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Biochemical Role of Folate in Cellular Metabolism

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I. INTRODUCTION

As described in the previous chapter, natural folate compounds exist in tissues as polyglutamates. These serve to keep the folates within the cell since only the monoglutamate forms are transported across membranes and only the monoglutamates are found in the plasma and urine. In most cases the polyglutamate forms of the folate coenzymes are better substrates for the enzymes than their monoglutamate counterparts. In this chapter all references to the folate coenzymes assume that it is the polyglutamates that are involved. An excellent review of the folate-vitamin B₁₂ relationship has been written [1], and a three-volume monograph [2,3] is available that contains articles on detailed aspects of folate metabolism. This chapter will emphasize some of the more recent studies with clinical significance.

The role of folate is, with a few rare exceptions, to carry one-carbon units. These one-carbon units exist at various levels of oxidation. The entire range of oxidation states for one-carbon compounds extends from methane, the most reduced, to carbon dioxide, the most oxidized. With the exception of carbon dioxide, one-carbon units are generated in the cell during metabolism and are carried from one reaction to another as folate derivatives. The oxidation levels differ from each other by the gain or loss of two electrons. When the one-carbon units are incorporated as folate derivatives, they may also be converted from one oxidation level to another by the gain (reduction) or loss (oxidation) of electrons. Table 1 lists the oxidation levels of the folate coenzymes.

One-carbon units are carried by folate only when the coenzyme is in the fully reduced tetrahydro form. It is important not to confuse the oxidation state of the folate coenzymes with the oxidation level of the one-carbon unit that is carried. As

Table 1 Oxidation Levels of the One-Carbon Substituents of the Folate Coenzymes

Oxidation level	Corresponding folate coenzyme
Methane, CH ₄	None
Methanol, CH ₃ -OH	5-Methyl-THF
Formaldehyde, CH ₂ =O	5,10-Methylene-THF
Formic acid, HCOOH	5,10-Methenyl-THF
	5,10-Formimino-THF
	5-Formyl-THF
	10-Formyl-THF
Carbon dioxide, CO ₂	None

noted in Chapter 1, folic acid is not a naturally occurring compound but can be converted to the dihydro- and tetrahydro- forms through the action of the enzyme dihydrofolate reductase. Dihydrofolate is generated during the synthesis of thymidylic acid and must be converted back to tetrahydrofolate in order to accept a one-carbon unit.

II. FOLATE AND THE ONE-CARBON POOL

The one-carbon pool is a functional concept. It describes the family of folate coenzymes that are in metabolic equilibrium [4]. Members of the pool are shown enclosed in large rectangles in Figure 1. Rectangles surrounded by a frame indicate that the folate carries a one-carbon unit. An unshaded frame indicates that the one-carbon unit is at the oxidation level of formate. The cross-hatched frame indicates that the one-carbon unit is at the oxidation level of formaldehyde, and the doubly cross-hatched frame indicates that the one-carbon unit is at the oxidation level of methanol.

Going from one oxidation level to another involves a redox reaction. The arrows indicate enzymatic reactions that convert one member of the pool to another or that move one-carbon units in and out of the pool. Before folate can carry a one-carbon unit, it must be reduced to the tetrahydro- reduction state. This is carried out by a single enzyme, dihydrofolate reductase, (1, Fig. 1), that reduces folic acid to dihydrofolate (DHF) and also reduces dihydrofolate to tetrahydrofolate (THF). Folic acid is not a natural compound, but it is much more stable than the majority of the reduced folates [5,6]. DHF is the natural substrate for the enzyme, and it is because folic acid is also reduced by the enzyme that it can serve as a vitamin.

A. Reactions That Bring One-Carbon Units Into the Pool

One-carbon units at the oxidation level of formate can enter directly as formic acid (reaction 8, Fig. 1). This is catalyzed by 10-formyl-THF synthase. This reaction uses ATP for synthesis of the formyl-amide bond with the production of ADP and

Substituents of the Folate Coenzymes

Corresponding folate coenzyme

None
5-Methyl-THF
5,10-Methylene-THF
5,10-Methenyl-THF
5,10-Formimino-THF
5-Formyl-THF
10-Formyl-THF
None

usually occurring compound but can be formed through the action of the enzyme. It is generated during the synthesis of tetrahydrofolate in order to accept

Folate Pool

cept. It describes the family of folate coenzymes [4]. Members of the pool are shown in Figure 1. Rectangles surrounded by a frame indicate that the compound is a reduced form. An unshaded frame indicates that the compound is at the one-carbon level of formaldehyde, and the doubly cross-hatched frame indicates that the one-carbon unit is at the oxidation level of

another involves a redox reaction. The conversion of one member of the pool to another is shown in Figure 1. Before folate can carry a one-carbon unit, it must be reduced to tetrahydrofolate (THF). This is carried out by dihydrofolate reductase (1, Fig. 1), that reduces folic acid to tetrahydrofolate (THF). THF is much more stable than the majority of folate coenzymes, and it is the natural substrate for the enzyme, and it is the only folate coenzyme that it can serve as a vitamin.

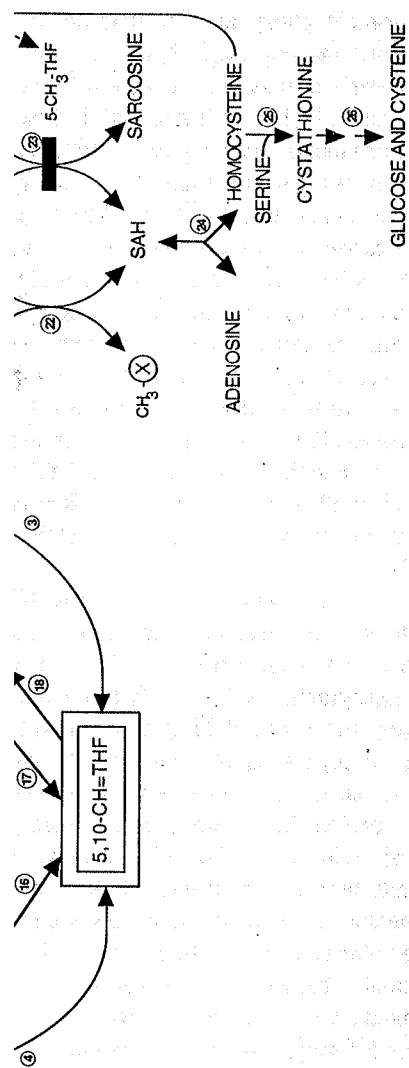
Entry of One-Carbon Units Into the Pool

Formate can enter directly as formic acid through the action of 10-formyl-THF synthase. This reaction involves the formation of a C-C bond with the production of ADP and

inorganic phosphate. In mammalian tissue this activity is found as part of a trifunctional enzyme [7,8] together with reactions 4 and 3, which serve to "channel" one-carbon units back and forth between the formate and formaldehyde levels of oxidation [9,10]. "Channeling" is a method of directing a metabolic pathway since the product of one enzymatic reaction can become the substrate of another without equilibrating with the medium. Entry at the formate level of oxidation can also take place via reactions 15 and 16. Again, in mammalian tissue, both enzymatic reactions are carried out by a single peptide, and "channeling" between the two enzymatic sites is believed to occur [11]. Formiminoglutamic acid (FIGLU) arises from the catabolism of histidine [12]. Reaction 15 in Figure 1 is catalyzed by glutamate formiminotransferase with the production of 5-formimino-THF. This is then rapidly converted to 5,10-methenyl-THF by formimino-THF cyclodeaminase (reaction 16). The third mode of entry at the formate level of oxidation involves formation of 5-formyl-THF, commonly known as folinic acid or leucovorin. This compound is the most stable of all the reduced folate coenzymes and is widely used clinically in chemotherapeutic protocols. It is rapidly converted to all the other forms of folate after cyclizing to 5,10-methenyl-THF via 5,10-methenyl-THF synthetase (reaction 17).

The metabolic origin of 5-formyl-THF has been a mystery for some time. For a long while there was doubt as to whether it was a natural product since it makes up only a small part (10–15%) of the total folate pool [13,14] and can be formed nonenzymatically from 5-formimino-THF [6]. Recent studies have shown, however, that 5-formyl-THF is not an artifact of isolation. The presence of an enzyme that catalyzes the utilization of 5-formyl-THF (reaction 17) supported the suggestion that it is a natural compound. The question of the enzymatic reaction that is responsible for the formation of 5-formyl-THF has now been conclusively answered [15]. Schirch and Stover have shown that 5-formyl-THF can be formed enzymatically by serine hydroxymethyltransferase. This enzyme's primary function is the interconversion of serine and glycine (reaction 14). Serine hydroxymethyltransferase can also convert 5,10-methenyl-THF to 5-formyl-THF (reaction 18) [16,17]. The actual substrate of the reaction may be a hydrated form of 5,10-methenyl-THF, called anhydroleucovorin B, first described in 1952. Schirch and Stover have shown that 5-formyl-THF is a potent inhibitor of many folate-utilizing reactions and suggest that it may play a regulatory role in one-carbon metabolism [17].

Entry of one-carbon units at the formaldehyde level of oxidation occurs primarily through the action of serine hydroxymethyltransferase (reaction 14, discussed above). Although this reaction is freely reversible, studies have shown that the β -carbon of serine is the major source of metabolically available one-carbon units and, in vivo, formation of glycine is predominant [18]. Another source of entry at the formaldehyde level of oxidation is the glycine cleavage reaction (reaction 13). This is a complex reaction requiring the participation of four separate proteins and found



There are no reactions that permit entry of a one-carbon unit at the methanol level of oxidation. As mentioned above, reactions 11 and 12 catalyze the oxidation of the one-carbon units of dimethylglycine and sarcosine from the methanol to the formaldehyde oxidation level before incorporation into the folate pool.

Figure 1 Reactions involving enzymes and coenzymes of the one-carbon pool. The circled numbers refer to the following enzymes: 1, dihydrofolate reductase; 2, thymidylate synthase; 3, 5,10-methylenetetrahydrofolate dehydrogenase; 4, 5,10-methenyltetrahydrofolate cyclohydrolase; 5, phosphoribosylglycinamide transformylase; 6, aminocarboxamide ribotide transformylase; 7, 10-formyltetrahydrofolate dehydrogenase; 8, 10-formyltetrahydrofolate synthase; 9, methionyl *t*-RNA formyltransferase; 10, nonenzymatic formation of 5,10-methylenetetrahydrofolate; 11, dimethylglycine dehydrogenase; 12, sarcosine dehydrogenase; 13, glycine cleavage enzyme system; 14, serine hydroxymethyltransferase; 15, glutamate formiminotransferase; 16, formiminotetrahydrofolate cyclodeaminase; 17, 5,10-methenyltetrahydrofolate synthetase; 18, minor reaction catalyzed by serine hydroxymethyltransferase; 19, 5,10-methylenetetrahydrofolate reductase; 20, methionine synthase; 21, methionine adenosyltransferase; 22, a variety of methyltransferase reactions; 23, glycine *N*-methyltransferase; 24, S-adenosylhomocystein hydrolase; 25, cystathionine synthase; 26, enzymes that convert cystathionine to cysteine and eventually glucose. DHF, dihydrofolic acid; THF, tetrahydrofolic acid; 10-HCO-THF, 10-formyltetrahydrofolic acid; 5-NH=CH-THF, 5-formiminotetrahydrofolic acid; 5,10-CH=THF, 5,10-methenyltetrahydrofolic acid; 5-HCO-THF, 5-formyl-tetrahydrofolic acid; 5-CH₃-THF, 5-methyltetrahydrofolic acid; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

B. Reactions That Move One-Carbon Units Out of the Pool

10-Formyl-THF synthase is reversible (reaction 8, Fig. 1), as discussed above. The energetically favorable direction of the reaction is the synthesis of 10-formyl-THF, which requires ATP and also produces ADP and inorganic phosphate [4]. This is in keeping with its role in removing formate that is generated metabolically and serves as the source of the one-carbon units needed for purine synthesis (reactions 5 and 6). In the presence of sufficient ADP and inorganic phosphate, however, ATP and formate are produced. Reaction 7 is catalyzed by 10-formyl-THF dehydrogenase [23,24]. During this reaction the formate is oxidized to CO_2 . Although this enzyme can also hydrolyze 10-formyl-THF to THF and formate, it appears to be a minor activity, and it is uncertain whether simple hydrolysis to THF and formate occurs in the cell [25]. The purpose of the 10-formyl-THF dehydrogenase reaction is probably to return any folate not needed for purine synthesis to THF where it is available for accepting other one-carbon units. Reactions 5 and 6 utilize one-carbon units at the oxidation level of formate for the synthesis of purines [26]. Glycinamide ribotide (GAR) and aminoimidazole-carboxamide ribotide (AICAR) each accept a one-carbon unit from 10-formyl-THF during the biosynthesis of the purine ring. The two enzymes responsible are GAR transformylase and AICAR transformylase. Reaction 9, restricted to mitochondria, is responsible for the formylation of methionine in tRNA-fMet needed to initiate protein synthesis in mitochondria [27]. This is not necessary for cytosolic protein synthesis in eukaryotes.

One-carbon units at the formaldehyde level of oxidation, i.e., 5,10-methylene-THF, are transferred in two reactions. Reaction 14, catalyzed by serine hydroxymethyltransferase, has been discussed. The formation of serine via this reaction is probably not physiologically important since serine can also be synthesized via glycolysis from phosphoserine. Reaction 2, however, is extremely important. It is responsible for the synthesis of thymidylic acid (TMP) from deoxyuridylic acid (dUMP). Thymine, the pyrimidine base needed for DNA synthesis, is made in this reaction from uridine, while both are deoxynucleotides (i.e., deoxyuridylate and thymidylate). An important feature of this reaction is the fact that the one-carbon unit transferred to form thymidylic acid appears at the methanol level of oxidation, while the source of the one-carbon unit, 5,10-methylene THF, is at the formaldehyde level of oxidation. No external electron donors are needed for this reduction. Instead, the electrons needed for this reduction come from the tetrahydrofolate portion of the molecule itself, and the product is dihydrofolate (DHF). Dihydrofolate reductase (reaction 1) is necessary to regenerate THF so that it can accept one-carbon units.

The only one-carbon unit at the methanol level of oxidation is 5-methyl-THF, and the only reaction in which it participates is the transfer of the methyl group to homocysteine for the synthesis of methionine (reaction 20). This might seem strange

Units Out of the Pool

on 8, Fig. 1), as discussed above. The main purpose of the synthesis of 10-formyl-THF, and inorganic phosphate [4]. This is a form of folate that is generated metabolically and is needed for purine synthesis (reactions 1 and 2). Inorganic phosphate, however, ATP is not needed for the synthesis of 10-formyl-THF. The reaction of 10-formyl-THF on the formate is oxidized to CO_2 . The reaction of 10-formyl-THF to THF and formate, it is not clear whether simple hydrolysis to THF is the purpose of the 10-formyl-THF. In any folate not needed for purine synthesis, it is not accepting other one-carbon units. The oxidation level of formate for the reaction is the same as for the reaction of 10-formyl-THF to THF and formate (GAR) and aminoimidazole-one-carbon unit from 10-formyl-THF. The two enzymes responsible are GAR (reaction 9, restricted to mitochondria) and methionine synthase (reaction 21, needed to initiate protein synthesis). Methionine is not necessary for cytosolic protein

level of oxidation, i.e., 5,10-methylene-THF. Reaction 14, catalyzed by serine hydroxymethyltransferase, is needed. The formation of serine via this reaction is important since serine can also be synthesized via reaction 2, however, is extremely important. The reaction of 5,10-methylene-THF to 5-methyl-THF (reaction 20), however, is extremely important. The reaction of 5,10-methylene-THF to 5-methyl-THF is catalyzed by the enzyme 5,10-methylene-THF reductase [28]. The electron donor for this reduction is tightly bound FADH_2 , and the reduction is essentially irreversible. It should also be noted that this reaction is inhibited by S-adenosylmethionine (SAM) [31,32]. SAM is the universal methyl donor for most reactions that occur in the cell. The methyl group of methionine can only be transferred after methionine has been converted to SAM in reaction 21. This has important regulatory implications.

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since methionine is an essential amino acid, but it is the homocysteine portion of the molecule that cannot be synthesized. As will be discussed below, the methyl group of methionine is metabolically labile. The enzyme that carries out this reaction is methionine synthase [28]. It requires a form of vitamin B_{12} (cyanocobalamin) as a cofactor. During the reaction the methyl group is transferred first from 5-methyl-THF to the cofactor to form methylcobalamin, and the methyl group is then transferred to homocysteine [29,30].

C. Enzymes That Interconvert One-Carbon Units Within the Pool

The interconversion of 10-formyl-THF and 5,10-methenyl-THF (reaction 4) is catalyzed by methenyl-THF cyclohydrolase. Both this reaction and the reduction of 5,10-methenyl-THF to 5,10-methylene-THF (reaction 3) are catalyzed by enzyme activities that are part of the trifunctional enzyme discussed earlier [7,8]. Reaction 16 is catalyzed by the cyclodeaminase that is found as part of a bifunctional enzyme along with the glutamate formiminotransferase that catalyzes reaction 15 [12]. The conversion of 5-formyl-THF to 5,10-methenyl-THF (reaction 17) is catalyzed by 5,10-methenyl THF synthetase, as described above. The formation of 5-formyl-THF from 5,10-methenyl-THF is a minor activity associated with serine hydroxymethyltransferase (reaction 14). As discussed earlier, this is probably the physiological source of 5-formyl-THF [15]. Reaction 19 carries out an extremely important interconversion, the reduction of 5,10-methylene-THF to 5-methyl-THF. This is catalyzed by the enzyme 5,10-methylene-THF reductase [28]. The electron donor for this reduction is tightly bound FADH_2 , and the reduction is essentially irreversible. It should also be noted that this reaction is inhibited by S-adenosylmethionine (SAM) [31,32]. SAM is the universal methyl donor for most reactions that occur in the cell. The methyl group of methionine can only be transferred after methionine has been converted to SAM in reaction 21. This has important regulatory implications.

D. Enzymes Regulated by Folate

In addition to those enzymes for which various forms of folate act as a substrate, there is one particular case in which folate acts as an allosteric regulator: the enzyme glycine *N*-methyltransferase (GNMT) catalyzes reaction 23 [33]. There are many examples of enzymes inhibited by a particular form of folate because of structural similarity with the substrate form of folate. In the case of GNMT, no form of folate is a substrate of the reaction. GNMT is a typical methyl transferase in which the methyl donor is SAM [34,35]. The methyl group is transferred to glycine to form *N*-methylglycine (sarcosine) and S-adenosylhomocysteine (SAH). The specific inhibitor of the reaction is 5-methyl-THF. There are more than 100 methyl transferase enzymes that act on a wide variety of substrates. Both small molecules and macromolecules are methylated, which results in the formation of compounds

that are metabolically and structurally important, e.g., epinephrine, phosphatidylcholine, and carnitine. The methylation of DNA is important in gene expression [36]. The different methyltransferases are collectively depicted in Figure 1 as reaction 22, where X denotes a compound that serves as a methyl acceptor. Each of these methyltransferases is present in small amounts. In contrast, GNMT is very abundant. In the livers of various species it makes up 1–3% of the soluble protein of the cytosol [35,37]. GNMT catalyzes a reaction that synthesizes sarcosine, a compound with no known metabolic role (it is rapidly oxidized back to glycine via reaction 12). Thus the methyl transferases that synthesize essential products are each present in small quantities, while the enzyme that synthesizes a nonessential product appears to be present in very large amounts. It has been suggested therefore that the function of GNMT is not to synthesize sarcosine, but to convert excess SAM to SAH through a reaction that does not form a product that might have physiological consequences. The ratio of SAM to SAH is important because most methyl transferase reactions are product-inhibited by SAH and their activities are governed by the SAM/SAH ratio [38]. GNMT provides an alternate route from SAM to SAH. Once SAH is formed it is cleaved to adenosine and homocysteine by SAH hydrolase (reaction 24). The homocysteine may be remethylated in reaction 20 or may combine with serine to form cystathionine (reaction 25) catalyzed by cystathionine synthase. Cystathionine can eventually be converted to cysteine and to glucose for gluconeogenesis. It is significant that this major pathway for methionine catabolism begins with activation to form SAM and requires methyl transfer to an acceptor in order for homocysteine to be produced [39].

Note that reactions 19 and 23 complement each other in that the synthesis of 5-methyl-THF is inhibited by SAM and the utilization of SAM is inhibited by 5-methyl-THF. This links the *de novo* synthesis of methyl groups (reaction 19) with regulation of the SAM/SAH ratio and availability of dietary methionine. When there is sufficient methionine in the diet, reaction 21 ensures that enough SAM will be made to inhibit reaction 19. The synthesis of 5-methyl-THF is unnecessary under these conditions since its only function is for remethylation of homocysteine. The decreased amount of 5-methyl-THF means that GNMT (reaction 23) is not inhibited and can proceed at a high rate to remove excess SAM and maintain the SAM/SAH ratio. When dietary methionine is low, however, there will be less SAM formed via reaction 21 and less inhibition of reaction 19. The increased levels of 5-methyl-THF contribute to the remethylation of homocysteine and inhibit GNMT (reaction 23). This acts to conserve the limited amount of methionine for the essential methylation reactions denoted by reaction 22.

III. COMPARTMENTATION OF FOLATE METABOLISM

Recent studies have shown that there is a compartmentation of folate metabolism between the cytosol and the mitochondria, at least in the liver and probably in other

ly important, e.g., epinephrine, methylation of DNA is important in gene expression. These processes are collectively depicted in Figure 1 and that serves as a methyl acceptor. It is found in small amounts. In contrast, GNMT is an enzyme that makes up 1–3% of the soluble cytosolic fraction. It catalyzes a reaction that synthesizes sarcosine, which is then rapidly oxidized back to glycine. The enzymes that synthesize essential products are distinct from those that synthesize nonessential products. It has been suggested therefore that the conversion of sarcosine, but not to convert excess SAM to SAH is important because most is inhibited by SAH and their activities are distinct. GNMT provides an alternate route from SAH to adenosine and homocysteine. Homocysteine may be remethylated in reaction 24 to methionine (reaction 25) catalyzed by methionine synthase. Eventually, methionine will be converted to cysteine and methionine is significant that this major pathway for the conversion of SAH to SAM and requires methyl groups to be produced [39].

Interrelated in that the synthesis of SAM is inhibited by the presence of methyl groups (reaction 19) with the availability of dietary methionine. When there is a deficiency of 5-methyl-THF, it ensures that enough SAM will be available for the remethylation of homocysteine. The deficiency of 5-methyl-THF is unnecessary under conditions of remethylation of homocysteine. The deficiency of GNMT (reaction 23) is not inhibited by excess SAM and maintain the SAM/SAH ratio, however, there will be less SAM available for reaction 19. The increased levels of homocysteine and inhibit GNMT, resulting in a limited amount of methionine for the synthesis of reaction 22.

FOlate METABOLISM

The compartmentation of folate metabolism is at least in the liver and probably in other

tissues as well. This was first shown by Cook and Blair [40], who demonstrated that radioactive folate injected into rats was distributed differently between the cytosol and mitochondria. Horne et al. [41] showed that most of the folate in liver is distributed between the cytosol and mitochondria and that these two pools are not in equilibrium. This was done by exposing rats to nitrous oxide gas. This agent destroys the cyanocobalamin cofactor needed for reaction 20, methionine synthase. It mimics many of the effects of vitamin B₁₂ deficiency. A functional folate deficiency develops because reaction 19 cannot be reversed and 5-methyl-THF is trapped and accumulates. It was found that although 5-methyl-THF accumulated in the cytosol fraction of the livers of nitrous oxide-treated rats, there was no change in the distribution of mitochondrial folates. Horne et al. [42] have now characterized the uptake of folate by intact mitochondria. They showed that the reduced folates, 5-methyl-THF and 5-formyl-THF, are taken up by a carrier-mediated process, while oxidized forms are not. This is consistent with the absence of dihydrofolate reductase in the mitochondria. Although folates are transported into the mitochondria by a carrier-mediated process, it is not energy dependent and is slower than the uptake of compounds such as serine and glycine.

In addition to the compartmentation of the folate coenzymes, there is a curious compartmentation of the enzymes. Some are found in the cytosol, some in the mitochondria, and some are found in both locations. A number of early studies that dealt with the isolation and characterization of these enzymes employed vigorous methods of tissue disruption which permitted mitochondrial contents to mix with the cytosol. Enzymes isolated in this way and found to be soluble were assumed, perhaps incorrectly, to have been derived from the cytosol.

Table 2 lists the probable location of each of the enzymes in Figure 1 that metabolize folate. Although many enzymes are present either in the cytosol or in the mitochondria, a number of activities (reactions 3, 4, 7, 8, and 14) are present in both compartments. In the cytosol, reaction 3, 4, and 8 are found as part of a trifunctional enzyme on a single peptide chain [7,8]. All three activities are present in rat liver mitochondria, but they have not been purified and it is not known whether they are on the same peptide [43]. Serine hydroxymethyltransferase (reaction 14) has been purified from both the cytosol and mitochondria. They are clearly different proteins with different primary structures but with identical active sites. Only the sequence of the cytosolic form of 10-formyl-THF dehydrogenase (responsible for reaction 7) is known.

Figure 2 shows the compartmentation of folate-mediated one-carbon metabolism [44]. The movement of reduced folates across the mitochondrial membrane is slow, but serine, glycine, and formate are rapidly equilibrated. Appling [45] has emphasized the interdependence of one-carbon metabolism between the two compartments. This was demonstrated in yeast, where it is much easier to obtain mutants lacking certain reactions but was also shown in higher animals. Certain mutants of Chinese hamster ovary (CHO) cells that are deficient in only the mitochondrial form

Table 2 Subcellular Localization of Folate Reactions

Reaction	Cytosol	Mitochondria
1	X	
2	X	
3	X	X
4	X	X
5	X*	
6	X*	
7	X	X
8	X	X
9		X
11		X
12		X
13		X
14	X	X
15	X	
16	X	
17	X	
18	X	
19	X	
20	X	
23	X	

Numbers refer to the reactions shown in Figure 1.

*Unpublished data, Glenn and Wagner.

of serine hydroxymethyltransferase (reaction 14) are unable to grow unless glycine is added to the medium [46]. Clearly the cytosolic form of the enzyme is unable to satisfy the glycine requirement of the cell. In other studies, it was shown that intact rat liver mitochondria could convert carbon 3 of serine or the *N*-methyl group of sarcosine to formate, which then was incorporated into purines by the addition of cytoplasmic 10-formyl-THF synthetase (reaction 8) and a cytoplasmic purine-synthesizing system [43]. It is believed that serine, the major source of one-carbon units, or dimethylglycine and sarcosine, which are products of choline catabolism in the liver only, enter the mitochondria and generate 5,10-methylene-THF. This can be converted to 10-formyl-THF via reactions 3 and 4 and used for mitochondrial protein synthesis. The glycine that is generated is degraded by the glycine cleavage reaction (reaction 13). Excess 10-formyl-THF can be converted to formate via reaction 8 in the mitochondria and transported back to the cytosol where it can reenter the one-carbon pool through reaction 8 in the cytosol.

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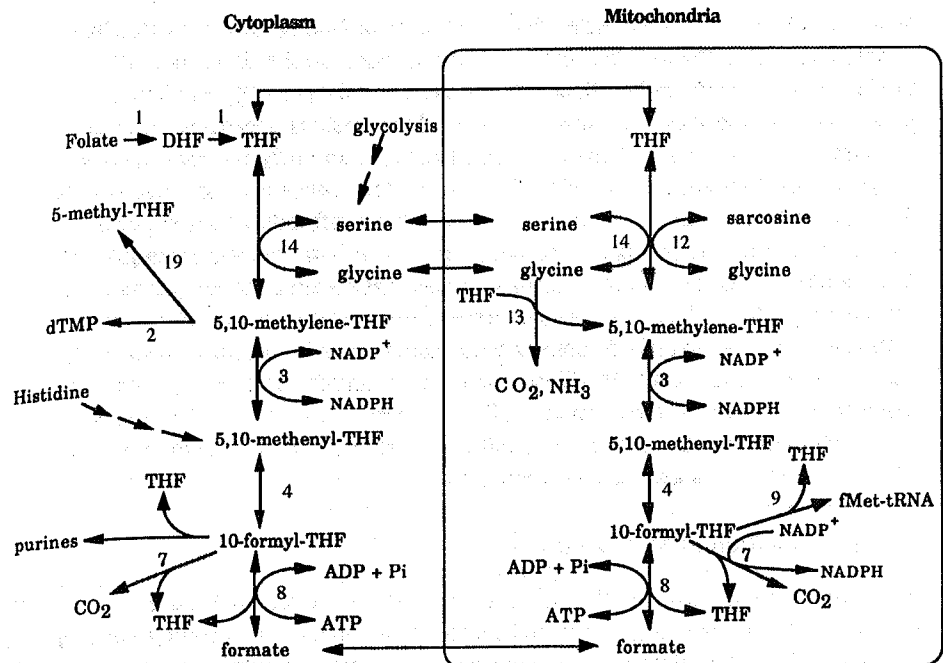
X
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X

Figure 2 Compartmentation of folate-mediated one-carbon metabolism. (Adapted from Garcia-Martinez and Appling [44].)

IV. MANIFESTATIONS OF FOLATE DEFICIENCY

A. Defective Cell Division

Folate deficiency results in a specific type of anemia, a megaloblastic anemia, in humans [47]. Megaloblasts are large, abnormal, nucleated cells that are precursors of erythrocytes, and in folate deficiency they accumulate and are found in the bone marrow. These cells arise as a result of a failure of the red cell precursors to divide. The anemia, the deficiency of erythrocytes, is only one manifestation of diminished cell division. There are also decreased numbers of white cells and platelets. It is possible to conclude that there is general impairment of cell division, which is more apparent in tissues which turn over rapidly, such as the hematopoietic system and the cells lining the digestive tract. These latter cells have "megaloblastic" changes in severe folate deficiency and appear abnormally large with enlarged nuclei. The impairment of cell division can be related to the role played by folate in nucleic acid synthesis. Two separate steps in the de novo pathway for purine biosynthesis utilize 10-formyl-THF, and the formation of thymidylic acid needed for DNA

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ia and transported back to the cytosol where
ugh reaction 8 in the cytosol.

synthesis utilizes 5,10-methylene-THF. A number of studies have indicated that it is the synthesis of thymidylate that is most susceptible to the deficiency of folate in the bone marrow rather than purine biosynthesis [48,49]. Studies on the pathogenesis of the anemia in folate deficiency have been hampered by the absence of a convenient experimental animal model. Until recently, only primates developed a typical megaloblastic anemia when fed a folate-deficient diet. Other species only demonstrated a decreased growth rate. Walzem and Clifford have developed a diet which, for the first time, rapidly produces anemia in both rats and mice [50,51]. This employs a mixture of amino acids to replace the casein previously used as the source of protein. Traces of folate present in the "vitamin-free" casein are eliminated, and the animals demonstrate decreased levels of red cells, white cells, and platelets in 3-5 weeks. Studies carried out on cultures of erythropoietic cells from the marrow of these folate-deficient mice showed that the block in erythropoiesis was corrected by thymidine but not by deoxycytidine or inosine (Koury and Horne, unpublished observations).

B. Accumulation of Metabolites

The overall metabolism of the folate coenzymes is shown in Figure 1. This interrelates reactions that generate and reactions that utilize one-carbon units and also shows how the different folate coenzymes are interconverted. In the case of folate deficiency, all of these reactions will be compromised to varying degrees depending on the relative affinities of the enzymes for their folate coenzymes and their subcellular location since, as described above, mitochondrial folates are not in rapid equilibrium with those in the cytosol. When reactions generating one-carbon units are affected by folate deficiency, substrates of those enzymes will accumulate and may be excreted. 5-Formiminoglutamic acid (FIGLU), a product of histidine breakdown, is such a compound and appears in the urine during folate deficiency [52]. It is easily measured and has been used as an indicator of folate deficiency. The accumulation of FIGLU has not been associated with any toxicity.

AICAR, the substrate for the incorporation of the second formate group in purine biosynthesis, likewise accumulates during folate deficiency. Both AICAR and its dephosphorylated product can be detected in the urine of folate-deficient humans and rats [53,54]. On the other hand, GAR, which is the substrate for the incorporation of the first formate, does not accumulate. When both of the enzymes from chicken liver were examined, as might be expected, the K_m for 10-formyl-THF, the formyl donor, was much lower for transfer of the formyl group to GAR than for transfer to AICAR, suggesting that the latter reaction is more easily affected by folate deficiency [55,56]. No toxic effects have been attributed to elevated levels of AICAR.

In contrast to the studies described above, accumulation of homocysteine, as a result of folate deficiency, may be a potential source of serious health problems:

number of studies have indicated that it is susceptible to the deficiency of folate in biosynthesis [48,49]. Studies on the mechanism have been hampered by the absence of a folate-deficient diet. Other species only recently developed a diet that causes anemia in both rats and mice [50,51]. The casein previously used as the sole protein source in the "vitamin-free" casein diet has decreased levels of red cells, white cells, and is based on cultures of erythropoietic cells. Recent studies showed that the block in hemoglobin synthesis is not by deoxycytidine or inosine.

The reactions of folate coenzymes is shown in Figure 1. This diagram illustrates the reactions that utilize one-carbon units and how these coenzymes are interconverted. In the case of folate, the reactions will be compromised to varying degrees if the folate coenzymes are deficient. As noted above, mitochondrial folates are not involved. When reactions generating one-carbon units are blocked, the intermediates of those enzymes will accumulate. For example, FIGLU, a product of histidine metabolism, appears in the urine during folate deficiency and is used as an indicator of folate deficiency. It is not associated with any toxicity.

The metabolism of the second formate group in folate is also affected during folate deficiency. Both AICAR and AICA are detected in the urine of folate-deficient animals, and GAR, which is the substrate for the conversion to AICAR, accumulates. When both of the enzymes are deficient, it might be expected that the K_m for 10-formyltransferase for transfer of the formyl group to GAR would be high, but the latter reaction is more easily affected. The effects have been attributed to elevated levels of homocysteine.

As noted above, accumulation of homocysteine, as a metabolic source of serious health problems.

As described above, the methyl group of methionine is lost during metabolism, and the homocysteine that eventually arises from this process is remethylated by the transfer of a methyl group from 5-methyl-THF. Failure to remethylate homocysteine results in elevation of both homocysteine and homocystine levels in the blood (homocystinemia) and in the urine (homocystinuria). A number of inherited metabolic defects are known to produce homocystinuria [39]: (1) methylene-THF reductase deficiency, which results in a failure to form 5-methyl-THF, (2) defects in processing of the cobalamin coenzyme needed for the transfer of 5-methyl-THF to homocysteine, and (3) absence of cystathionine synthase. During folate deficiency it might be expected that the remethylation of homocysteine would be compromised. This has been confirmed in a number of studies, and there is an inverse correlation between the levels of folate and homocysteine in blood in humans [57]. The elevation of homocysteine levels in the blood is not benign, however. Homocystinemia is associated with an increased risk of vascular disease [58]. Clinical studies have established that increased plasma homocysteine is associated with premature atherosclerosis in the coronary, cerebral, and peripheral vessels. There is thus some concern that folate deficiency itself might contribute to the development of atherosclerosis. This topic is dealt with in more detail in a later chapter.

C. Neural Tube Defects

There is now convincing evidence that folate supplementation reduces the incidence of neural tube defects in certain high-risk populations. There is no conclusive evidence, however, that individual mothers who have given birth to children with such defects were folate deficient. Although folate is necessary for cell division, the metabolic link between folate or one-carbon metabolism and development of the neural tube is unknown. This topic is dealt with in detail in a Chapter 12.

D. Defective Methylation

The importance of methylation reactions has been mentioned above. Not only are these reactions needed for the biosynthesis of many important products, but the methylation of DNA has been shown to regulate the expression of genes in eukaryotic cells. Methyl groups are transferred to the N5 position of cytosine in DNA by a specific DNA methylase. In most species about 3–5% of the cytosine in DNA appears as methylcytosine [59]. The extent of methylation of specific genes varies from tissue to tissue and changes during development. It has been shown that, in most cases, undermethylation favors gene expression and increased methylation is associated with gene "silencing" [59]. It is therefore of considerable interest that when rats were fed a diet containing no methyl groups, there was a very high incidence of spontaneous hepatic cancer [60]. This was a synthetic amino acid diet containing no choline in which methionine had been replaced with homocysteine. Animals fed the methyl-deficient diet were shown to have

undermethylated DNA when compared with animals fed a control diet [61]. The decrease in DNA methylation was accompanied by altered gene expression, which resembled the pattern of gene expression seen in animals exposed to carcinogens and in hepatomas. It is suggested that the undermethylation of the DNA causes increased expression of certain oncogenes.

In the diet described above all of the methyl groups needed for normal metabolism must be synthesized *de novo* from the one-carbon folate pool. Presumably *de novo* synthesis is not sufficient to provide enough SAM for methylation of DNA, and perhaps for other reactions as well. In addition, the high levels of homocysteine in the diet led to elevated amounts of SAH (due to reversibility of reaction 24) and thus decreased SAM/SAH ratios, which might be expected to inhibit methyltransferases generally [62]. Even when a normal diet containing choline is fed the amount of methionine it is not enough to satisfy the requirements for protein synthesis and for metabolic methylations. In humans, between 50 and 80% of the homocysteine generated is remethylated, depending on the dietary content of methionine and choline [63,64]. It might be expected, therefore, that folate deficiency itself could impair homocysteine metabolism by decreasing the amount of 5-methyl-THF available for reaction 20 and generally depressing the size of the one-carbon folate pool. This would also result in the elevation of homocysteine levels and decrease the SAM/SAH ratio. Both effects would be expected to impair cellular methylation reactions. This is indeed the case. When rats were fed the amino acid-defined folate-deficient diet developed by Clifford for 2 weeks, the SAM/SAH ratio in the liver decreased from 9.7 to 1.8 [65]. After 4 weeks the methylation of total liver DNA was significantly reduced [66].

The negative effect of folate deficiency on cellular methylation reactions may also be apparent in other ways. A number of recent studies have suggested that one-carbon compounds may play an important role in the function of the exocrine pancreas. The major function of this organ is the secretion of enzymes used in the digestion of a variety of nutrients. Ethionine, an analog of methionine that blocks cellular methylation reactions, has a specific toxic effect on the exocrine function of the pancreas [67]. The metabolic defect is an inability to release preformed zymogen granules from the pancreas [68]. A possible role for methylation in the secretion process is suggested by recent evidence showing that hormone-stimulated secretion in a number of tissues requires the interaction of G-proteins with components in the plasma membrane [69]. The carboxyl-terminal amino acid of these G-proteins is cysteine, which is first prenylated and then must be methyl esterified on the free carboxyl by reaction with SAM in order for interaction with the membrane to take place. Because secretion in the exocrine pancreas is such an active process, one might expect this to place a high demand on tissue levels of SAM. Alternatively, the extensive turnover of plasma membrane components during secretion may place a large burden on the synthesis of phosphatidylcholine from phosphatidylethanolamine. The enzyme, GNMT, is concerned with the regulation

l with animals fed a control diet [61]. The mpanied by altered gene expression, which on seen in animals exposed to carcinogens the undermethylation of the DNA causes nes.

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of the SAM/SAH ratio and the de novo synthesis of methyl groups, as described above. Although GNMT is very abundant in the liver, it is almost as high in the exocrine pancreas [70], where it is also subject to allosteric inhibition by 5-methyl-THF [71], suggesting that it may have a function similar to that in the liver. The level of total folate in the pancreas is also very high, about 25% of the level in the liver, which is higher than any other extrahepatic organ [72]. Folate deficiency produces many of the same changes in the pancreas as it does in the liver, and the SAM/SAH ratio is decreased about 50% after 4 weeks [72]. It has recently been shown that after feeding the folate-deficient diet for 5 weeks the secretory response of the pancreas to cholecystokinin was significantly decreased (Balaghi and Wagner, unpublished observations). It is not clear whether this is a direct result of impaired methylation in the folate-deficient animals or whether it is due to a more general effect on amino acid and nucleic acid metabolism.

There is a well-known association between alcohol consumption and impaired folate absorption [73,74]. Alcoholism is the highest single cause of nonobstructive pancreatitis in humans [75,76]. If the pancreas is particularly sensitive to folate deficiency because of its high requirement for methyl groups, it might explain the high incidence of pancreatitis among alcoholic patients.

V. SUMMARY

The principal function of the folate coenzymes is to carry one-carbon units. Folic acid is converted to the active coenzyme by reduction to tetrahydrofolate. One-carbon units are carried at the formate, formaldehyde, or methanol levels of oxidation. Interconversion of these forms is accomplished by a series of enzymes. Many of these enzymes are multifunctional and are capable of "channeling" the one-carbon unit from one reaction to the next without equilibrating with the medium. 5-Formyltetrahydrofolate, previously believed to be an unnatural, but pharmacologically important, form of folate, has been shown to arise physiologically as a minor product of the enzyme serine hydroxymethyltransferase.

In addition to enzymes that metabolize folate, glycine N-methyltransferase is an enzyme whose activity is regulated by folate. This enzyme is important in controlling the methylating capacity of the cell.

Folate coenzymes and folate enzymes are compartmentalized between the cytosol and the mitochondria. Metabolic products are readily transported between compartments, but the folate coenzymes are not.

Folate deficiency leads to defective cell division and accumulation of metabolites, some of which, e.g., homocysteine, may be toxic. Folate deficiency can also lead to defective methylation since the de novo generation of methyl groups requires folate and the dietary supply of methyl groups coming from choline and methionine are not ordinarily sufficient to supply the needs of body.

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