

Modern Nutrition in Health and Disease

SEVENTH EDITION

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Chapter 21

FOLIC ACID AND VITAMIN B₁₂

Victor D. Herbert and Neville Colman

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HISTORY

The unraveling of the relation of vitamin B₁₂ and folic acid to anemia traces back almost two centuries.¹⁻³ In 1822, physician Combe reported to the Royal Medical and Surgical Society of Edinburgh on the "history of a case of anemia" which he surmised was due to "some disorder of the digestive and assimilative organs."⁴ Thus was launched the study of pernicious anemia in particular and megaloblastic anemia in general, after restimulation by physician Thomas Addison's description, in 1849 and 1855,⁵ of what his contemporaries evidently recognized as pernicious anemia, even though he did not mention the characteristic glossitis, jaundice, or nerve damage. He did mention "the disease having uniformly occurred in fat people," which would imply deficiency of vitamin B₁₂ rather than of folate; deficiency of the latter tends to be associated with wasting.

The nutritional basis of pernicious anemia was suspected by the American physician Austin Flint

in 1860, when he stated that "in these cases there exists degenerative disease of the glandular tubuli of the stomach."⁶ Another two thirds of a century had passed when the classic work of physician William Castle and his associates demonstrated that normal human gastric juice contains an "intrinsic factor" (i.e., within the body) that combines with an "extrinsic factor" (i.e., outside the body—in food) contained in animal protein to result in absorption of the "antipernicious anemia principle."⁷ Vitamin B₁₂ was finally reported as isolated in 1948 in the United States,⁸ and three weeks later, entirely independently in England,⁹ Berk and his associates¹⁰ showed that this vitamin was "extrinsic factor" and "antipernicious anemia principle."

Early reports of disease now recognizable as probable folate deficiency include those of Channing¹¹ and Barclay,¹² who reported a severe form of anemia in pregnancy and the puerperium, which was often fatal when it resulted from the nutritional drains of several pregnancies superimposed on a nutritionally inadequate diet. Osler

postulated that megaloblastic anemia of pregnancy and the puerperium was "caused by an agent which differs from that which causes the anemia of Addison."¹³ Support for this hypothesis emerged a decade later when Wills and her associates described a macrocytic anemia in Hindu women in Bombay, usually associated with pregnancy,¹⁴ that responded to therapy with a commercial preparation of autolyzed yeast called marmite (still a favored home remedy in Commonwealth countries). By feeding the same type of diet ingested by their patients to monkeys, they produced in them a similar macrocytic anemia that responded to a "Wills factor" present in crude but not purified liver extracts.

We now know that the more purified extract consisted of a fairly pure solution of vitamin B₁₂, and that the Wills factor removed from the crude liver extract in the process of purification is folic acid. This fact gradually became clear after the purification of pteroylglutamic acid in 1943 by Stokstad,¹⁵ its crystallization from liver in the same year by Pfaffner and associates,¹⁶ its synthesis and structural identification by Angier and his co-workers,¹⁷ and the 1948 isolation of crystalline vitamin B₁₂. The rapid isolation of vitamin B₁₂ by the American workers was greatly aided by a microbiologic assay based on Shorb's¹⁸ discovery that "LLD factor," a growth factor required by *Lactobacillus lactis Dorner*, was not only the "animal protein factor" necessary for proper growth and function in animals fed an all-vegetable diet. It was also present in liver extracts in amounts that closely paralleled their potency in the treatment of pernicious anemia.

Folic acid proved to be not only the Wills factor but also the vitamin M contained in dried brewer's yeast that corrected the deficiency anemia, leukopenia, diarrhea, and gingivitis of monkeys studied by Day and associates.¹⁹ It also proved to be the vitamin B_c contained in yeast that corrected the deficiency syndrome in chicks characterized by anemia and growth failure. Furthermore, folic acid proved to be the norite eluate factor (i.e., it could be adsorbed on and eluted from charcoal) of liver, described by Snell and Peterson²⁰ as essential to the growth of *Lactobacillus casei* (and therefore also called the "*L. casei* factor"). The term *folic acid* was coined by Mitchell and co-workers²¹ because they found this material in a leafy vegetable (spinach). At that time, it was not recognized that vitamin B₁₂, and not folic acid, was the active ingredient in the oral liver therapy that Minot and Murphy reported in 1926 as successful in treating pernicious anemia²² (for which work they received the Nobel Prize in Medicine in 1934).

CHEMISTRY^{3,23,23a,23b,23c,24,24a,24b,24c,24d}

Neither cyanocobalamin nor pteroylglutamic acid, the common pharmaceutical forms of vitamin B₁₂ and folic acid, is present as such in significant quantity in either the human body or the various foods from which these agents were isolated. In the body and in foods, they are present in various reduced metabolically active coenzyme forms, often conjugated (in the case of vitamin B₁₂ to peptide and in the case of folate to one or more glutamates) in peptide linkage. During the extraction procedure, these labile active forms are either destroyed by oxidation (particularly folates) or oxidized and converted to cyanocobalamin or pteroylglutamic acid, which are the stable forms of the respective vitamins. These stable forms are partially oxidized and not known to be metabolically active; not until they are reduced by metabolic systems present within gut and other tissue cells do they become metabolically active cobalamins or folates.

Vitamin B₁₂^{3,23,23a,23b,23c,25-27}

Figure 21-1 shows the structural formula of vitamin B₁₂ (cyanocobalamin): Delineation of this structure, using x-ray crystallography, by Hodgkin and her co-workers was partly responsible for her winning the 1964 Nobel Prize in Chemistry. The chemistry of the vitamin has been repeatedly reviewed.^{23,23a,23b,23c} The two major portions of the molecule are the corrin nucleus (a planar group) and a "nucleotide" lying in a plane nearly at right angles to the corrin nucleus and linked to it by D-1-amino-2-propanol. The "nucleotide" (5-6-dimethylbenzimidazole) is attached to ribose by an alpha-glycoside linkage. A second bond between the two major parts of the molecule is the coordinate linkage of the cobalt atom to one of the nitrogen atoms of the "nucleotide."

In cyanocobalamin, the anionic (-R) group in coordinate linkage with the cobalt is cyanide. Fortunately, the original isolation of B₁₂ from liver yielded a stable product, cyanocobalamin, because the unstable linkage of the 5'-deoxyadenosyl anionic group to the rest of the molecule in coenzyme B₁₂ (the form naturally dominant in liver) was ruptured and replaced by cyanide, which leached from the charcoal columns used in the isolation procedure.

Cyanocobalamin crystals are dark red and the substance absorbs water. The official product in the USP contains 12% absorbed moisture. Cobalamins are destroyed by heavy metals and strong oxidizing or reducing agents, but not by autoclaving for short periods at 121°C. Ascorbate and other nutrients that are strong reducing agents, when placed in solution with cobalamins, destroy co-

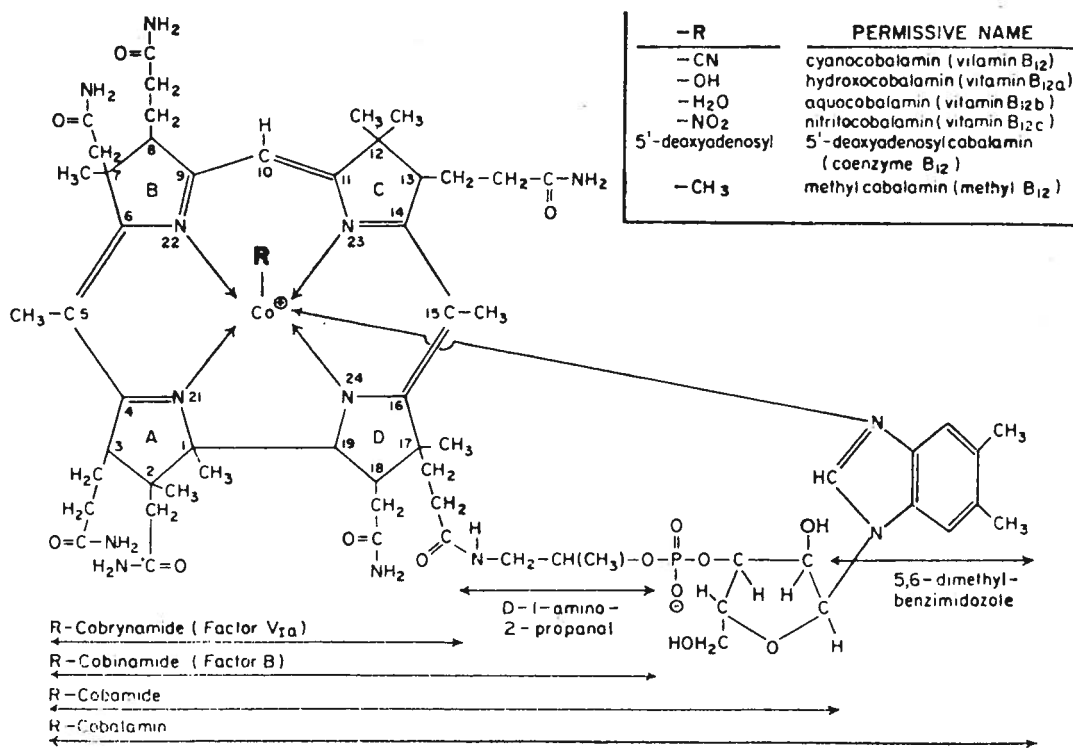


Fig. 21-1. Structural formula of vitamin B₁₂ (cyanocobalamin). The numbering system for the corrin nucleus is made to correspond to that of the porphin nucleus by omitting the number 20. (Modified from Brown and Reynolds: *Annu. Rev. Biochem.* 32:419, 1963.)

balamins by converting them to various analogues,^{27a} some of which may actually block cobalamin metabolism (i.e., function as antimetabolites).^{27b} Cyanocobalamin is soluble 1:80 in water, and stable in solution. Aqueous solutions are neutral; maximal stability is at pH 4.5 to 5, making this the favored pH for extracting the vitamin from foods and tissues.

Coenzyme B₁₂ (5-deoxyadenosylcobalamin) and methylcobalamin (methyl-B₁₂) are the two vitamin B₁₂ coenzymes known to be metabolically active in mammalian tissues. Both should be extracted in the dark^{27c} because they are unstable in light and undergo photolysis with formation of aquocobalamin or, in the presence of potassium cyanide, cyanocobalamin. Under the rules of the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biochemical Nomenclature,²⁸ cyanocobalamin is a permissive (semisystematic) name for vitamin B₁₂, and the term vitamin B₁₂ without qualification means cyanocobalamin exclusively. However, the term is also entrenched in the literature as a generic term for all the cobalamins active in man. The permissive term cobalamin (or vitamin B₁₂) is used to describe the vitamin B₁₂ molecule minus the cyanide group, and is prefixed by the designation

of the anionic R group (Fig. 21-1) attached to the cobalt. The terms coenzyme B₁₂ and vitamin B₁₂ coenzyme are not interchangeable; the former means 5'-deoxyadenosylcobalamin exclusively, and the latter applies to any coenzyme form of B₁₂.

Figure 21-1 delineates some of the family of natural and semisynthetic cobalamins; others include dicyanocobalamin, thiocyanatocobalamin, chlorocobalamin, and sulfitecobalamin. The alphabetic congeners of vitamin B₁₂ (B_{12a}, B_{12b}, B_{12c}) listed on this figure are believed to be equipotent in treatment of vitamin B₁₂ deficiency (unless that deficiency is due to a congenital or acquired defect in enzymes involved in converting one B₁₂ form to another). However, minimal dose therapy (0.1 µg daily) suggests²⁹ that coenzyme B₁₂ is more potent therapeutically than cyanocobalamin. Therapy with doses greater than minimal is always used clinically, and all forms of B₁₂ appear equipotent when used in greater than minimal doses.

Folic Acid^{3,24,24a,24b,24c,24d,30}

Figure 21-2 presents the structural formula of folic acid (pteroylglutamic acid [PteGlu or PteGlu₁]), the reference compound of the folate vi-

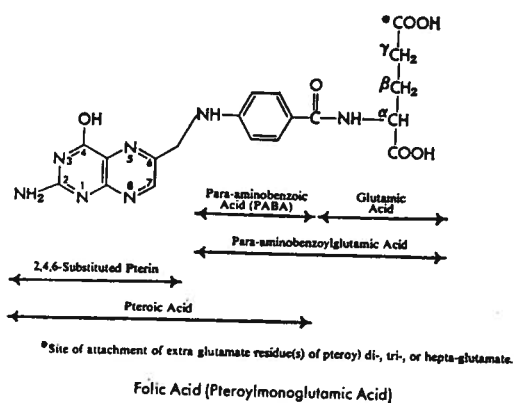


Fig. 21-2. Structural formula of folic acid (pteroylglutamic acid). (From Herbert,³ with permission of Macmillan Publishing Co., Inc.)

tamin forms. Because it is an oxidized and therefore more stable form, it is the pharmaceutical form usually used therapeutically. Oxidized forms of folate cannot function in one-carbon metabolism in humans until converted by tissue enzymes to reduced forms. Reduced forms are the ones normally found in food and tissues. The major subunits of the molecule are the pteridine moiety linked by a methylene bridge to para-aminobenzoic acid, which is joined by peptide linkage to glutamic acid.

Crystalline folic acid is yellow. The free acid is almost insoluble in cold water, but the disodium salt is more soluble (about 1.5 g/dl). Injectable solutions are prepared by dissolving folic acid in isotonic sodium bicarbonate solution, or by using the disodium salt. Folic acid is destroyed at pH below 4, but is relatively stable above pH 5, with no destruction in one hour at 100°C. The molecule usually splits into pteridine and para-aminobenzoyl glutamate.

The nomenclature of folic acid and its derivatives is currently undergoing revision. Recommendations of an advisory panel to Commissions on Nomenclature of the International Union of Biochemistry (IUB) and the International Union of Pure and Applied Chemistry (IUPAC)²⁸ are that

"Folate and folic acid are the preferred synonyms for pteroylglutamate and pteroylglutamic acid, respectively...The term folates may also be used in a generic sense to designate any member of the family of pteroylglutamates, or mixtures of them, having various levels of reduction of the pteridine ring, one-carbon substitutions, and numbers of glutamate residues."

The term "folacin" is no longer to be used.

Pteroylglutamic acid, an oxidized compound, is not normally found as such in foods or in the human body in significant concentrations. The

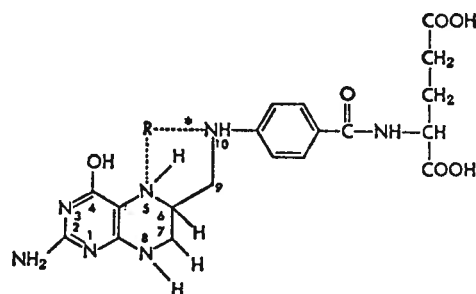
forms that are found in such sources are the reduced forms indicated in Figure 21-3. They differ from the parent compound by virtue of one to three structural modifications. First, all are reduced folates and, except for 7,8-dihydrofolate, all are 5,6,7,8-tetrahydrofolates (THF). Second, as indicated in Figure 21-3, various 1-carbon adducts may be linked to THF at the N-5, N-10, or 5,10 position, conferring on folates their role as 1-carbon carriers. It has been suggested that only five natural 1-carbon substitutions are on THF, and that N⁵ formyl THF (folinic acid, citrovorum factor) represents nonenzymatic conversion of folate during processing of natural materials.³⁰ Third, the number of glutamate residues may vary from one to seven, and sometimes up to 11, each linked by peptide bonds between its amino group and the gamma-carboxyl group of the preceding glutamate (see Fig. 21-2).

UNITS OF MEASUREMENT AND METHODS OF ASSAY

Vitamin B₁₂^{3,23,23a,23b,23c,32-35}

Human serum levels of vitamin B₁₂ are measured in picograms (pg: 10⁻¹² grams, also called μμg [micromicrograms]) per ml of serum. Normal values range from 200 to 900 pg/ml; below 80 pg/ml represents unequivocal B₁₂ deficiency according to the WHO Scientific Group on Nutritional Anemias.^{31,31a,31b} However, the level below which one can state there is unequivocal deficiency actually varies from laboratory to laboratory depending on microbiologic or radioassay methodology used; there is no "gold standard" method for assay.^{31,31a,31b,32a,35h} In fact, as vitamin B₁₂ deficiency develops, B₁₂ analogue levels may rise,^{32b} making assays that measure total corrinoids instead of cobalamins alone unreliable.^{32c} The tiny quantity of vitamin B₁₂ activity in human serum may be measured only microbiologically or by radioassay. Many microorganisms require vitamin B₁₂ in order to grow, and therefore there are many microbiologic assays for vitamin B₁₂. Radioassay has the advantage over microbiologic assay that false low results do not occur if serum contains antibiotics or other substances that inhibit growth of microorganisms, causing the microbiologic assays to yield false low results. Additionally, one can perform differential radioassay to separate cobalamins from other corrinoids,^{32c,32d} which cannot be adequately done by differential microbiologic assay.^{32e,32f,32g} Radioassay for vitamin B₁₂ was first described by Herbert in 1958.³³ The most widely employed assay uses coated charcoal^{34,35} to separate free from bound vitamin B₁₂, but many

	R	OXIDATION STATE
N ⁵ formyl THFA	—CHO	formate
N ¹⁰ formyl THFA	—CHO	formate
N ⁵ formimino THFA	—CH=NH	formate
N ^{5,10} methenyl THFA	>CH	formate
N ^{5,10} methylene THFA	>CH ₂	formaldehyde
N ⁵ methyl THFA	—CH ₃	methanol



*Broken lines indicate the N⁵ and/or N¹⁰ site of attachment of various 1-carbon units for which THFA acts as a carrier.

5,6,7,8-Tetrahydrofolic Acid [THFA] (R=—H)

Fig. 21-3. Structures and nomenclature in the folate field. The table above the formula lists some of the possible 1-carbon adducts formed with THFA. (From Herbert³ with permission of Macmillan Publishing Co., Inc.)

other satisfactory separation methods exist.^{23,23a,23b,23c,31,31a,31b,32a}

Human serum and tissues contain not only biologically active "true B₁₂" (i.e., cobalamins), but also analogues (i.e., noncobalamin corrinoids) of varying to no activity; some may even be anti-metabolites.^{32a-g,35b-g} Radioassays that do not use pure intrinsic factor or impure intrinsic factor with its nonintrinsic factor blocked by an analogue (which binds only "true B₁₂," i.e., "true cobalamins")^{35a} measure "total B₁₂" ("total corrinoids," i.e., cobalamins plus noncobalamin analogues) rather than "true B₁₂." Thus they may not pick up early vitamin B₁₂ deficiency, since in early deficiency only that portion of "total B₁₂" that is "true B₁₂" may fall, and analogues may still be in the normal range^{32c,35b,35c} or may even rise.^{32b} In addition, heat plus the presence of large amounts of vitamin C or other nutrients with strong reducing activity may destroy vitamin B₁₂ unless the B₁₂ is protected by cyanide, —S, or another protective mechanism.^{27a,27b,35d}

Differential radioassay is necessary to distinguish cobalamin from noncobalamin corrinoids in human serum,^{32c} erythrocytes,^{35e} liver,^{35e} nervous system,^{35e} and bile.^{35f} Differential microbiologic assays are incapable of doing so. Workers unaware

of this fact represented that the early data of Herbert et al. on B₁₂ destruction by vitamin C was incorrect. In fact, the vitamin C converted the vitamin B₁₂ to noncobalamin corrinoids that non-differential microbiologic assays and radioassays were unable to distinguish from true B₁₂.^{35d-f}

Larger quantities of vitamin B₁₂ than those present in human serum may be assayed colorimetrically, spectroscopically, fluorometrically, or chemically. A serum standard was proposed.^{35h}

The USP assay for vitamin B₁₂ in pharmaceutical preparations is spectrophotometric. Although liver extract for injection was deleted from the USP in 1960, such largely obsolete often allergenic products are still being used, assayed by a non-differential microbiologic method for their "vitamin B₁₂" content, despite the fact that some of this "B₁₂" is noncobalamin analogues.^{35e,f} Differential radioassay demonstrates that 10 to 30% of "B₁₂" in multivitamin-mineral pills may be such analogues.^{32g}

Folic Acid^{3,35-37}

Normal human serum contains 5 to 16 nanograms (ng; 10⁻⁹ grams; mμg [millimicrograms]) of folic acid activity (i.e., PteGlu equivalents) per ml. These tiny quantities could be measured only mi-

crobiologically, as originally described in 1959,³⁸⁻⁴² until a radioisotopic assay was described in 1970.^{43,43a} The dominant folate in serum and red cells is 5-methyltetrahydrofolate, and folate assays must therefore be capable of measuring this derivative. Because *Lactobacillus casei* is the only microorganism known to grow well on this folate, the only microbiologic assay that adequately measures serum and red cell folate uses *L. casei*. Similarly, the only radioassays that accurately measure serum folate are those that measure 5-methyltetrahydrofolate; these are mainly done at pH 9.3, where folic acid (used as a stable standard) and serum folate have similar reactivity.⁴⁴

Simultaneous radioisotope determinations of serum vitamin B₁₂ and folate can be done in the same test tube. The results correlate well with those of other radioassays. This procedure considerably simplifies assay of the two vitamins.^{45,46} It may be the method of choice for use in most laboratories.

Because serum folate is labile, false low values for serum folate activity occur if the serum has not been protected against oxidative destruction before assay. Such protection is brought about by storing the serum frozen, storing it in the presence of a reducing agent such as ascorbate, or both. It must be remembered that ascorbate may destroy B₁₂ in storage.^{35d}

Larger quantities of folate activity than those normally present in human serum may be measured chemically, fluorometrically, by paper and thin-layer chromatography, enzymatically, or by animal assay.^{35a}

CAUSES OF NUTRITIONAL DEFICIENCY

In the final analysis, nutritional deficiency means there is inadequate usage of a nutrient in one or more intracellular systems to sustain normal biochemical functions. Such inadequate usage falls in one or more of six basic categories: inadequate ingestion, inadequate absorption, inadequate utilization, increased requirement, increased excretion, and increased destruction. Any one or combination of these three inadequacies and three excesses may result in nutritional deficiency. Table 21-1 presents the currently known possible etiologic factors in each of these six categories that may produce nutritional deficiency of vitamin B₁₂ or folic acid. The ensuing sections will discuss in more detail mechanisms of inadequate absorption and utilization of these two vitamins.

ABSORPTION

Vitamin B₁₂^{3,23,23a,23b,23c,47-51a-e}

There are two separate and distinct mechanisms for the absorption of vitamin B₁₂. The *physiologic* mechanism, the derangement of which accounts for much human vitamin B₁₂ deficiency, is capable of handling a maximum of 1.5 to 3 µg of free vitamin B₁₂ at any one time. This mechanism operates as follows: (1) Ingested vitamin B₁₂ is freed from its polypeptide linkages to food by gastric acid and gastric and intestinal enzymes. (2) The free vitamin B₁₂ attaches to salivary R binder in preference to the gastric intrinsic factor (IF) of Castle (a glycoprotein of molecular weight in the range of 50,000, produced by normal gastric parietal cells, which dimerizes on combination with vitamin B₁₂ so that a complex is formed containing two molecules of vitamin B₁₂). (3) The salivary R binder is destroyed by pancreatic trypsin, releasing B₁₂ onto IF to create the B₁₂-IF complex, which is carried down to the ileum, where it attaches to receptors for B₁₂-IF on the brush border of the ileal mucosal cells in the presence of ionic calcium and a pH above 6. (4) Via a currently uncertain mechanism, the vitamin B₁₂ is released from the B₁₂-IF complex probably at (but possibly within) the ileal enterocyte (epithelial cell). (5) The vitamin then finds its way across the enterocyte into the portal venous blood, at which point it is attached to serum vitamin B₁₂-binding proteins, perhaps having attached to transcobalamin (TC) II in the enterocyte.^{93b} Bicarbonate and trypsin from the pancreas facilitate this mechanism by selectively destroying R binders.^{51b,51c,78c} Pancreatic enzymes similarly effect transfer of biliary cobalamin from R binder to intrinsic factor, creating much of the enterohepatic cobalamin circulation^{78b-d} so important to the vitamin B₁₂ economy of the vegetarian. R binder (particularly TC III) delivers B₁₂ and analogues to the liver but not to other tissues. TC II preferentially binds cobalamins and delivers them to all tissues that need them. TC II is to cobalamin economy what transferrin is to iron economy. Holo-TC III is more a reflection of cobalamin stores.

The other, or *pharmacologic*, mechanism of vitamin B₁₂ absorption appears to be diffusion.^{48,48a} It accounts for the absorption along the entire length of the small intestine of approximately 1% of *any* quantity of *free* vitamin B₁₂ in the small bowel. This mechanism makes possible oral (rather than parenteral) therapy for vitamin B₁₂ deficiency caused by vitamin B₁₂ malabsorption. However, such therapy is less reliable than parenteral therapy.³

Table 21-1. Etiologic Classification of Megaloblastic Anemias: A Tabular Lexicon

- I. Vitamin B₁₂ deficiency (normal B₁₂ body stores last 3 to 6 years after cessation of B₁₂ absorption, but 20 to 30 years after cessation of only B₁₂ ingestion, because of continuation of reabsorption of the 3 to 6 µg of B₁₂ excreted daily in bile)
 - A. Inadequate ingestion
 1. Poor diet (lacking microorganisms and animal foods, which are the sole B₁₂ sources)
 - a. Strict vegetarianism (eating no meat, fowl, seafood, eggs, milk, or any products thereof)
 - b. Chronic alcoholism (no B₁₂ or folate in hard liquor; folate deficiency occurs first, and is more common, partly because body stores of B₁₂ last much longer than those of folate)
 - c. Poverty, religious tenets (Hinduism, Seventh-Day Adventism,* certain Catholic orders), dietary faddism
 - B. Inadequate absorption
 1. Gastric disorder, producing inadequate or absent secretion by gastric parietal cells of intrinsic factor
 - a. Addisonian pernicious anemia (PA—that form of B₁₂ deficiency disease due to inadequate intrinsic factor secretion of uncertain etiology)
 - (1) Hereditary absence of normal intrinsic factor secretion; absent secretion at birth (circulating antibody to intrinsic factor never present; supports theory that antibody only occurs when antigenic stimulus is produced by intrinsic factor, which enters blood from damaged parietal cells and is recognized as foreign by the immunologic surveillance system); rare
 - (2) Congenital production of defective intrinsic factor molecule (three published cases)
 - (3) Autoimmunity-associated gastric atrophy. These patients usually have nondiagnostic-for-PA circulating parietal cell antibody which is index only of past or present gastric damage and *not* of amount of intrinsic factor secretion (circulating diagnostic-for-PA antibody to intrinsic factor is always present under age 21; there is a gradual decrease in measurable antibody, so that by age 65 only two thirds of patients present with measurable circulating antibody to intrinsic factor)
 - (a) Juvenile pernicious anemia (usually presents between ages 3 and 14)
 - (b) Hereditarily determined degenerative gastric atrophy (gradually progressing with increasing age; almost half of all adult PA cases fall in this category)
 - (c) Acquired gastric atrophy as the end result of superficial inflammatory gastritis; superficial gastritis with atrophy (almost half of all adult PA cases fall in this category, which includes acquired gastric damage related to iron deficiency or alcohol)
 - (d) Endocrine disorders (hypothyroidism, polyendocrinopathy) associated with gastric damage
 - b. Gastrectomy
 - (1) Total
 - (2) Subtotal (approximately 20% develop PA within 10 years after surgery, associated with atrophy of remaining parietal cells)
 - (a) Proximal
 - (b) Distal
 - c. Lesions that destroy the gastric mucosa (ingested corrosives, linitis plastica)
 - d. Intrinsic factor inhibitor in gastric secretion
 - (1) Antibody to intrinsic factor (in saliva or gastric juice)
 - (a) "Blocking" antibody (attaches to intrinsic factor to block ability of intrinsic factor to take up B₁₂)
 - (b) "Binding" antibody (attaches to intrinsic factor at site distal to site of B₁₂ attachment)
 2. Small intestinal disorder (affecting ileum, which is the main site of B₁₂ absorption)
 - a. Gluten-induced enteropathy (childhood and adult celiac disease); idiopathic steatorrhea; nontropical sprue
 - b. Tropical sprue (B₁₂ is often the first nutrient to be subnormally absorbed and the last to return to normal absorption)
 - c. Regional enteritis
 - d. Strictures or anastomoses of the small bowel, other "stagnant bowel" syndromes
 - e. Intestinal resection
 - f. Cancers and granulomatous lesions involving the small intestine
 - g. Other conditions characterized by chronically disturbed intestinal function
 - h. Drugs damaging B₁₂ absorption
 - (1) Para-aminosalicylic acid (PAS) (therapy of tuberculosis)
 - (2) Colchicine (therapy of gout)
 - (3) Neomycin (antimicrobial)
 - (4) Ethanol (societal)
 - (5) Metformin (and other biguanide oral antidiabetic agents?)
 - (6) Oral contraceptive agents?
 - i. Specific malabsorption for vitamin B₁₂
 - (1) Due to long-term ingestion of calcium-chelating agents (ionic calcium required for B₁₂ absorption)

*Only 1–2% of Seventh-Day Adventists eat no animal foods.

Table 21-1. *Continued*

- (2) Due to inadequately alkaline pH in ileum (Zollinger-Ellison syndrome, pancreatic disease) (pH >6 required for B₁₂ absorption)
- (3) Unknown causes (lack of intestinal receptors for B₁₂-intrinsic factor complex? absence of "releasing factor"?)
 - (a) Congenital (Imerslund-Gräsbeck syndrome; receptors probably functioning)
 - (b) Acquired (forme fruste of sprue; receptors absent or nonfunctioning?)
3. Competition for vitamin B₁₂ by intestinal parasites or bacteria
 - a. Fish tapeworm (*Diphyllobothrium latum*; decreasing in Finland because of pollution)
 - b. Bacteria: The blind loop syndrome (B₁₂ adsorbing bacteria)
4. Pancreatic disease (normal pancreatic exocrine secretion of trypsin and bicarbonate required for normal B₁₂ absorption)
- C. Inadequate utilization
 1. Vitamin B₁₂ antagonists
 - a. Substituted B₁₂ amides and anilides (experimental agents)
 - b. Cobaloximes (experimental agents)
 - c. Anti-B₁₂ analogues?
 2. Congenital or acquired enzyme deficiency or deletion
 - a. Methylmalonyl-CoA mutase
 - b. Methyltetrahydrofolate-homocysteine methyltransferase
 - c. B_{12a} reductase
 - d. B_{12r} reductase
 - e. Deoxyadenosyltransferase
 - f. Other enzyme reduction or deletion
 3. Abnormal B₁₂-binding protein in serum, irreversibly binding B₁₂ and making it unavailable to tissues
 - a. Increased TC I or TC III glycoprotein (myeloproliferative disorders—"granulocyte-related" B₁₂ binders)
 - b. Increased TC II protein (liver disease; "liver-related" B₁₂ binders)
 - c. Other abnormal B₁₂ binding (a glycoprotein in some hepatoma cases)
 4. Inadequate serum B₁₂-binding protein (congenital or acquired)
 - a. TC II protein (lack produces megaloblastic anemia; it delivers B₁₂ to blood cells, as transferrin delivers iron)
 - b. TC I glycoprotein (lack not known to produce megaloblastic anemia; it is mainly a storage protein for B₁₂, somewhat akin to ceruloplasmin for copper)
 - c. TC III (larger amounts produced in vitro by granulocytes)
 5. Protein malnutrition?
 6. Cancer?
 7. Liver disease?
 8. Renal disease?
 9. Thiocyanate intoxication?
- D. Increased requirement (normal adult daily requirement for exogenous sources is 0.1 µg)
 1. Hyperthyroidism
 2. Increased hematopoiesis?
 3. Infancy (increased requirement for growth)
 4. Parasitization
 - a. By fetus
 - b. By malignant tissue?
- E. Increased excretion
 1. Inadequate B₁₂-binding protein in serum (inadequate TC II particularly?)
 2. Liver disease (inadequate storage capacity for B₁₂)
 3. Renal disease?
- F. Increased destruction by antioxidants
 1. By pharmacologic doses of ascorbic acid
- II. Folic acid deficiency (normal folate body stores will last only 3 to 6 months after cessation of folate ingestion or absorption)
 - A. Inadequate ingestion
 1. Poor diet (lacking unprocessed fresh, uncooked, or slightly cooked food or fruit juices—folates are heat-labile)
 - a. Nutritional megaloblastic anemia
 - (1) Tropical
 - (2) Nontropical
 - (3) Scurvy (diets poor in vitamin C are also poor in folate)
 - b. Chronic alcoholism with or without cirrhosis
 - B. Inadequate absorption (affecting upper third of small intestine, which is the main site of folate absorption; since most food folates are in polyglutamate forms, biliary and intestinal gamma glutamyl conjugases are necessary to split off excess glutamates to make folates absorbable)
 1. Malabsorption syndromes
 - a. Gluten-induced enteropathy (childhood and adult celiac disease; idiopathic steatorrhea, nontropical sprue; coincident B₁₂ malabsorption rare)
 - b. Any other chronic functional or structural disorder involving the upper small intestine
 - (1) Tropical sprue (coincident B₁₂ malabsorption almost invariably present)
 - (2) Associated with herpetic and other skin disorders
 - c. Drugs
 - (1) Diphenylhydantoin (Dilantin—anticonvulsant)
 - (2) Primidone (anticonvulsant)
 - (3) Barbiturates
 - (4) Oral contraceptive agents (?)
 - (5) Cycloserine (tuberculosis)
 - (6) Ethanol (societal)
 - (7) Metformin (diabetes therapy)
 - (8) Dietary amino acid excess of glycine or methionine
 - (9) Nitrofurantoin? (antimicrobial)
 - (10) Glutethimide? (sedative)
 - (11) Cholestyramine
 - (12) Salicylazosulfapyridine (Azulfidine)

Table 21-1. *Continued*

2. Specific malabsorption for folate
 - a. Congenital nonconjugase defects (four cases published)
 - b. Acquired nonconjugase defects
 - c. Inadequate biliary or intestinal conjugase
 - d. Conjugase inhibitors (such as contained in some beans)
3. Blind loop syndrome (folate-greedy bacteria; more commonly, bacteria make folate and actually raise serum folate level of host)
- C. Inadequate utilization (metabolic block)
 1. Folic acid antagonists (dihydrofolate reductase inhibitors)
 - a. 4-amino-4-deoxyfolates (Chemotherapy, (i.e., methotrexate) immuno-suppression, psoriasis)
 - b. 2,4-Diaminopyrimidine

Pyrimethamine	(Malaria, toxoplasmosis)
Trimethoprim	(Antibacterial)
 - c. Triamterene (Diuretic)
 - d. Diamidine (*Pneumocystis carinii*, compounds (i.e., pentamidine protozoacidal) isothionate)
 2. Diphenylhydantoin and possibly other anticonvulsants (possibly block cell uptake or use of folate)
 3. Enzyme deficiency
 - a. Congenital
 - (1) Formiminotransferase
 - (2) Dihydrofolate reductase
 - (3) Methyltetrahydrofolate transmethylase
 - (4) Other enzymes (some secondarily affect folate)
 - b. Acquired
 - (1) Liver disease
 - (a) Formiminotransferase
 - (b) Other enzymes
 4. Vitamin B₁₂ deficiency (reduces folate uptake and retention by cells)
 5. Alcohol (both specific and nonspecific damage to folate metabolism)
 6. Ascorbic acid deficiency (increased hematopoiesis associated with bleeding reduces folate stores; may also decrease ability of body to retain folates in their metabolically active reduced state)
 7. Dietary amino acid excess (glycine, methionine)
- D. Increased requirement (normal adult daily requirement from exogenous sources is 50 µg)
 1. Parasitization
 - a. By fetus (especially in multiple and twin pregnancies)
 - b. By malignant tissue (especially lymphoproliferative disorders)
 - c. By breast-fed infant
 2. Infancy (increased requirement for growth)
 3. Increased hematopoiesis (hemolytic anemias; chronic blood loss, including scurvy)
 4. Increased metabolic activity (hyperthyroidism, chronic temperature elevations)
 5. Lesch-Nyhan syndrome
 6. Drugs (L-Dopa?)
 - E. Increased excretion
 1. Vitamin B₁₂ deficiency? (? of obligatory excretion of folate in urine and bile; possible inability to reabsorb methylfolate excreted in bile because B₁₂ required for it)
 2. Liver disease?
 3. Kidney dialysis
 4. Chronic exfoliative dermatitis
 - F. Increased destruction
 1. Oxidant in diet?
- III. *Interference with purine ring synthesis and interconversion of purine bases*
 - A. Purine antagonists
 1. 6-Mercaptopurine (6-MP) Chemotherapy, immunosuppression
 2. Thioguanine Chemotherapy, immunosuppression
 3. Azathioprine Immunosuppression
 - B. Enzymatic defects in ability to make purine nucleotide from preformed bases
 1. Lesch-Nyhan syndrome (there is an associated increased requirement for folate, which is needed at two steps in the increased de novo biosynthesis of purine)
- IV. *Interference with pyrimidine synthesis*
 - A. Pyrimidine antagonists
 1. 5-Fluorouracil (5-FU) Chemotherapy (blocks thymidylate synthetase)
 2. 6-Azauridine Chemotherapy, psoriasis
 - B. Enzymatic defects in ability to make pyrimidine
 1. Hereditary oroticaciduria (not responsive to therapy with vitamin B₁₂ or folic acid, but responsive to yeast extract or its active ingredients, uridine or the pyrimidine nucleotides, cytidylic, and uridylic acids)
- V. *Inhibition of ribonucleotide reductase (cytidylic to deoxycytidylic acid)*
 - A. Cytosine arabinoside (inhibits DNA polymerase also) Chemotherapy, antiviral
 - B. Hydroxyurea Chemotherapy, psoriasis
 - C. Iron deficiency: Iron is required for ribonucleotide reductase, but it is not yet established that lack of iron can produce megaloblastosis; some workers have reported hypersegmented polys in iron deficiency that disappear with iron therapy, but coincident folate deficiency has not been excluded fully
 - D. Procarbazine (depolymerizes DNA)
- VI. *Inhibition of protein synthesis*
 - A. L-Asparaginase Chemotherapy

Table 21-1. *Continued*

VII. Mechanism unknown

- | | |
|--|--------------------|
| A. Benzene | Solvent |
| B. Azulfidine | Ulcerative colitis |
| C. Arsenic | Poison |
| D. Pyridoxine-responsive megaloblastic anemia (only about 10% of patients with pyridoxine-responsive sideroblastic anemia have megaloblastic morphology) | |
| E. Thiamin-responsive megaloblastic anemia (one case reported) | |
| F. Megaloblastoid anemias (differentiated from megaloblastic anemias by bizarre morphology, including marked polyploidy, few to no orthochromatic megaloblasts, tendency to hyposegmentation, and sometimes karyorrhexis in nucleated red cells) | |

1. DiGuglielmo syndrome (erythremic myelosis—a myeloproliferative disorder usually presenting as refractory anemia and eventuating in death from "erythroleukemia" or myelogenous leukemia); preleukemia
2. Occasional cases of polycythemia vera and other myeloproliferative disorders
3. Occasional cases of aplastic anemia (which may subsequently develop myeloproliferative disorders)
4. Occasional cases of miliary tuberculosis (in such cases, the megaloblastoid marrow is often accompanied by monocytosis and leukopenia or leukocytosis)

Folate^{3,24,24a,24b,24c,24d,48,48a,52,53}

Current evidence suggests that food folate is absorbed primarily from the proximal third of the small intestine, although it is capable of being absorbed from the entire length of the small bowel. Folate in food is present primarily in polyglutamate form. Before absorption, the "excess" glutamates must be split off the side chain of the vitamin molecule by conjugases (pteroylpolyglutamate hydrolase). The products of conjugase action are detectable in the intestinal lumen before absorption,⁵⁴ and preliminary evidence suggests that this occurrence may be due to a surface-active brush border conjugase functionally and chromatographically distinguishable from intracellular conjugase.⁵⁵ Conjugase action may be specifically inhibited by food factors described in yeast and beans⁵⁶ and may be nonspecifically impaired at acid pH.⁵⁷

However, impaired mucosal transport of monoglutamyl folates after deconjugation probably accounts for most instances of folate malabsorption. Active mucosal transport is accelerated by glucose⁵⁸ and galactose and impaired by unidentified factors present in many foods.^{59,60} However, studies using milk folate binder suggest that folate uptake in the gut is facilitated by prior binding.^{60a}

It is probable that a small but relatively unchanging percentage of ingested folate is absorbed by passive diffusion after deconjugation,⁶¹ as is the case with vitamin B₁₂.

TRANSPORT, DISTRIBUTION, STORAGE, FATE, AND EXCRETION**Vitamin B₁₂ (Fig.****21-4A)**^{3,25,49,49a,62,62a,62b,63}

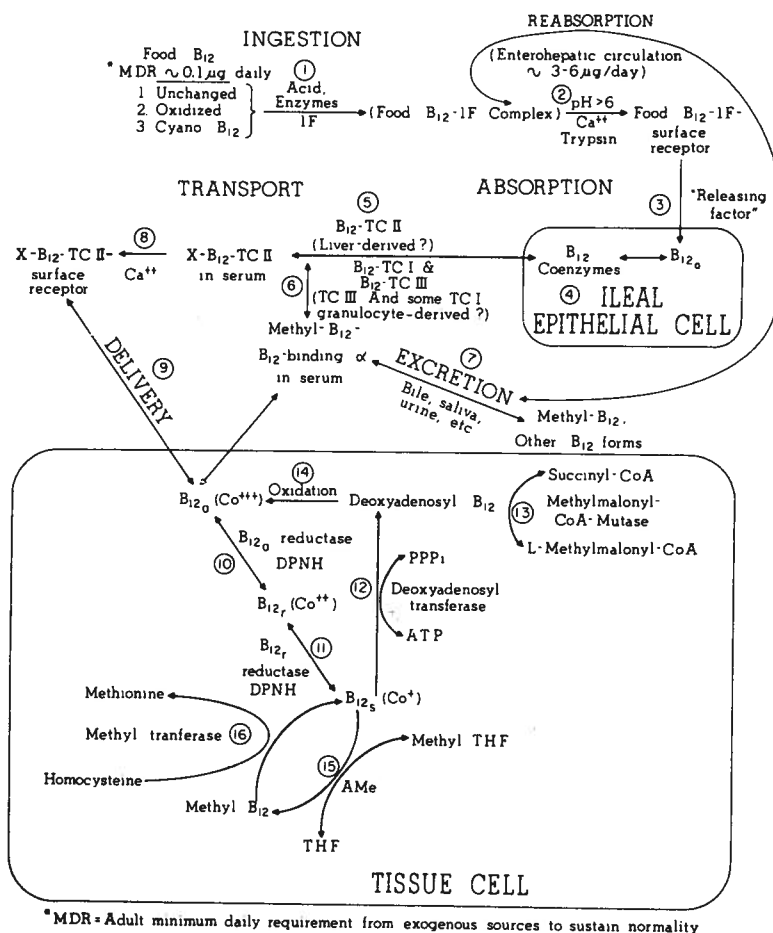
Vitamin B₁₂ in human serum is bound to three different vitamin B₁₂-binding proteins: transcobalamins I, II, and III (TC I, TC II, TC III). The normal total vitamin B₁₂-binding capacity (TBBC)

of serum ranges from about 1000 to 1800 pg/ml, and the unsaturated vitamin B₁₂-binding capacity (UBBC) ranges from 600 to 1400 pg/ml, normally largely (450 to 1000 pg/ml) due to TC II.⁶⁴ TC I and TC III are glycoproteins that are synthesized largely in granulocytes,^{63,63a,63b,63c} but possibly also in salivary glands, gastric mucosa, and some hepatomas.^{65,65a,65b} They have a molecular weight of about 60,000, and appear to have largely a storage function, although TC III may be involved in redelivery of vitamin B₁₂ to the liver, to which TC III may attach via a terminal galactose.^{63,63a,63b,63c,65a,65b} The amounts of unsaturated TC I and TC III in serum range from 30 to 110 and 120 to 300 pg/ml respectively.⁶⁴ These glycoproteins are referred to by some as cobalophilins (R proteins). They are microheterogeneous mixtures of various isoproteins in different proportions.^{65,65a,65b,65c}

TBBC, UBBC, and serum vitamin B₁₂ levels all tend to be elevated in any situation in which the total body neutrophil pool is increased (as in myeloproliferative disorders). This elevation is caused by an increase in the amounts of unsaturated TC I and TC III.⁶⁶ These glycoproteins, however, do not deliver vitamin B₁₂ to the bone marrow, as illustrated by the following: one patient with chronic myelogenous leukemia and pernicious anemia had a normal serum vitamin B₁₂ level because of these glycoproteins and yet the tissues were starved of the vitamin to the point where biochemical deficiency was severe.⁶⁷

Further evidence that TC I and TC III do not participate in transport of the vitamin to bone marrow and nervous tissue is provided by the report of two brothers with congenital lack of these proteins who had low serum vitamin B₁₂ levels but no hematologic or other manifestations of tissue B₁₂ deficiency.^{63,63b,63c}

Unlike the cobalophilins (TC I and TC III), which are glycoproteins, TC II is a pure protein.



*MDR = Adult minimum daily requirement from exogenous sources to sustain normality

Fig. 21-4. A, Cobalamin (B₁₂) metabolism in man. (From Herbert¹⁴⁹ with permission of W.B. Saunders Co.)

It has an apparent molecular weight of 38,000,^{65,65a,65b} is synthesized largely in the liver,⁶⁸⁻⁷⁰ is a betaglobulin, and is chiefly responsible for transport and delivery of the vitamin. Relatively little vitamin B₁₂ is attached to it in vivo because of the rapidity with which it is degraded as it delivers B₁₂ to tissues. When a sample of blood is drawn, only 10 to 20% of plasma vitamin B₁₂ (which is mainly methyl-B₁₂) is bound to TC II; the rest is bound to cobalophilin, largely TC I (which is 80 to 100% saturated with B₁₂). The plasma survival time of TC III is very short (minutes) compared to TC II, which in turn is short (hours) compared to TC I (days) and the cobalophilins in chronic myelogenous leukemia and hepatoma.^{65,65a,65b}

When it enters the blood, vitamin B₁₂ is bound by TCs in proportion to their binding capacity and delivered by TC II to liver, bone marrow cells, reticulocytes, lymphoblasts, fibroblasts, and tumor cells via a mechanism similar to that by which intrinsic factor delivers the vitamin to ileal

mucosal cells. Bone marrow cells, reticulocytes, and many other cells contain on their surfaces "receptor sites" for the TC II-B₁₂ complex. These sites will take up the complex only in the presence of ionic calcium and a pH greater than 6⁷¹; liver cells may contain receptor sites for TC III.^{63,63a,63b,63c,65,65a,65b} Thus, delivery of B₁₂ to the gut enterocyte and the immature erythrocytes both require (1) a transport protein, (2) pH above 6, (3) ionic calcium, and (4) a receptor site for the protein-vitamin B₁₂ complex on the surface of the cell.

The importance of TC II in the absorption, transport, and delivery of vitamin B₁₂ to the tissues is emphasized by the finding of individuals with congenital absence of TC II who have defective vitamin B₁₂ delivery and therefore require treatment with large frequent parenteral doses of the vitamin.^{72,73} The possible "acute phase reactant" status of TC II may be important,^{73a} including elevated TC II in autoimmune disease.^{73b}

"Normal" stores of vitamin B₁₂ range between 1 and 10 mg,^{32,74,75} with the liver containing 50 to

damage to the stomach, ileum, or pancreas, that vitamin B₁₂ deficiency disease develops more rapidly (i.e., in 3 to 6 years).^{91a}

The effect of the enterohepatic circulation is to remove noncobalamin vitamin B₁₂ analogues from the body, because about 60% of the corrinoids excreted in bile are analogues,^{78a,b} and cobalamin is normally preferentially reabsorbed over analogues.^{78c} The role of noncobalamin vitamin B₁₂ analogues in human vitamin B₁₂ economy is unknown, but some may act as antivitamin and could prove harmful.^{27a,b,32d} Although some have suggested that all noncobalamin corrinoids in humans derive from vitamin supplements fed to livestock and taken by humans, we found analogue levels of Americans similar to those in Kalahari Bushmen never exposed to supplements.^{35f}

Folate (Fig. 21-4B)^{3,30,79-82}

Plasma transport of folate remains far less clearly elucidated than that of vitamin B₁₂. Folate in plasma appears to be distributed in three fractions. Free folate and that loosely bound to low-affinity binders are similar in magnitude, whereas much less is bound to high-affinity binders.

Low-affinity binding is a nonspecific property of many different plasma proteins and is similar to nonspecific binding of bilirubin and various drugs. It was detected many years ago using ultrafiltration (which overestimates the amount bound), equilibrium dialysis, and gel filtration. The potential binding capacity is several hundred times greater than the amount of folate in serum. Early data showing failure to half-saturate these binders at high concentrations of free folate indicate that the affinity constant of the system is less than 10⁴ liters per mole, which is less than the 10⁶ liters per mole affinity constant of the carrier-mediated cell transport systems for folate.⁸²

Study of the high-affinity serum folate binders was initiated by Rothenberg's serendipitous discovery of such binders in chronic myeloid leukemia cells,⁸³ and subsequently in the serum of the same patients. They are demonstrable in granulocytes of a proportion of nonleukemic subjects, and are released into the serum by these cells.⁸⁴ Although their release has not yet been detected in most normal subjects, all human (and animal) serum contains these binders,⁸⁰ which more often than not are largely saturated.

Current data suggest that there is only one class of high-affinity serum folate binders—glycoproteins with a molecular weight of about 40,000 probably granulocyte-derived but modified in liver and perhaps kidney.⁸⁰ They have binding constants (K_d) of approximately 10⁻¹⁰ M folic acid and of the order of 10⁻⁸ M 5-methyltet-

rahydrofolate, the main folate present in serum. Although these affinities indicate that binder would only be half saturated at usual serum methyltetrahydrofolate concentrations, they normally average 67% saturation. This phenomenon may be due to the presence of small quantities of non-methyl unsubstituted folates on binders. These binders carry less than 5% of serum folate. Their physiologic function has not yet been demonstrated, but at least three lines of inquiry are currently being pursued: (1) The binders may play a role in folate delivery to liver similar to the role of transcobalamin I in vitamin B₁₂ transport. (2) They may be important in controlling folate distribution, breakdown, and excretion in deficient states. (3) Their presence in cerebrospinal fluid,^{85,85a} their increased affinity for oxidized folate, and the active transport of oxidized folates out of cerebrospinal fluid⁸⁶ all suggest that they may function as carriers important in transporting oxidized folates from cerebrospinal fluid to blood. As discussed earlier, they are also present in milk, where they may enhance folate absorption from gut, and they may be related to a membrane protein-mediated uptake of folate.

Folate is delivered to bone marrow cells, reticulocytes, liver, cerebrospinal fluid, and renal tubular cells against a concentration gradient in a manner that suggests energy-dependent carrier-mediated transport.⁸² The transport constant of these systems is about 10⁻⁶ M. Methyltetrahydrofolate, which accounts for almost all serum folate, appears to be transported more efficiently than folic acid across the intestinal cell and into the cells of the body. A red cell membrane protein that binds folates (including methyltetrahydrofolate) and is immunologically cross-reactive with folate binders in placenta, milk, and serum,^{82a} increases in quantity when cells are grown in low-folate medium.^{82b} This phenomenon suggests that it may be important in cellular folate transport.

Normal total-body folate stores range from 5 to 10 mg, of which approximately half is in the liver.³¹ It has been suggested that the enterohepatic circulation, which transports about 0.1 mg of biologically active folate daily,³ is important in the maintenance of serum folate levels.⁸⁷

Most stored folate is present as polyglutamates,³⁰ which have far greater molecular size and charge than monoglutamates. Transport across cell walls probably requires hydrolysis to monoglutamates, and the enzymes responsible for polyglutamate synthesis and hydrolysis are thus thought to play a major role in folate storage. These enzymes, which have been incompletely characterized, are known respectively as polyglu-

tamate synthetase and conjugase (pteroylpolyglutamate hydrolase; gamma-glutamylcarboxypeptidase). Conjugase, which has been more fully studied than the synthetase, is present in almost all mammalian tissue and is under partial hormone control. Some of the folate-dependent reactions shown in Figure 21-4B are altered by varying glutamyl chain lengths, and it is thus probable that conjugase and polyglutamate synthetase effect the metabolism role of folate as well as its storage.

Folate is excreted in urine and bile in metabolically active and inactive forms. Urinary excretion of the biologically active material occurs after glomerular filtration of the free fraction and reabsorption of some filtered folate by active transport across the tubular cell wall. The principal breakdown product of folate in urine, acetamidobenzoylglutamate, suggests that the principal route of folate catabolism occurs through oxidative cleavage of the folate molecule at the 9-10 bond, with acetylation of the para-aminobenzoyl moiety in the liver before excretion.⁸¹ Some workers believe that scorbutic patients may lose large amounts of folate through irreversible oxidation of 10-formyltetrahydrofolate to 10-formylfolate and excretion of the latter in the urine.⁸⁸

As mentioned previously, about 100 µg of biologically active folate is excreted in the bile daily. In addition, studies with radioactive tracer folates⁸⁹ indicate that a large proportion of injected radioactivity is excreted in the bile as a biologically inactive compound that is not a product of 9-10 cleavage, but that has not been well characterized. Alcohol interferes with the folate enterohepatic cycle.⁸⁷

NUTRITIONAL REQUIREMENTS AND NATURAL SOURCES

The term *minimal daily requirement* (MDR), as used in this chapter, means the minimum from *exogenous sources* required to sustain normality, with normality defined as the absence of any biochemical hypofunction that is correctable by addition of greater quantities of the vitamin. By this definition, the minimal daily requirement for vitamin B₁₂ of a normal subject would be only 0.1 µg, since this quantity will sustain normality in a normal subject.^{90,91} The minimum daily requirement for vitamin B₁₂ of a patient with gastric or ileal structural or functional damage would be greater because such damage eliminates not only the normal absorption of vitamin B₁₂ from exogenous (food) sources, but also the normal daily reabsorption from the ileum of almost all the vitamin B₁₂ normally excreted each day in bile.

The minimal daily requirements can be reduced

to a formula⁹² applicable generally to essential (i.e., required from exogenous sources) nutrient deficiency, as follows:

$$\text{MDR (units/day)} = \frac{\text{UBS (units)}}{\text{D (days)}}$$

where MDR = minimal daily requirement of nutrient from exogenous sources, UBS = utilizable body stores of nutrient, and D = number of days required to develop tissue deficiency after cessation of absorption from exogenous sources of nutrient (with appropriate correction for *incomplete* cessation of absorption).

This formula may also be written as:

$$D = \text{UBS/MDR}$$

or

$$\text{UBS} = D \times \text{MDR}$$

As suggested previously, one can predict the time it would take any given nutrient deficiency to develop in any given person after reduction or cessation of absorption of the nutrient if one knows (or can reasonably estimate) the MDR for the nutrient and the utilizable body stores thereof.

The Recommended Dietary Allowance (RDA) "are the levels of intake of essential nutrients considered, in the judgment of the Food and Nutrition Board (of the National Academy of Sciences of the US) on the basis of available scientific knowledge, to be adequate to meet the known nutritional needs of practically all healthy persons."⁹¹ The RDA for each nutrient is intentionally substantially greater than the MDR in order to produce some measurable amount of body stores to allow for normal variation in utilization and transient increased requirements. There is a tendency to err on the side of larger body stores when information is incomplete. Small storage surpluses of nutrients are rarely detrimental, whereas small deficits may result in deficiency over a long period of subtle negative balance. Other countries often use the term "RDI" (recommended dietary intake) to describe the concept termed "RDA" in the United States.^{91a}

Vitamin B₁₂

Nutritional Requirements.^{3,29-31,74,90,91,91a-b} Vitamin B₁₂ requirements have been estimated from three different types of study:³¹ (1) those designed to determine the minimal amount needed to prevent or to cure megaloblastic anemia resulting from vitamin B₁₂ deficiency, (2) those correlating

the relationship between the levels of vitamin B₁₂ in serum and in liver in deficient and healthy subjects, and (3) those correlating body stores and turnover rates of vitamin B₁₂.

The results of such studies³⁰ demonstrated that: (1) The minimum quantity of vitamin B₁₂ that would produce a hematologic response in patients with uncomplicated vitamin B₁₂ deficiency was approximately 0.1 µg daily, and 0.5 to 1 µg of the vitamin daily produced maximum hematologic responses, with similar amounts maintaining a normal picture. (2) Patients with moderate vitamin B₁₂ deficiency resulting from B₁₂ malabsorption had an average liver vitamin B₁₂ content of 0.16 µg/g wet weight of liver, associated with serum vitamin B₁₂ levels ranging from 80 to 130 pg/ml and an average total body B₁₂ of approximately 250 µg. (A second group of individuals who were also suffering from vitamin B₁₂ malabsorption, but who had not yet developed morphologic evidence of blood damage due to vitamin B₁₂ deficiency, all had serum levels between 130 and 200 pg/ml, associated with approximately 0.28 µg of vitamin B₁₂/g wet weight of liver and an average total body vitamin B₁₂ content of approximately 525 µg.) (3) The daily whole-body turnover of vitamin B₁₂ measured with tracer doses of radioactive vitamin indicates a radioactivity turnover of between 0.1 and 0.2% daily, regardless of whether the body vitamin B₁₂ stores are normal or reduced.

Loss of 0.1 to 0.2% of radioactive vitamin B₁₂ daily means less than that quantity of vitamin is lost from the body stores daily because the radioactive B₁₂ excreted in the bile mixes with nonradioactive B₁₂ in the diet, and some of the radioactive B₁₂ that would otherwise be reabsorbed in the ileum is replaced by absorbed nonradioactive vitamin. The net result is a gradual reduction in the radioactivity of the body vitamin B₁₂ stores, but a much lesser reduction in the actual vitamin B₁₂ content of those stores.

The Food and Nutrition Board of the National Academy of Sciences (NAS) set the RDA for vitamin B₁₂ in 1980 at 3 µg/day for adolescents and normal adults.⁹¹ This amount is much higher than the intake of millions of healthy vegetarians, is associated with gradually increasing body stores each decade of life and, for this and other reasons, it was reduced to 2 µg/day by the 1980-1985 RDA Committee.^{91a} The Joint FAO/WHO Expert Group recommended in 1987 a daily intake of 1 µg of vitamin B₁₂ for the normal adult and 2 µg for those with achlorhydria.^{31b} The 1980 recommendation of the NAS group was based almost entirely on studies of the body turnover of radioactive vitamin B₁₂—studies that seem to have no relation to

minimal daily requirements, since such turnover tends to be a fixed percentage of body stores regardless of their size. On the other hand, the 1 µg daily intake recommended in 1987 for adults by the FAO/WHO group^{31b} is based not only on the same radioactivity turnover studies on which the NAS relied almost exclusively, but also on studies of minimal amounts needed to prevent or to cure megaloblastic anemia resulting from vitamin B₁₂ deficiency, and on studies of the relationship between the levels of vitamin B₁₂ in serum and in liver in deficient and in healthy subjects. The daily allowance of 2 µg of vitamin B₁₂ for adults recommended by the 1980-1985 RDA Committee^{91a} carries a greater margin above normal physiologic requirements for absorption of the vitamin by the nonachlorhydric adult than does the FAO/WHO recommendation, resulting in larger tissue stores. There is no clear evidence that these larger stores constitute a benefit (or a harm).

Vitamin B₁₂ deficiency does not occur in breast-fed infants unless their mothers are deficient in the vitamin. Infants showing such deficiency respond hematologically to 0.1 µg of vitamin B₁₂ orally.⁹³ In the economically advanced countries, breast milk with a content of about 0.4 µg/L supplies about 0.3 µg of vitamin B₁₂ daily, which is clearly adequate as manifested by lack of evidence of any deficiency in the infant. Available evidence suggests that the milk from mothers whose serum contains vitamin B₁₂ concentrations close to the lower limit of normality (i.e., 200 pg/ml) is also adequate, but that of vegetarian mothers with lower serum levels is not adequate when it falls to 0.07 µg/L of breast milk or less.³¹ The Joint FAO/WHO Expert Group in 1987^{31b} therefore recommended a daily intake of 0.1 µg of vitamin B₁₂ in breast milk as adequate for infants. The 1980-1985 RDA Committee recommended for those on artificial feeding 0.3 µg at age 0 to 2.9 months, 0.4 µg at 3 to 5.9 months, 0.5 µg at 6 to 11.9 months, 0.7 µg at 1 to 1.9 years, 1 µg at 2 to 5.9 years, 1.5 µg at 6 to 9.9 years, and 2.0 µg thereafter.^{91a}

Vitamin B₁₂ deficiency does not occur in normal children on adequate calorie and animal protein intakes. The Joint FAO/WHO Expert Group in 1987 calculated desirable intakes of vitamin B₁₂ for children as 0.04 µg/kg body weight/day to a maximum of 1 µg/day.^{31b}

Pregnancy produces an increased requirement for vitamin B₁₂ owing to the fetal drain on maternal stores. The fetus removes approximately 0.2 µg of vitamin B₁₂ daily from the maternal stores in the latter half of pregnancy. The Joint FAO/WHO Expert Group in 1987 calculated the desirable daily intake of vitamin B₁₂ in pregnancy to be the adult 1 µg plus an additional 0.4 µg, and

in lactation an additional 0.3 µg on top of the adult 1 µg.^{31d}

Total recommended intake during lactation is 2.5 µg/day (4 µg RDA) based on the recommended daily intake of 2 µg for the normal adult (3 µg RDA) plus an additional 0.5 µg to accommodate the approximately 0.3 µg lost in the milk of nursing mothers.^{91a}

The sequence of events in developing vitamin B₁₂ deficiency^{74,74a,74b} is delineated in Figure 21-5 and involves the concept of four stages of depletion for vitamins using vitamin B₁₂ and folate as models.^{93a,101b} The first stage, negative vitamin B₁₂ balance, begins when vitamin B₁₂ absorption falls low enough to deplete the amount of vitamin B₁₂ on its primary delivery protein, transcobalamin II (TC II), resulting in a low holo-TC II level.^{93b,93c} For any laboratory test, because of individual variability, deviation from the value normal for the

individual often precedes deviation from the range of normal for the laboratory,^{93d} i.e., the individual's low holo-TC II may be low for him and may indicate that he is in negative vitamin B₁₂ balance before his holo-TC level falls below the laboratory range of normal. The same is true for other tests, such as the granulocyte nuclear lobe average, which can be high enough to indicate slowed DNA synthesis in granulocytes of the individual owing to vitamin B₁₂ or folate deficiency even when the lobe average is still within the laboratory normal range.^{93d}

When one understands this normal sequence of events, it is not surprising to learn that about 10% of Hindu Indian vegetarians with early cobalamin deficiency did not yet have an elevated mean corpuscular volume (MCV) (particularly with their frequent iron deficiency) or anemia.^{93e}

Natural Sources.^{2,3,23,23a,23b,23c,25-27,32,74,91,94} The
















Stage:	Normal	Negative B ₁₂ Balance	B ₁₂ Depletion	B ₁₂ -Deficient Erythropoiesis	B ₁₂ -Deficiency Anemia
Liver B ₁₂					
HoloTC II					
RBC+WBC B ₁₂					
HoloTC II	>30 pg/ml	<20 pg/ml	<20pg/ml	<12 pg/ml	<12 pg/ml
TC II % sat.	>5%	<5%	< 2%	< 1%	< 1%
Holohap	>150 pg/ml	>150pg/ml	<150 pg/ml	<100 pg/ml	<100 pg/ml
dU suppression	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	No	No	No	Yes	Yes
TBBC*% sat.	>15%	>15%	>15%	<15%	<10%
Hap % sat.	>20%	>20%	>20%	<20%	<10%
RBC Folate	>160 ng/ml	>160	>160	<140	<140
Erythrocytes	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Low
TC II	Normal	Normal	Normal	Elevated	Elevated
Methylmalonate	No	No	No	?	Yes
Myelin damage	No	No	No	?	?

Fig. 21-5. Sequential stages in the development of vitamin B₁₂ deficiency. Biochemical and hematologic sequence of events as negative vitamin B₁₂ balance progresses. Holo TC II = holotranscobalamin II (i.e., transcobalamin II with cobalamin on it). Without cobalamin, it is apotranscobalamin II. TC II % sat = % of total TC II that has cobalamin on it. Holohap = holohapatocorrin (i.e., haptocorrin with cobalamin on it). Synonyms for haptocorrin are cobalaphilin and transcobalamin (I and II).

dU suppression = diagnostic test of ability of cells (bone marrow cells, peripheral blood lymphocytes) to make DNA. Hypersegmentation = increase in number of lobes in granulocytic white cell nuclei.

TBBC % sat = % of total B₁₂ binding capacity of plasma that has B₁₂ on it.

RBC = red blood cell.

MCV = mean corpuscular volume of red cells.

sole source of vitamin B₁₂ in nature is synthesis by microorganisms. The vitamin is not found in plants except when they are contaminated by microorganisms. Fruits, vegetables, and grains and grain products are usually devoid of vitamin B₁₂. However, the root nodules of certain legumes contain microorganisms that make vitamin B₁₂, and unwashed vegetable products may also contain vitamin B₁₂ because of contamination by soil or fecal matter. These small amounts of vitamin B₁₂ in legumes and in contaminated food may provide the only dietary source of vitamin B₁₂ for strict vegetarians. The usual dietary sources of vitamin B₁₂ are meat and meat products (including shellfish, fish, and poultry) and, to a lesser extent, milk and milk products. Rich sources of vitamin B₁₂ (greater than 10 µg/100 g of wet weight) are organ meats such as lamb and beef liver, kidney, and heart and bivalves (clams, oysters) that siphon large quantities of vitamin B₁₂-synthesizing microorganisms from the sea. Moderately high amounts (3 to 10 µg/100 g of wet weight) are present in nonfat dry milk, some seafood (crabs, rock fish, salmon, sardines), and egg yolk. Moderate amounts (1 to 3 µg/100 g of wet weight) are found in fluid milk products, cream, cheddar cheese, and cottage cheese. Bacteria in the knobby growth of some seaweeds make the vitamin,^{94a,94b} and some manufacturers add the vitamin to breakfast cereals.

The vitamin B₁₂ molecule is resistant to heat unless exposed in an alkaline medium to temperatures in excess of 100°C; the molecule splits at 250°C. Thus, hamburgers cooked on a hot griddle may lose some of their B₁₂ from the surface flat against the hot griddle, but the vitamin B₁₂ deep within the patty is preserved. Of the vitamin B₁₂ in liver, 8% is lost by boiling at 100°C for five minutes. Boiling muscle meat at 170°C for 45 minutes results in a loss of 30% of the vitamin from the meat. Milk pasteurized for two to three seconds loses 7% of its available vitamin B₁₂; when boiled for two to five minutes, it loses 30%. Sterilization in a bottle for 13 minutes at 119 to 120°C causes a loss of 77%; rapid sterilization (3 to 4 seconds) with superheated steam at 143°C destroys only about 10% of the vitamin.⁷⁴ In the presence of ascorbic acid, vitamin B₁₂ is less heat-stable, and substantial amounts in food may be destroyed by 0.5 g of ascorbic acid.^{35c,93,95,96,97} In light of this, if ascorbic acid is added to food, as has been suggested in some schemes of iron fortification,^{88,98} vitamin B₁₂ intake may be significantly reduced. In some circumstances, large doses of ascorbic acid may reduce vitamin B₁₂ absorption.^{99a}

The enzymatically active forms of vitamin B₁₂

(coenzyme B₁₂ and methylcobalamin) are the dominant forms in foodstuffs, in which they are generally attached to polypeptides. Cyanocobalamin per se is probably not present to a significant extent in any natural source. This stable form of vitamin B₁₂ results from the action of cyanide on natural vitamin B₁₂ coenzymes. Thiocyanate ingestion and absorption of cyanide from tobacco smoke may convert a small amount of enzymatically active cobalamins in blood and tissue to cyanocobalamin.

Folic Acid

Nutritional Requirements.^{3,30,31,74,90,91,91a,100,100a,100b} The minimal daily requirement (MDR) for folate is approximately 50 µg for adults. The FAO/WHO Expert Group in 1987 recommended a daily dietary intake for adults of 3.1 µg/kg of body weight, to equal a daily intake of 200 µg for a 65-kg man and 170 µg for a 55-kg woman.^{31e} This amount will provide stores sufficient to prevent deficiency for 3 to 4 months of zero intake. Because of the possibility of an acute drain on stores from acute blood loss, or pregnancy, a smaller daily intake is not recommended. To meet the added needs of pregnant women, WHO/FAO recommends a supplement of 200 to 300 µg daily, so that the daily folate intake is no less than 350 µg (or 7 µg/kg body weight), and a supplement of 100 µg/day during lactation (i.e., a total of 5 µg/kg body weight). The 1980 RDA of the Food and Nutrition Board of the NAS ranged from 50 µg for infants (under 1 year) through 100 µg (1 to 3 years), 200 µg (4 to 6 years), and 300 µg (7 to 10 years) to 600 µg for lactating women and 800 µg in pregnancy.⁹¹

The 1980-1985 NRC RDA Committee for folate, based on considerably more data correlating food folate intake with red cell and liver folate stores, proposed a daily intake of 16 µg for ages 0 to 2.9 months, 24 µg for ages 3 to 5.9 months, 32 µg for ages 6 to 11.9 months, 3.3 µg/kg body weight for ages 1 to 9.9 years, and 3 µg/kg body weight thereafter, with a total of 500 µg/day during pregnancy and 3 µg/kg plus 100 µg/day during lactation.^{91a}

Because the daily folate requirement is hinged to the daily metabolic and cell turnover rates, it is increased by anything that increases metabolic rate (such as infection and hyperthyroidism), and anything that increases cell turnover (such as hemolytic anemia and rapid tissue growth in the fetus and malignant tumors). Folate consumption by individual cells is proportional to their rate of one-carbon-unit transfer. Alcohol interferes with folate utilization, and thereby increases folate requirement.¹⁰¹

The sequence of events^{74,101a,101b} in developing

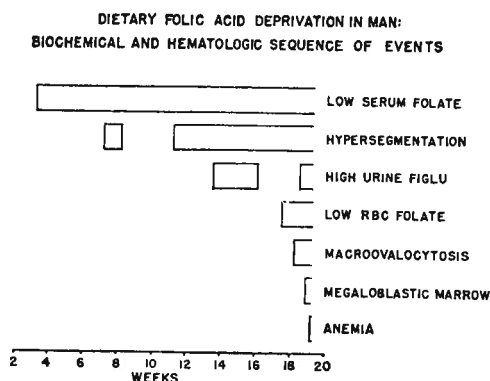


Fig. 21-6. Biochemical and hematology sequence of events in developing dietary folate deficiency in man. (From Herbert, V.: *Trans. Assoc. Am. Physicians* 75:307, 1962.)

folate deficiency in man is depicted in Figures 21-6 and 21-7.* The concept in Figure 21-7 was first presented in slightly different form at the 1985 International Congress of Nutrition.^{93a,101b,101c,101d} As Figure 21-7 depicts, the stages are: negative folate balance (characterized by a fall in erythrocyte folate to below 3 ng/ml); tissue folate depletion (characterized by a fall in erythrocyte folate to below 160 ng/ml (and a *pari passu* fall in hepatic folate); defective DNA synthesis (characterized by an abnormal diagnostic dU suppression test and by granulocyte nuclear hypersegmentation); and finally, gross macroovalocytosis, elevated mean corpuscular volume (MCV), and anemia. More than half of folate-depleted individuals have not yet reached the stage of anemia and will be missed by screening tests that do not recognize that folate depletion may precede anemia by months.

Natural Sources.^{24,91,102-105} Unlike vitamin B₁₂, which is present only in animal protein, folates are ubiquitous in nature, being present in nearly all natural foods. Unlike vitamin B₁₂, folate is highly susceptible to oxidative destruction; 50 to 95% of the folate content of foods may be destroyed by protracted cooking or other processing, such as canning, and all folate is lost from refined foods such as hard liquor and hard candies. Foods with the highest folate content per unit of dry weight include yeast, liver and other organ meats, fresh green vegetables, and some fresh fruits.

The naturally occurring folates are active metabolic forms, usually in polyglutamate linkage³⁰ (with pteroylheptaglutamates dominant in yeast). Conjugates present in vegetable and mammalian

tissues¹⁰⁶ (including human intestine) liberate pteroyldiglutamates and pteroylmonoglutamates from the conjugates, thereby making the folate available for absorption. About 90% of monoglutamate is absorbed by humans when ingested on its own, but this proportion is markedly decreased in the presence of many foods, irrespective of whether the folate was derived from or added to the food.^{59,60} Prior data suggested that polyglutamate was poorly absorbed compared with monoglutamate; however such data were probably affected by other factors in the foods from which polyglutamate was derived. The efficiency of polyglutamate hydrolysis in intestine^{53,54} appears to rule out this process as a rate-limiting step in folate absorption unless conjugase inhibitors are present.

The pharmaceutical product pteroylglutamic acid (PGA), like the pharmaceutical product cyanocobalamin, is not usually found as such in natural sources. Its isolation from natural sources, like the isolation of cyanocobalamin, was the result of oxidation and deconjugation of the naturally occurring conjugated forms to a stable form.

METABOLIC FUNCTIONS AND INTERRELATIONSHIPS^{2,3,25,107-109}

DNA Synthesis

As illustrated in Figure 21-8, both vitamin B₁₂ and folic acid are required for synthesis of thymidylate and, therefore, of DNA. A vitamin B₁₂-containing enzyme removes a methyl group from methyl folate and delivers it to homocysteine, thereby converting homocysteine to methionine (methyl-homocysteine) and regenerating tetrahydrofolic acid (THFA) from which the 5,10-methylene THFA involved in thymidylate synthesis is made. Methyl folate is the dominant form of folate in human serum and liver, and probably also in other body storage depots for folate. Because methyl folate may only return to the body's folate pool via a vitamin B₁₂-dependent step, a patient with vitamin B₁₂ deficiency has much of his folate "trapped" as methyl folate, which is metabolically useless. This "folate trap" hypothesis^{32d,107-110} may provide much of the explanation for the hematologic damage of vitamin B₁₂ deficiency that is not clinically distinguishable from that of folate deficiency. In both instances, the hematologic defect results from lack of adequate 5,10-methylene THFA, which delivers its methyl group to deoxyuridylate to convert that substance to thymidylate, and thus makes DNA during the S (synthesis) phase. In either deficiency, lack of adequate DNA synthesis causes many hematopoietic cells to die in the bone marrow, possibly without ever com-

*Note: Herbert has introduced a stage of negative nutrient balance between the stages of normality and tissue nutrient depletion.^{93a,101b,150}
















	Normal	Negative Folate Balance	Folate Depletion	Folate Deficient Erythropoiesis	Folate Deficiency Anemia
Liver folate					
Plasma folate					
Erythron folate					
Serum folate	>5 ng/ml	<3 ng/ml	<3 ng/ml	<3 ng/ml	<3 ng/ml
RBC folate	>200 ng/ml	>200 ng/ml	<160 ng/ml	<120 ng/ml	<100 ng/ml
Diagnostic dU suppression	Normal	Normal	Normal	Abnormal*	Abnormal*
Lobe average§	< 3.5	< 3.5	< 3.5	> 3.5	> 3.5
Liver folate	>3 µg/g	>3 µg/g	<1.6 µg/g	<1.2 µg/g	<1 µg/g
Erythrocytes	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	> 12 g/dL	> 12 g/dL	> 12 g/dL	> 12 g/dL	< 12 g/dL
Plasma clearance of intravenous folate	Normal	Normal	Normal	Increased	Increased

Fig. 21-7. Sequential stages in the development of folate deficiency. In the first three lines, the area of the shaded boxes in the normal individual shows the relative quantities of folate in each compartment. The sequentially increasing unshaded areas show its proportional disappearance during successive stages of folate deficiency development. In our laboratory, in 100 normal adults, arithmetic mean serum folate = 8.125 ± 5.06 ng/ml, and geometric mean serum folate = 6.92 with range (± 2 s.d.) = 1.89 to 21.8. In the same 100, arithmetic mean red cell folate = 359.5 ± 158.2 ng/ml, and geometric mean red cell folate = 329.7 with range (± 2 s.d.) = 129.8 to 773.3. Boxes enclose laboratory abnormalities which characterize onset of the stage indicated in the column heading.

RBC = red blood cell.

Diagnostic dU suppression = the test of DNA synthesis normality described in other references.^{32d,108,128a,128b,128d}

MCV = mean corpuscular volume of erythrocytes.

*Abnormal in bone marrow first and subsequently in lymphocytes, except with concomitant iron deficiency, when the reverse occurs.

§Normal lobe average = 3.2 ± 0.15 .^{93d,128b}

pleting the S phase of cell replication (i.e., a form of "ineffective erythropoiesis").

Megaloblastosis (the presence of giant germ cells) is the end product of deranged DNA synthesis of any cause. It is most easily understood as an arrest in the S phase of cell replication, usually because of inadequate availability of vitamin B₁₂ or folate, with most replicating cells in the body, instead of being in a resting phase, in the process of attempting (with poor success) to double their DNA in order to divide. The underlying biochemical defect that translates poor thymidylate synthesis (due to folate and/or vitamin B₁₂ deficiency) into morphologic megaloblastosis may be failure to elongate DNA chains in the presence of a relatively normal capacity to initiate DNA synthesis. This process occurs, presumably, because the lowered thymidylate concentrations remain adequate to serve as substrate for "initiat-

ing" but not for "elongating" DNA polymerase.¹¹¹ Alternatively, the mechanism of the defect may be "illicit" incorporation of thymidylate precursors (such as deoxyuridylate) into DNA, with subsequent cleavage of the DNA containing the "illicit" nucleotide.^{111a}

Morphologic changes are most striking in bone marrow cells, with the "ineffective hematopoiesis" resulting in peripheral blood pancytopenia (anemia, leukopenia, and thrombopenia). However, megaloblastosis is also present in all other duplicating cells of the body^{3,112} and may be strikingly noted in the epithelial cells of the entire alimentary tract, producing glossitis and variable degrees of megaloblastosis along the entire alimentary tract epithelium. It is not yet clear why gut changes associated with vitamin B₁₂ deficiency are often related to constipation whereas those associated with folate deficiency are more

sulting from the inadequate myelin synthesis caused by vitamin B₁₂ deficiency include paresthesia, especially numbness and tingling in the hands and feet, diminution of vibration sense and/or position sense (usually but not always occurring first in the ankles and feet), unsteadiness, poor muscular coordination with ataxia, moodiness, mental slowness, poor memory, confusion, agitation, depression, and central scotomata (sometimes with dim vision due to optic atrophy and tobacco amblyopia). Delusions, hallucinations, and even overt psychosis (usually with paranoid ideas) may occur. The wide variety of sensory and motor changes tend to be symmetrical, especially if present for weeks or months.

Early subtle nerve damage (inability to sense position in index toes, inability to perceive the vibrations of a 256-vps tuning fork) precedes by months grosser nerve damage (inability to sense position in great toes, inability to perceive the vibrations of a 128-vps tuning fork).¹¹² Correction by vitamin B₁₂ therapy confirms that the damage is due to vitamin B₁₂ deficiency rather than to the normal process of aging.

Comparing "private" with "ward" patients in large hospital populations, we observed that economically advantaged people with vitamin B₁₂ deficiency tend to have relatively severe neurologic damage and relatively mild hematologic damage, whereas economically deprived people with vitamin B₁₂ deficiency tend to have relatively equal severity of neurologic and hematologic damage.¹¹² A major explanation for the variable degree of hematologic damage with fixed amount of neurologic damage in vitamin B₁₂ deficiency is the quantity of folate in the diet. Well-to-do people tend to eat better diets, richer in high-folate foods such as fresh fruits, vegetables, and meats. The folate retards development of hematologic damage while allowing neurologic damage to progress.

Folate deficiency does not damage myelin, but it is associated with a high frequency of irritability, forgetfulness and, often, hostility and paranoid behavior. These phenomena often strikingly improve within 24 hours of the start of therapy with folic acid. Other neurologic sequelae attributed by some to folate deficiency have been reviewed elsewhere,¹²¹ but the association has not been convincingly demonstrated.⁸⁵

Cerebration may improve rapidly when vitamin B₁₂ deficiency is appropriately treated, but the neurologic damage resulting from inadequate myelin synthesis heals slowly. Because the nerve damage is related to deterioration of the axon underneath the deteriorated myelin, healing is related to the speed of regeneration of damaged

axons; this regeneration creeps peripherally from the nerve head at the rate of 0.1 mm/day.

B₁₂ in Fat and Carbohydrate Metabolism^{3,23,23a,23b,23c,25,27,30,122}

Because coenzyme B₁₂ is required for the hydrogen transfer and isomerization whereby methylmalonate is converted to succinate,^{23,25} B₁₂ is involved in both fat and carbohydrate metabolism. As indicated previously, although it is an attractive speculation that the neurologic damage of patients with vitamin B₁₂ deficiency is caused by inability to make the lipid portion of the lipoprotein myelin sheath (due to inadequate propionic acid utilization related to inadequate interconversion of methylmalonate and succinate), there is not yet evidence to associate this with the neurologic damage of vitamin B₁₂ deficiency in man.

More recently, researchers have shown that rat glial cells in vitamin B₁₂-deficient media produce two odd-chain fatty acids that are not found when the cells are grown in the presence of vitamin B₁₂.¹²³ Further, nerve biopsies from subjects with vitamin B₁₂ deficiency incubated in ¹⁴C-labeled propionate produced labeled ¹⁵C and ¹⁴C fatty acids.¹²⁴ The explanation for this finding and its relationship to the functional and histologic changes have not yet been elucidated.

B₁₂ in Protein and Fat Metabolism^{23,23a,23b,23c,25,27,30}

Vitamin B₁₂ is involved in protein synthesis through its role in the synthesis of the amino acid methionine, and possibly in other ways as well. Because methionine is involved in making available more of the lipotropic substances choline and betaine, this is another area where cobalamin may play a role in lipid metabolism.

B₁₂ as a Reducing Agent^{3,23,23a,23b,23c,125}

Vitamin B₁₂ appears to be involved in maintenance of sulfhydryl (SH) groups in the reduced form necessary for function of many SH-activated enzyme systems. Vitamin B₁₂ deficiency is characterized by a decrease in reduced glutathione (which is changed from reduced glutathione [GSH] to oxidized glutathione [GSSG]) of erythrocytes and liver. This condition may be corrected by administration of vitamin B₁₂. In vitro, vitamin B₁₂ derivatives catalyze the nonenzymatic oxidation of sulfhydryl derivatives.

Folate in One-Carbon Unit Transfers^{24,30,126}

Folate coenzymes are concerned with mammalian metabolic systems involving transfer of a one-carbon unit. These reactions include (1) de

novo purine synthesis (formylation of glycynamide ribonucleotide [GAR] and 5-amino-4-imidazole carboxamide ribonucleotide [AICAR]), (2) pyrimidine nucleotide biosynthesis (methylation of deoxyuridylic acid to thymidylic acid), (3) three amino acid conversions: the interconversion of serine and glycine (which also requires vitamin B₆), catabolism of histidine to glutamic acid, conversion of homocysteine to methionine (which also requires vitamin B₆), (4) generation of formate into the formate pool (and utilization of formate), and (5) methylation of small amounts of transfer RNA. The suggestion that folate may be involved in physiologic methylation of biogenic amines¹²⁷ has been refuted.¹²⁸

Role of Ascorbic Acid

It is possible that ascorbate *in vivo* may aid in the protection against oxidative destruction of reduced folates in the body. There is no evidence that ascorbate plays any role in the reduction of pteroylglutamic acid to tetrahydrofolic acid, a reaction mediated by folate reductases. The original such evidence proved to be protection by ascorbate of the end product against oxidative destruction.

Selective Nutrient Deficiency

An important area of nutrition research is selective nutrient deficiency in one cell line or one tissue and not another in the same patient. This condition can result from one tissue obtaining food folate first, as do intestine cells when food folate is decreased.^{101a} Another cause may be the presence of more machinery and/or more efficient machinery for folate uptake and less for folate rejection and loss in relation to one cell line as compared to another. Selective folate deficiency can occur, for example, in white cells but not in red cells.^{128a,128b} Therefore, lymphocytes (which in their resting form in the human blood appear impervious to the vitamins B₁₂ and folic acid, and also other nutrients, such as nicotinamide^{128c}) can be used to measure past nutrient deficiency up to two months after therapy,^{128a} and also to measure covert folate deficiency in patients who lack macro-ovalocytosis because of coincident iron-deficiency or hemoglobinopathy.^{128d,128e,128f} Only when lymphocytes are making DNA do they appear to be pervious to nutrients.^{128a,128b,128c}

THERAPY

The only established therapeutic use of vitamin B₁₂ or folic acid is in treating deficiency of the respective vitamins. Claims made for nutritional value of either of these vitamins in clinical situ-

ations in which deficiency of the vitamin does not exist are without foundation in fact.

When the deficiency is of vitamin B₁₂, only this vitamin should be used for therapy; conversely, when the deficiency is of folate, only that vitamin should be used for therapy. The use of folic acid in the treatment of a patient whose deficiency is of vitamin B₁₂ often produces temporary hematologic improvement, but allows the neurologic damage of the underlying vitamin B₁₂ deficiency to progress, often to an irreversible state.

Although liver extract for injection was deleted from the USP in 1960, such products are still being used by some practitioners and appear in the National Formulary. For clinical purposes, the injectable unit is approximately the equivalent of 1.33 µg of vitamin B₁₂. Because preparations of liver extract contain hematopoietic materials other than vitamin B₁₂ (e.g., folic acid, folinic acid), they constitute "shotgun therapy," like many other multi-ingredient therapies, and such use is to be avoided.

The signs and symptoms of deficiency of vitamin B₁₂ or folate in humans^{1-3,111,129-131} are listed in Table 21-2.

Therapy of Critically Ill Patients

It is rarely necessary to institute immediate therapy before determining the cause of megaloblastic anemia. Major indications for emergency therapy include severe thrombocytopenia (platelet count less than 50,000/mm³) associated with bleeding, severe leukopenia (white cell count less than 3,000/mm³) associated with infection, infection itself, coma, severe disorientation, marked neurologic damage, severe hepatic disease, uremia, or other debilitating illness complicating the anemia. The anemia itself is not a problem because the dyspnea and occasional angina that may accompany a hematocrit of less than 15 volumes % are relieved by a transfusion of one to two units of packed erythrocytes. Transfusion is unwarranted in the absence of symptoms of anemia. When venous pressure is elevated, transfusion of packed erythrocytes should be accompanied by withdrawal of equivalent or slightly smaller quantities of whole blood, which will reduce rather than raise the venous pressure. Transfusion of whole blood without withdrawal of blood has been responsible for acute rises in venous pressure with resultant irreversible congestive failure in elderly patients with megaloblastic anemia and unrecognized elevated venous pressure. Ideally, venous pressure should be determined before transfusion and monitored during both the transfusion of packed cells and the simultaneous withdrawal of whole blood. An alternate to exchange transfusion

Table 21-2. Clinical Picture of the Megaloblastic Anemias

1. Symptoms
 - Weakness, tiredness
 - Dyspnea
 - Sore tongue
 - Paresthesia (B₁₂ deficiency only)
 - Diarrhea (especially folate deficiency)
 - Constipation (especially B₁₂ deficiency)
 - Irritability and forgetfulness (especially folate deficiency)
 - Anorexia
 - Syncope
 - Headache
 - Palpitation
2. Signs
 - Megaloblastic bone marrow (orthochromatic megaloblasts, giant metamyelocytes)
 - Anemia, leukopenia, thrombocytopenia, with macro-ovalocytes (normal MVC = 87 ± 5 cu μ) and "hypersegmented polys" (normal Arneth count 2 lobes = 20 to 40%, 3 lobes = 40 to 50%, 4 lobes = 15 to 25%, 5 lobes = 0 to 5%, 6 lobes = 0 to 0.1%, more than 6 lobes = 0) (normal "lobe average" = 3.17 ± 0.25). (Rule of fives: When 100 neutrophils are counted, the presence of more than 5% containing 5 or more lobes means hypersegmentation.)
 - Morphologic red herrings: congenital hypersegmentation (approximately 1% of population), hypersegmentation with renal disease; twinning deformities; macrocytes of pyruvate kinase deficiency, aplastic anemia, reticulocytosis, hypothyroidism, neoplasia.
 - Fever
 - Icterus plus pallor (lemon-yellow skin)
 - Glossitis
 - Acute
 - Chronic atrophic
 - Neurologic damage (only proven in B₁₂ deficiency, which damages myelin)
 - Vibration sense diminished
 - Position sense diminished, ataxia, "combined systems disease"
 - Impaired mentation, paranoid ideation (seen in both deficiencies)
 - Malabsorption
 - Achylia gastrica (primary with B₁₂ deficiency, secondary with folate deficiency) (reduced intrinsic factor)
 - Splenomegaly (in approximately one third of cases, if looked for radiologically)
 - Weight loss (especially folate deficiency)
 - Pigmentation; vitiligo
 - Postural hypotension (especially B₁₂ deficiency)
 - Low serum vitamin B₁₂ or folate level
 - Elevated serum lactic dehydrogenase (LDH)
 - Elevated urine formiminoglutamate
 - Methylmalonic aciduria (B₁₂ deficiency only)
 - High serum iron, increased saturation of iron-binding capacity of serum, increased bone marrow iron stores, normal free erythrocyte protoporphyrin (findings that may obscure occult Fe deficiency)
 - Low red cell folate is present in either deficiency
 - Circulating antibody to intrinsic factor in two thirds of pernicious anemia patients
 - Circulating antibody to gastric parietal cells in most patients with gastric damage, regardless of cause
 - Abnormal "dU suppression test" (corrected by adding vitamin in vitro)
 - Abnormal liver function tests
 - Subnormal intestinal absorption
 - Low red cell B₁₂ or folate level; low lymphocyte B₁₂ or folate

is to precede the administration of blood by the parenteral injection of a diuretic.

When, for one of the reasons discussed previously, immediate vitamin therapy is necessary before etiologic diagnosis, 100 μ g of cyanocobalamin and 15 mg of folic acid are given intramuscularly and 5 mg of folic acid by mouth and 100 μ g of vitamin B₁₂ intramuscularly daily, for a week. Such treatment produces excellent hematologic response except in patients in whom hematopoiesis is suppressed by infection, uremia, chloramphenicol administration, or some other factor.

Vitamin B₁₂ Therapy

Vitamin B₁₂ deficiency in man is nearly always the result of inadequate ingestion and/or inadequate absorption, and therapy is guided by adequate etiologic diagnosis (see Table 21-1).

Inadequate ingestion of vitamin B₁₂ is corrected by adding to the daily diet any food containing vitamin B₁₂ (i.e., animal products, including meat, milk, fish, shellfish, or poultry). If the patient, because of poverty, cannot afford animal products or, for religious or other reasons, is a strict vegetarian, adequate treatment consists of 1 μ g of vi-

tamin B₁₂ orally supplied daily as a liquid or tablet or B₁₂-containing food supplement.

When the vitamin B₁₂ deficiency is the result of inadequate absorption, 1 µg of vitamin B₁₂ parenterally (subcutaneously or intramuscularly) daily constitutes adequate therapy. A single injection of 100 µg or more of vitamin B₁₂ produces a complete therapeutic remission in any patient whose deficiency is not complicated by unrelated systemic disease or other factors. The remission is sustained for life by monthly injections of 100 µg of vitamin B₁₂. It is important to inform the patient who has permanent gastric or ileal damage that he must receive monthly injections of vitamin B₁₂ for life.

For simultaneous differential diagnosis and therapy, patients should be treated with an injection of 1 µg of vitamin B₁₂ daily for 10 days, after a control period of a few days to establish the constancy of the reticulocyte level, and the elimination of dietary sources of vitamin B₁₂ and folic acid (by provision of a diet consisting exclusively of well-cooked finely particulate grains or vegetables, such as rice and beans, and beverages devoid of vitamin B₁₂ and folate, such as tea, coffee, and soft drinks).¹²⁹

Initial therapy with doses of vitamin B₁₂ greater than 1 µg daily is desirable when the vitamin B₁₂ deficiency is complicated by other debilitating illness such as infection, hepatic disease, uremia, coma, severe disorientation, or marked neurologic damage. In such patients, 30 µg or more of vitamin B₁₂ is given daily parenterally for 5 to 10 days. Daily parenteral doses larger than 30 µg have no proven therapeutic advantage, and much of the excess is rapidly excreted in the urine.¹³⁰

Vitamin B₁₂ can also be administered in a nasal gel, facilitating self-administration. Preliminary data indicate that nasal B₁₂ is more effectively absorbed than oral B₁₂ in normal individuals, that it is effective in B₁₂ malabsorption, and that it can be used to treat B₁₂-deficient patients.^{130a,b}

Hydroxocobalamin and other depot preparations of vitamin B₁₂ may be retained longer at the site of injection and in serum, but this possible slight therapeutic advantage over cyanocobalamin does not warrant their greater cost; they also may have undesirable side effects. More detailed discussion of vitamin B₁₂ preparations, routes of administration, dosage, and therapeutic responses may be found in *The Pharmacological Basis of Therapeutics*.³

Folic Acid Therapy

For combined differential diagnosis and therapy, the patient is treated with 100 µg of folic acid orally daily (if the suspected diagnosis is folate

malabsorption). This dosage produces a maximal hematologic response in patients with folate deficiency, but does not produce hematologic response in patients with vitamin B₁₂ deficiency.¹²⁹ As in treated vitamin B₁₂ deficiency, treatment of folate deficiency returns subnormal leukocyte and platelet levels to normal within a week after the start of therapy, at approximately the time of the reticulocyte peak.

Therapy with doses of folic acid larger than 0.1 mg daily is desirable when the folate deficiency state is complicated by conditions that may suppress hematopoiesis (such as unrelated systemic disease), conditions that increase folate requirement (pregnancy, hypermetabolic states, alcoholism, hemolytic anemia), and conditions that reduce folate absorption. Therapy should then consist of 0.5 to 1 mg daily. There is no evidence that doses greater than 1 mg daily have any greater efficacy; additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

Maintenance therapy is normally 0.1 mg of folic acid daily for one to four months, and then should be discontinued only if the diet contains at least one fresh fruit or fresh vegetable daily. If the daily folate requirement is increased owing to an increased metabolic or cell-turnover rate, the maintenance dose should be 0.2 to 0.5 mg daily.

Ideal nutritional therapy for dietary folate deficiency is the ingestion of one fresh fruit or one fresh vegetable daily. Such a diet would probably eliminate nutritional folate deficiency from the earth.¹³⁰⁻¹³² At present, nutritional folate deficiency probably encompasses approximately a third of all the pregnant women in the world.⁷⁴

PREVENTION OF FOLATE DEFICIENCY

It is the consensus that pregnant women should receive folate supplements,³ and they have been recommended in clinical disorders that increase the risk of folate deficiency. Major problems have been encountered in the delivery of such supplements to patients. Significant numbers of pregnant women do not ingest iron tablets given to them.¹³³ This problem may not be as severe when tablets containing both iron and folate are given, because a separate report suggests that the adverse gastrointestinal effects of iron ingestion may be decreased when folic acid is simultaneously ingested.¹³⁴ However, the iron in the supplement should not exceed the 30-mg iron daily pregnancy supplement recommended by the 1980-1985 RDA Committee.^{133a} Tablets containing 100 mg iron and 350 µg folate significantly interfere with the absorption of needed zinc.^{133b} The largest com-

ponent of the problem is that antenatal care is not available to, or taken advantage of, by large numbers of pregnant women, particularly in populations in which folate deficiency is common.¹³⁵ This phenomenon has again been highlighted by the observation that of 110 consecutive women seeking prenatal care in New York City, 61 were in the second trimester and 26 already in the third trimester of pregnancy.^{136,136a}

As an alternative approach to the alleviation of the problem, a series of studies was devised to determine the feasibility of fortifying staple foods with folic acid.^{60,135,137-140} When the data generated in these studies are judged against criteria delineated by an Expert Committee of the World Health Organization (WHO) and Food and Agricultural Organization (FAO),¹⁴¹ such fortification appears feasible, inexpensive, effective, and safe in populations with a demonstrable need for increased dietary levels of folic acid. It has been suggested that such elevations may increase the incidence and/or severity of neurologic damage in subjects with pernicious anemia, but the literature contains little data to support this view. This reservation thus seems minor when weighed against the primary object of food fortifications, namely, the elevation of dietary folate levels in large undernourished populations to amounts present in the diet of more affluent people who rarely suffer folate deficiency, i.e., to levels approaching the RDA. A joint meeting of the WHO and other bodies has recommended that authorities concerned with populations in which folate deficiency is common should initiate trials to determine the feasibility and effectiveness of food fortification with folate in those populations.¹⁴²

TOXICITY

There is no evidence that either vitamin B₁₂ or folic acid, in the human-active forms necessary for life, is toxic to humans in quantities close to minimal daily requirements. These substances in their human-active forms are nontoxic in man not only in small doses but also in doses that exceed the minimal daily adult human requirement by 10,000 times for vitamin B₁₂ and several hundred times for folic acid. Being water-soluble, excesses of these vitamins tend to be excreted in the urine rather than, like fat-soluble vitamins, being stored in tissues. Vitamin B₁₂ and folic acid both appear to require binding to polypeptides as a precondition of storage; excesses above the limited serum- and tissue-available-binding capacity tend to be excreted rather than retained.

A rare allergic reaction has been reported, possibly due to impurities in a rare preparation of crystalline cyanocobalamin.²⁵ Hydroxocobalamin

injections and injections of various depot preparations of cyanocobalamin have been associated with the appearance of antibody to plasma vitamin B₁₂-binding protein.¹²⁷ Although the significance of this antibody is not yet clear, such preparations offer no clear advantage over cyanocobalamin to warrant their use, especially in view of their greater cost and the pain on injection of some of the depot preparations. The pharmaceutical preparation, cyanocobalamin, is not metabolically active in humans. Tissue enzymes remove the cyanide to make the cobalamin active. The rare infant who is born with a congenital lack of this cyanide-removing mechanism can, of course, be harmed by cyanocobalamin, because for him it is an antimetabolite of vitamin B₁₂.^{142a}

The efficacy and safety of the vitamin B₁₂ analogues created by nutrient-nutrient interaction in vitamin-mineral supplements is unknown.^{32g}

One questionable instance of an allergic reaction to folic acid has been reported in man.² Daily doses of up to 15 mg in healthy humans without convulsive disorders are without known toxic effects; this daily dose is well below that which could lead to precipitation of crystalline folic acid in the kidneys (such precipitation produces renal toxicity in rats given massive doses of folic acid). Very large amounts of folic acid in its pharmaceutical oxidized form (pteroylglutamic acid) may be noxious to the nervous system and can reverse the antiepileptic effects of phenobarbital, diphenylhydantoin, and primidone, and have provoked seizures in patients otherwise under control on anticonvulsant therapy.^{121,144,144a,144b,144c}

Although no such effect has been observed in controlled studies using oral doses of 15 mg folic acid daily, experimental and clinical evidence suggests that very high concentrations of folic acid can have a convulsant effect.¹²¹ The convulsant dose in normal rats was 45 to 125 mg if administered intravenously and 15 to 30 mg if preceded by the induction of a focal cortical lesion.¹⁴⁵ Convulsions have been reported in one of eight epileptics given parenteral folic acid under electroencephalographic monitoring.¹⁴⁶ This reaction occurred after the rapid intravenous infusion of 14.4 mg folic acid, which presumably elevated serum folate concentration in the cerebral vessels several times higher than would be the case after folic acid ingestion.^{85,121} Anticonvulsants and folic acid compete with each other for absorption across the intestinal epithelial cells,^{146a} and probably also compete at the brain cell wall.^{107,144,144a,144b,144c} However, at present, despite evidence in uncontrolled studies suggesting increased fit frequency in epileptics given oral ther-

apeutic doses of folic acid, no such effect of oral folic acid has so far been demonstrated in carefully conducted controlled trials.

Oral folic acid supplements of 350 µg daily reduce zinc absorption and may be a problem where maternal zinc depletion and intrauterine growth retardation are common.^{91a,133b}

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