesis of thymidylate, CH\textsubscript{2} is transferred from the folate coenzyme by thymidylate synthetase and simultaneously reduced to CH\textsubscript{3}; concurrently the tetrahydrofolate initially formed is oxidized to dihydrofolate which is then reduced to tetrahydrofolate by dihydrofolate reductase and NADPH\textsubscript{2}. Recyling of the folic acid and the continuation of pyrimidine synthesis thus depends upon the activity of dihydrofolate reductase. Folic acid antagonists which inhibit the action of this enzyme inhibit thymine synthesis; further, there is an accumulation of dihydrofolate and a decrease in the amount of tetrahydrofolate available for conversion into other folate coenzymes. Thus, other metabolic functions of folic acid are impaired by the antagonists. The synthesis of thymidylate is believed to be the rate limiting step in DNA synthesis.

Vitamin B\textsubscript{12}

Vitamin B\textsubscript{12}, although found in most animal tissues, is almost exclusively synthesized by micro-organisms, either commensals in the animal’s digestive tract or ingested with animal food. The human is wholly dependent on ingested B\textsubscript{12}, being unable to utilize any of the vitamin which might be synthesized in the intestine.

Vitamin B\textsubscript{12} probably occurs in nature in its coenzyme form linked to a specific protein. Cyanocobalamin, the form of the vitamin containing a cyanide group, does not occur in the body, but is formed during the extraction and purification procedures for obtaining the vitamin (active
charcoal is used, which contains cyanide). However, most assay procedures, whether microbiological or radioisotopic, use cyanocobalamin as the reference material; the various forms of the vitamin are first converted to it during the preliminary extraction procedures.

**Chemistry of Vitamin B₁₂**

Vitamin B₁₂ has the structure shown in figure 5. It consists of two major portions: a planar group closely, but not completely, resembling the porphyrins, and a nucleotide lying in a plane almost at right angles to it. The porphyrin-like structure is termed the corrin nucleus; unlike the porphyrins, there is a direct linkage between the two α-carbon atoms of rings A and D. The central cobalt atom, which is trivalent and positively charged, is linked to the four nitrogen atoms of the reduced pyrrol rings, to the nitrogen atom of the nucleotide and to the cyanide group. Thirteen of the nineteen carbon atoms of the corrin nucleus are fully substituted with methyl groups or acetamide or propionamide residues. The groups attached to the bridge carbon atoms are in the plane of the corrin nucleus; the other groups lie above or below the plane of the nucleus.

The nucleotide in vitamin B₁₂ differs from the nucleic acid nucleotides in two respects: the base is neither a purine nor pyrimidine, but 5,6-dimethylbenzimidazole; the ribose linkage is α-glycosidic, not β-glycosidic as in the nucleic acids. The ribose is phosphorylated at C-3. The phosphate is esterified with 1-amino-2-propanol, which is combined through an amide link with the propionic acid residue of ring D at C-17. The third hydroxyl group of the phosphate is ionized; the negative charge on the O atom and the positive charge on the Co atom make vitamin B₁₂ an inner salt.

The B₁₂ structure without the cyanide group is termed a cobalamin. Other groups may take the place of cyanide. Important cobalamins are: hydroxocobalamin, which contains an OH linked to the Co atom; aquocobalamin, which has H₂O linked to the cobalt (it is the form in which hydroxocobalamin occurs in neutral or acid solution); sulphito, chloro, nitrito, bromo and thiocyanato groups may be attached to the Co by appropriate means.

Coenzyme B₁₂, linked to a specific protein, is the principal form in which B₁₂ occurs in human and animal tissues. Two coenzymes are known: 5'-deoxyadenosylcobalamin in which adenosine (minus its OH at C 5') is linked at this atom to Co, and methylcobalamin, in which a methyl group is directly attached to the cobalt atom. Both coenzymes are light sensitive in the presence of oxygen, yielding hydroxocobalamin. Cyano-, hydroxo- and other forms of B₁₂ are rapidly converted in vivo into the coenzymes.

---

*Figure 5: Structure of cyanocobalamin.*
Biochemical Reactions of $\text{B}_{12}$ Coenzymes

These may be divided into two major groups: those requiring deoxyadenosylcobalamin and those requiring methylcobalamin. As with folic acid, most of our knowledge of the biochemistry of $\text{B}_{12}$ has been obtained through the study of microorganisms. Those reactions known or believed to occur in the human will be discussed in some detail; the remaining reactions will be described briefly.

Reactions requiring 5'-deoxyadenosylcobalamin

In these reactions, the coenzyme-enzyme complex combines with $H$ from the substrate and transfers it to the adjacent C atom. A group, originally attached to this C atom, migrates to the C atom from which the H has been detached. These changes result in an intramolecular rearrangement of the substrate. In some cases, water or ammonia may be eliminated. In the case of the ribonucleotide reductase reaction, which is more complex, the substrate is reduced in addition (figure 6).

1) Glutamate mutase reaction

Glutamic acid is rearranged to form L-threo-$\beta$-aspartic acid.

2) Dioldehydrase reaction

Ethylene or propylene glycol is converted to acetaldehyde or propionaldehyde respectively.

3) Ethanolamine deaminase reaction

Ethanolamine is converted to acetaldehyde.

4) $\beta$-Lysine isomerase

Lysine is converted to butyrate, acetate and ammonia by some clostridia. An intermediate, $\beta$-lysine, is converted to 3,5-diaminohexanoic acid. The further stages in the degradation are not clear.

These four reactions occur in bacteria, not in humans. The next two reactions are believed to occur in man, as well as in micro-organisms.

5) L-methylmalonyl-CoA mutase reaction*

This enzyme catalyzes the interconversion of L-methylmalonyl-CoA (an inter-

* Co in this enzyme refers to coenzyme A, not cobalt

![Figure 6. Reactions requiring deoxyadenosylcobalamin.](image-url)
mediate in the enzymatic conversion of propionate to succinate) and succinyl-CoA. The reaction involves the transfer of the H from C-3 of the substrate to C-5' of the deoxyadenosyl moiety of the coenzyme. The CoA-thioester carboxyl group migrates from C-2 to C-3 of the substrate and the H is transferred from the coenzyme to C-2. There is decreased activity of this enzyme system in vitamin B₁₂ deficiency; urinary excretion of methylmalonic acid is increased from the normal value of less than 4 mg in 24 hours to over 6 mg methylmalonic acid excretion in normals is unaffected by an oral dose of 10 gm of valine; this dose causes an increased excretion in patients with pernicious anemia. Increased excretion of methylmalonic acid is not consistently found in patients with other forms of B₁₂ deficiency. It is normal in patients with folic acid deficiency who do not have concurrent B₁₂ deficiency (these two deficiencies often occur together). Normal methylmalonic acid excretion is restored in patients with B₁₂ deficiency who are treated with parenteral B₁₂. Folic acid administration in these patients is without effect. These clinical observations point strongly to the existence of methylmalonyl-CoA mutase in the human.

6) Ribonucleotide reductase reaction

This enzyme catalyzes the reduction of ribonucleotides to deoxyribonucleotides, which are required for DNA synthesis. The substrate is a ribonucleotide diphosphate or triphosphate. The reaction involves the substitution of an OH group by H in the 2’ position of the ribosyl moiety by a complicated mechanism involving transfer of H from a thiol group of a low molecular weight protein to the C-5’ of the coenzyme-adenosyl moiety. Here it is exchanged for an OH. This is not an intramolecular rearrangement, as occurs in the other reactions described, but transfer of H, via the coenzyme, from a hydrogen donor.

Study of this reaction in bacteria has shown that two different reductases exist; one is B₁₂ dependent, the other is independent of B₁₂. Mammalian ribonucleotide reductase requires Mg⁺⁺ and acts on ribonucleotide disphosphate. It is not stimulated by B₁₂ coenzyme and is probably not B₁₂ dependent. B₁₂-depleted bone marrow, obtained from patients with pernicious anemia, showed no stimulation by B₁₂ or the deoxyadenosyl coenzyme.⁵ Thus, there is no evidence to show that ribonucleotide reductase activity in man is dependent on deoxyadenosylcobalamin.

Reactions Requiring Methylocobalamin

These reactions involve transfer of a methyl group from 5-methyltetrahydrofolate to an appropriate substrate. Methylocobalamin, bound to a reducing enzyme, acts as intermediate transfer agent in the reaction. Only the first reaction described below is known to occur in man.

1) Methionine synthesis

In this reaction, methionine is formed from homocysteine by transfer of a methyl group from 5-methyltetrahydrofolate; FADH₂, S-adenosylmethionine and methylocobalamin are required. The de novo synthesis of the methyl group takes place as described earlier through the formation of 5-methyltetrahydrofolate. Methionine synthesis serves as the means of renewing the tetrahydrofolate.

\[
\text{Homocysteine + 5-CH}_3\text{H}_4\text{PteGlu} \rightarrow \text{Methionine + H}_4\text{PteGlu}
\]

The reaction mechanism is complex. Initially, the reducing enzyme is combined with a cobalamin. In the presence of the other factors, the Co is reduced to the monovalent state, in which it readily accepts the CH₃ group from the S-adenosylmethionine, forming enzyme-bound methyl-
cobalamin. The methyl group is transferred from this as a carbonium ion to homocysteine and the cobalamin is remethylated by a methyl group donated by the 5-methyltetrahydrofolate. In the reaction, methyl groups are donated by 5-methyltetrahydrofolate; S-adenosylmethionine acts as a primer (i.e., it initiates the reaction) and methylcobalamin is the transfer agent. A B₁₂-independent pathway for methionine synthesis exists in some bacteria. It is of fundamental importance to determine whether methionine synthesis can occur independently of B₁₂ in the human. This question is discussed in the next section.

2) Methane formation

Methanol is converted to methane by bacteria in sewage sludge. Methylcobalamin is involved. The mechanism is similar to that described for methionine synthesis.

3) Actetate synthesis

Both 5-methyltetrahydrofolate and B₁₂ are involved in the total synthesis of acetate from CO₂. As in the two reactions described above, a protein-bound methylcobalamin complex is formed as an intermediate.

Interrelationships of Vitamin B₁₂ and Folic Acid

Folic acid deficiency and pernicious anemia produce the same cellular changes in the human bone marrow. Tissue culture studies of normal human marrow and megaloblastic marrow, involving incorporation of tritium-labelled precursors into DNA and RNA of megaloblasts and erythroblasts, suggest that, in these deficiency states, there is a failure to complete DNA synthesis prior to mitosis, i.e. there is prolongation of the S and G-2 phases of the cell cycle. These observations suggest a close association between B₁₂ and folate during hematopoiesis.

Of the many biochemical reactions involving B₁₂, only two are believed to occur in man: the isomerisation of L-methylmalonyl-CoA to succinyl-CoA (not affected by folate; disturbance of this enzyme action is postulated as the cause of the degenerative disease of the nervous system due to B₁₂ deficiency), and the methylation of homocysteine to methionine, which is folate dependent. It is this latter reaction which forms the common ground between folate and vitamin B₁₂. The folate coenzymes are interconvertible, with the exception of 5-methyltetrahydrofolate. The probable way in which tetrahydrofolate is regenerated is through the synthesis of methionine.

The “methyltetrahydrofolate trap” hypothesis postulates that, in fact, 5-methyltetrahydrofolate can be converted to other folate derivatives only by the cobalamin-dependent methyltransferase reaction; this reaction is seriously diminished in B₁₂ deficiency. The result is that the total body pool of folate is largely trapped in the form of methyltetrahydrofolate and reactions depending on the other folate coenzymes are diminished. In particular, purine and pyrimidine biosynthesis are diminished and therefore DNA production is impaired. Megaloblastosis results. The hypothesis depends upon the demonstration that the cobalamin-independent pathway, known to exist in some bacteria, does not exist in man, or is of little significance. This has not, as yet, been satisfactorily determined by direct methods.

Some indirect evidence supports the hypothesis. Methyltetrahydrofolate is the principal monoglutamate coenzyme of plasma and liver. In B₁₂ deficiency associated with a normal intake of folate and methionine, there is often an increase in plasma methyltetrahydrofolate; this is consistent with the hypothesis. The crucial test of the methyltetrahydrofolate trap hypothesis would be the demonstration of tissue deficiency of folate derivatives, other
than methyltetrahydrofolate, in patients with pernicious anemia in relapse, on ade­quate dietary folate and methionine. Direct measurements on marrow and liver have not been performed. Current methods have inadequate sensitivity.

However, indirect evidence of folate enzyme levels in tissues can be obtained by studying in these patients enzyme reactions which are folate dependent. The increased excretion of FIGlu and aminomimidazolecarboxamide in the urine of B₁₂ deficient patients, diminished by treatment with folate, is in keeping with the hypothesis. In vitro experiments on the thymidylate synthetic activity of cells from bone marrow aspirates suggest that there is a deficiency of methylenetetrahydrofolate in the marrow cells of cobalamin-deficient as well as folate deficient patients. Although there are some contradictory experiments, the methyltetrahydrofolate trap hypothesis provides at present the best explanation for the clinically documented relationship between vitamin B₁₂ and folic acid in cell metabolism.

References


SPRING MEETING
of the
ASSOCIATION OF CLINICAL SCIENTISTS

Topic:
Advances in Clinical Science
Elkhart, Indiana
April 27-30, 1972