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BIOCHEMICAL ASSESSMENT OF VITAMINS AND TRACE METALS

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Modern biochemical methods for assessing nutritional status provide advantages of sensitivity, specificity, and objectivity as compared with gross clinical observations. Although nutritional biochemistry and metabolism are often seen as daunting to the clinical laboratorian, understanding several basic concepts will help in the selection and interpretation of nutritional assessment tests.

Nutrients partition throughout the body in patterns that are unique for a particular nutrient or class of nutrients. The form(s) and concentration of a nutrient in a particular tissue depend largely on its function. Various body compartments or "pools," e.g., plasma, leukocytes, or muscle, accumulate (require) different amounts of a nutrient, and deplete or replete at different rates. This means that a "static" test (simply measuring the *level* rather than the *function* of a nutrient) provides information on the degree of deficiency of the particular body pool sampled rather than the whole body status. Thus, over the course of developing folate deficiency, serum folate levels drop initially (within weeks), while red cell and tissue folate levels remain adequate. In the next stage, red cell and tissue folate levels decrease over a period of months. Continuing deficiency then results in overt clinical symptoms, i.e., megaloblastic anemia, which is nonresponsive to iron supplementation. Thus, biochemical tests theoretically can define the entire process of nutrient deficiency, from reduced body stores to clinical symptoms. This should be considered when interpreting, and sometimes when selecting, a nutritional assessment test. A complete profile of tests for assessing the various stages of nutrient deficiency are available for just a few nutrients, however, such as for iron and folic acid. The determination of whole-body nutrient pools provides a unified test and allows for simpler interpretations, but few practical methods for this currently exist.

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For clinical biochemistry assessment tests, interpretive guidelines, i.e., reference ranges, for interpreting nutritional tests, may depend on individual variables, such as age and sex. Often precise guidelines are not available for nutritional tests, however. Functional tests that monitor a biochemical or physiologic function, such as reduced B vitamin–dependent enzyme activities or poor dark adaptation due to vitamin A deficiency, have the advantage of being less confused by individual variables, such as age or gender. Few such tests are now available for use in the clinical laboratory, however. Providing biochemical tests that can define the entire breadth of nutrient deprivation, assess whole-body nutrient pools, and assess deficiency on the basis of functions are three important frontier areas for nutritional assessment research.

As is true for interpretive guidelines, relatively few standards and quality control materials are available for nutritional assessment tests compared with clinical tests. Often the analyst must rely on performing tests of analyte recovery from the specimen matrix, repeating the analysis of frozen aliquots from large sample pools to assess intraday and interday variance, using reference controls from healthy subjects, and sharing specimens with other laboratories to obtain comparative analyses.

The following sections provide brief descriptions of current methodologies for the biochemical assessment of vitamins and essential trace metal status. Other sources provide expansive and detailed coverage of nutritional biochemistry and metabolism, nutritional diagnostics, and interpretive guidelines.[5,29,31,34,39,40,47] The Tietz reference[47] provides information on chemical and clinical factors that affect or interfere with many of the established nutritional diagnostic tests.

WATER-SOLUBLE VITAMINS

Vitamins B_1, B_2, and B_6

Traditional tests for assessing B-vitamin status involve the determination of urinary excretion of the vitamin in a 24-hour collection, in creatinine-corrected random samples, or after an oral loading dose.[31,39,47] These methods, tedious to perform and often difficult to interpret, have been largely supplanted by HPLC techniques to determine blood vitamers and functional tests based on the degree to which a vitamin-dependent red cell enzyme activity is stimulated by in vitro addition of the vitamin cofactor to a red cell hemolysate.[4,31,51] In the latter type of test, an *activity coefficient* (AC) is calculated as the ratio of the rate of the vitamin-stimulated enzyme reaction to that of the nonstimulated reaction:

$$AC = \text{rate (stimulated)}/\text{rate (nonstimulated)}$$

Where a patient's red cells are deficient in vitamin cofactor, the nonstimulated rate is considerably smaller than the stimulated rate, and the AC becomes greater than unity.

Calculating the ratio of enzyme activity in a split hemolysate equals that of each test subject supplying their own internal control so that interpretation of results from these functional tests of B-vitamin status are less affected by patient variables, such as age and sex. The erythrocyte enzyme activity tests provide a better measure of long-term dietary vitamin intake and tissue vitamin stores, whereas urine and blood vitamer levels can be strongly influenced by recent dietary intakes. Because of metabolic conservation, urinary excretion of B vita-

mins generally falls off to minimal levels even with marginal deficiency; hence, urinary vitamin levels are not useful for discriminating between marginal and severe vitamin depletion.

Vitamin B_1 (Thiamin)

Thiamin levels in urine and blood can be determined after conversion of thiamin to the fluorescent compound thiochrome by treatment with potassium ferricyanide.[52] More recent HPLC methods permits the separate determination of thiamin and its phosphate esters.[3,38] Reference ranges for serum thiamin, whole blood total thiamin plus ester forms, and urine thiamin per 24-hour and per gram creatinine are tabulated.[47]

The erythrocyte transketolase stimulation test is a useful functional test that may be less prone to metabolic interferences and difficulties of interpretation than thiamin assays. The activity of erythrocyte transketolase, a thiamin-dependent enzyme, is determined in red cell hemolysates with and without added thiamin pyrophosphate.[42,46] Activity coefficients (as described previously) greater than 1.3 represent deficiency. Some variants of the procedure calculate a *percentage of stimulation* or *TPP effect* (normal is <25%), and a whole blood hemolysate has been suggested as a better sample.[6] The transketolase enzyme is particularly labile; therefore, blood samples should be kept cold and analyzed promptly.[36]

Vitamin B_2 (Riboflavin)

Measurement of urinary riboflavin and the erythrocyte glutathione reductase test are the two most effective laboratory tests currently available for assessing riboflavin status. Riboflavin and related flavins, including flavin mononucleotide and flavin adenine dinucleotide (FAD), are best determined in biologic specimens by fluorometric and HPLC-fluorometric methods.[25,31,47] Urinary riboflavin excretion in healthy adults should be greater than 266 nmol/d (100 μg/d), or greater than 19.8 μmol/mol creatinine (66 μg/g creatinine).[29] As for most water-soluble vitamins, the urinary excretion after an oral dose of the vitamin can be used to assess the extent of tissue or whole-body vitamin deficit. Adults excreting less than 1 mg of a 5 mg dose of riboflavin over 4 hours postdose are considered deficient.[31] Urinary riboflavin excretion, however, may be increased during fasting or in catabolic/inflammatory states often encountered in the clinical setting.

The erythrocyte glutathione reductase test provides an excellent functional test of riboflavin status that avoids some of the difficulties of urine riboflavin tests. The principle is similar to that of the erythrocyte transketolase assay for thiamin status: the activity of glutathione reductase, a riboflavin-dependent enzyme, is compared with and without exogenous stimulation with FAD, and AC, or percentage of stimulation, is calculated.[29,31] Generally, AC values greater than 1.3 (>30% stimulation) are considered to represent deficiency[29,31]; however, the upper limit of normal has also been listed as 1.4.[47] Reports of studies showing AC values greater than 1.3 along with paradoxically high urine riboflavin excretion[11] prompted Horwitt[16] to advise caution when interpreting AC values below 1.5 and to assess urinary riboflavin for confirmation. Direct measurements of riboflavin or other flavins in blood have not been shown to be reliable markers of riboflavin status.

Vitamin B_6 (Pyridoxine)

A greater variety of tests are available for assessing pyridoxine status as compared with thiamin and riboflavin. The tests reflect the complex and varied biochemistry of the vitamin and include direct measures of B_6 vitamers in blood and urine, amino acid load tests, and red cell enzyme activity tests.[27,41] Reference ranges for these tests are available,[9,27,39,47] but users should be aware that considerable variation in the ranges exist.

Plasma pyridoxal 5-phosphate (PLP) is one of the most useful measures of vitamin B_6 status. PLP is the principal active form of the vitamin and the major form in the plasma. Plasma PLP generally reflects dietary intake of the vitamin, but caution in its use has been suggested because its level may be affected by protein intake (inversely), drug ingestion, or other factors.[27] Plasma PLP values less than 20 nmol/L (5 ng/mL) represent deficiency.[47] HPLC methods for quantitating the major B_6 vitamers (pyridoxine, pyridoxal, pyridoxamine) and their phosphate esters have now been published,[10,37] but no other vitamers have as yet been established as reliable status markers as has judicious use of the PLP test. Another direct measure of B_6 status is the determination of the major urinary metabolite, 4-pyridoxic acid. This assay has the drawback of requiring a 24-hour urine collection and reflects primarily recent dietary intake rather than overall body/tissue status.

Erythrocyte enzyme activity tests, based on the B_6-dependent alanine (ALT or GPT) or aspartic acid (AST or GOT) transaminases, are commonly used measures of B_6 status. The methodology is fairly simple to implement, and the tests provide a time-integrated measure of B_6 status. Many versions of the methodology have been published,[27,31,41] including adaptions to automated chemistry analyzers.[41,51] Test results have been reported as activity coefficients, percentage of stimulation, or enzyme activity levels; hence some problems with standardization and interpretation of results exist. Some studies suggest that the erythrocyte ALT test is preferable to AST as a measure of B_6 status.[27]

Another function-based test of B_6 status, the tryptophan load test, measures the excess urinary excretion of xanthurenic acid due to the lack of B_6 cofactor required for tryptophan metabolism.[27,31] The test is sensitive and provides a measure of tissue B_6 deficit, but collection of a timed urine specimen precludes its use for population surveys, and tryptophan metabolism may be affected by factors other than B_6 nutriture, such as protein intake. The methionine load test has also been used as a functional measure of B_6 status, but considerably less often than the tryptophan load test.[27]

Folic Acid and Vitamin B_{12}

Folic Acid

Folic acid is one of the few nutrients whose nutritional status in humans can be defined from low dietary intakes and marginal deficiency to depleted tissue stores and clinical symptoms (megaloblastic anemia).[1,5,12,13] Serum folate is the most sensitive measure of early folate depletion of which values less than 7 nmol/L (3 ng/mL) represent a low dietary intake or negative folate balance (dietary intake is insufficient to make up for whole body losses). At the stage of tissue folate depletion, erythrocyte folate levels fall below 340 nmol/L (150 ng/mL). Although serum folate levels may change within weeks in response to

dietary intakes, red cell folate levels reflect the folate balance over several months. This is due to the approximately 3-month life span of red cells and the fact that folate is taken up primarily by the young red cells synthesizing DNA in the bone marrow. The final stage of folate deficiency produces megaloblastic anemia and hypersegmented neutrophils, which are described by Herbert[12,13] and hematology texts.

The deoxyuridine suppression test provides a measure of functional folate deficiency and can provide a distinction between folic acid and cobalamin (vitamin B_{12}) deficiencies. The test is based on the folate requirement for conversion of deoxyuridine to deoxythymidine. The suppression of radioactive thymidine incorporation into DNA in cultured bone marrow or blood lymphocyte cells by exogenously added deoxyuridine is less effective in folate deficiency. The test is not popular for routine clinical use, however, because it is tedious to perform while not offering diagnostic advantage over red cell folate measures. The histidine load test, based on the increased urinary excretion of formiminoglutamate after an oral histidine load, is not widely used because it is rather tedious and responds to vitamin B_{12} as well as folate deficiencies. More recently, reports of homocysteinemia in folate-deficient patients and in subjects with marginal serum folate levels (4.5–9 nmol/L) indicate that total plasma homocysteine can provide a functional measure of folate deficiency.[5] The remethylation of homocysteine to methionine is both cobalamin and folate dependent; therefore, homocysteinemia may result from cobalamin as well as from folate deficiency (and from inborn errors of folate or cobalamin metabolism). Levels of plasma total homocysteine in adults are normally in the range of 7 to 22 μmol/L (0.9–3.0 mg/L).[5] Factors that antagonize folate status include smoking and the use of alcohol and a wide variety of drugs.[47]

Folate chemistry is complex because of the many different molecular forms and the presence of polyglutamate-conjugated forms. Folic acid can be easily determined in blood plasma by competitive protein-binding radioassays, which are commercially available in kit form. Dual isotope kits are available that allow simultaneous determination of plasma folate and vitamin B_{12}. The biochemistry and metabolic functions of these two vitamins are closely linked; hence they should be evaluated together. Whereas plasma folate exists predominantly in the monoglutamate form, polyglutamate forms in red cells, tissues, and foods must be hydrolyzed before radioassay. Although more tedious to perform than the radioassay procedure, microbiologic assay using *Lactobacillus casei* remains as the reference technique, especially for the determination of tissue and food folate, which contain varied forms of the vitamin.[31,39]

Vitamin B_{12}

The determination of serum vitamin B_{12} levels provides the best test for routine screening of cobalamin status. Competitive protein-binding assays for serum vitamin B_{12} are simple to perform and commercially available. Levels less than 111 pmol/L (150 pg/mL) are considered to represent deficiency. Red cell B_{12} levels are also low during folate deficiency and therefore are not specific for cobalamin deficiency. Microbiologic assays for vitamin B_{12}, using *Lactobacillus leichmanii*, are also available but are considerably more tedious to perform than radioassays. The classic Schilling test diagnoses B_{12} malabsorption due to lack of the intrinsic factor. Urinary excretion of methylmalonic acid and homocysteine is greatly increased in B_{12} deficiency, but increases in methylmalonic acid are specific for B_{12} as opposed to folate deficiency.

Niacin

The choice of methods for assessing niacin status is relatively limited in that blood tests have not been well established, leaving measurement of urinary metabolites of niacin, N^1-methylnicotinamide and 2-pyridone, as the tests of choice.[20,31] Daily urine excretions of less than 10 μmol/d (1.5 mg/d) for either metabolite represent low dietary intake and deficient niacin status in adults.[20,21,47] Niacin metabolite excretion in children is dependent on age. Analogous to other water soluble vitamins, load tests based on decreased urinary excretion of niacin metabolites after an oral dose of nicotinamide or tryptophan have been suggested as measures of tissue niacin depletion.[21,39]

More recently, a drop in blood or erythrocyte NAD, the functional form of niacin, or a ratio of NAD/NADP of less than unity has been suggested as indicative of niacin deficiency.[20] No tests of niacin compounds in plasma have yet been established as reliable markers of deficiency; however, 2-pyridone has been suggested as possibly useful.[21]

Vitamin C (Ascorbic Acid)

As no functional indicators of ascorbic acid (AA) deficiency have been established, the determination of plasma or leukocyte AA remain as the methods of choice for assessing AA status. No practical tests of the AA body pool are available. The lack of increase in urine or serum AA levels after an oral AA load indicates AA-depleted tissue or body stores,[39] but the test is tedious to perform.

Plasma AA is the most popular test choice because it is easy to perform and generally provides a reliable measure of low AA dietary intake or deficient body status. Plasma AA is more sensitive to recent dietary intake than is leukocyte AA, which provides a better measure of tissue AA and the body pool. Generally, plasma AA is found to correlate with leukocyte AA levels.[18] The determination of leukocyte AA is more complex than plasma AA assay because of the heterogeneous nature of peripheral leukocytes and the need to take larger blood samples to harvest leukocytes. Different types of leukocyte cells and platelets vary greatly in their AA content, mononuclear cells containing severalfold higher levels than neutrophils. Various clinical and physiologic factors have been cited as affecting the relative proportions of the leukocyte cell distributions. Hence, assay of the buffy coat cell mixture, though simpler to perform, may reflect the particular distribution of cell types as well as AA status.[26] Significant contamination of an isolated cell fraction with platelets will increase the apparent AA level significantly. Reported ranges of AA in human blood cells have been summarized, but considerable variation exists due to the technical difficulties and other factors.[18] For leukocyte assay, the determination of AA in isolated fractions of mononuclear or polymorphonuclear neutrophil cells appears to be the best choice currently, with insufficient data available to recommend one over the other.

Many reliable analytic procedures for determining AA in biologic specimens are available.[18,19,31] Colorimetric methods based on the reducing property of AA and HPLC techniques that use electrochemical detection are most commonly used. The HPLC techniques have the advantage of sensitivity (which is most important when determining leukocyte AA levels) and of specificity. The HPLC methods allow specific determination of the reduced (ascorbic acid) or oxidized (dehydroascorbic acid, or DHAA) forms of the vitamin. Both forms are

Table 1. GUIDELINES FOR INTERPRETING BIOCHEMICAL MEASURES OF ASCORBIC ACID STATUS*

	Plasma (μmol/L)†	Mixed Leukocytes (nmol/10^8 cells)‡	Mononuclear Leukocytes (nmol/10^8 cells)
Adequate	> 23	> 114	> 142
Low	11.4–23	57–114	114–142
Deficient	<11.4	<57	<114
Normal Range	23–84	114–301	142–250

*Upper end of ranges may be higher in subjects taking vitamin C supplements.
†μmol/L ÷ 56.8 = mg/dL.
‡ nmol/10^8 cells ÷ 5.68 = μg/10^8 cells; mixed leukocytes are buffy coat or mixed cell fraction containing neutrophils and mononuclear cells; 10^8 cells approximately 100 μL.

biologically available and plasma ascorbate exists almost entirely in the reduced form. Whether an appreciable fraction of ascorbate in leukocytes exists in vivo as DHAA is not known currently. The colorimetric method using dinitrophenylhydrazine measures total ascorbate (reduced and oxidized forms) and is recommended as a reliable procedure; however, this method has not been successfully automated. Other methods, such as the colorimetric method using dichloroindophenol and certain HPLC methods, assay only the reduced form.

Because AA in biologic specimens is easily oxidized, it is most important that the vitamin be stabilized by the addition of preservatives soon after specimen collection. Acids, such as metaphosphoric acid and trichloroacetic or perchloric acid, or reducing agents, such as dithiothreitol, are added depending on the method.

Guidelines for interpreting plasma and leukocyte AA levels are listed in Table 1. Women generally carry higher plasma AA levels than men, and smokers have lower levels than nonsmokers. No definitive relationship between age and plasma AA levels has been established for adults.[19] The relations of various individual factors to leukocyte AA levels is not well known at present.

FAT-SOLUBLE VITAMINS

Vitamin A

Serum retinol is the most commonly used measure for assessing vitamin A status, but because the serum retinol level is homeostatically regulated, it does not fall to low levels until liver stores of the vitamin are nearly exhausted.[31,48] Hence, the test does not differentiate vitamin A status over a wide range of nutriture from marginally deficient to near tissue saturation. The test is most useful for detecting overt tissue depletion of vitamin A in individuals (uncommon in the United States population) and for assessing the prevalence of vitamin A deficiency in populations. Serum retinol values less than 0.70 μmol/L (20 μg/dL) generally indicate low dietary intake and tissue stores of the vitamin, whereas values between 0.70 and 1.05 μmol/L are indeterminate and warrant further investigation.[47] Guidelines for interpreting serum retinol levels within sex- and age-specific subgroups are not well defined. Plasma retinol-binding protein levels correlate strongly with retinol levels and may be used as an alternative to retinol assays if HPLC instruments are not available. Plasma RBP

levels can be determined easily with commercially available radial immunodiffusion plates. The reference range for plasma RBP in healthy adults is 30 to 60 mg/L.[47]

A variety of analytic methods for determining serum retinol are available[31,47]; however, the traditional spectrophotometric and fluorometric methods have been largely supplanted by HPLC methods, which provide increased specificity and sensitivity. Many recent HPLC methods also provide the advantage of simultaneous quantitation of many fat-soluble vitamins and forms of the vitamins, including retinoids, tocopherols, and carotenoids.[14,17,31,47,49]

Serum retinyl esters (primarily retinyl palmitate) can also be determined through HPLC techniques, and this test has been suggested to be useful for diagnosing vitamin A toxicity, e.g., values exceeding 11 μg/dL of retinyl esters or greater than 10% of total circulating vitamin A indicating excessive liver stores of the vitamin.[31,47] The determination of serum or tissue *carotenoids* is not useful as a measure of vitamin A status because the conversion of specific carotenoids into retinol is variable and poorly understood.

The relative dose-response (RDR) test is based on the increased ability of the liver to mobilize vitamin A for peripheral tissue needs when vitamin A stores of the liver are low.[48] Hence, the test is useful as a measure of marginal as well as severe vitamin A deficiency. Serum or plasma retinol is measured before, and at 5 hours after, an oral dose of 450 to 1000 μg of retinyl palmitate. The RDR is calculated as:

$$\%RDR = \text{vit } A_{5hr} - \text{vit } A_{0hr} / \text{vit } A_{5hr} \times 100$$

where vit A_{5hr} is the serum retinol level 5 hours after dosing and vit A_{0hr} is the level just before dosing. An RDR value of greater than 20% indicates low hepatic stores of vitamin A. In the clinical setting, conditions of liver disease or severe protein malnutrition may interfere with test results. Recently, a modified RDR test that doses with vitamin A_2 (3,4-didehydroretinol) has been reported.[48] This test has the advantage of requiring only a single blood sampling at 5 hours postdose, but is not widely used now because the vitamin A_2 compound is not readily available. Other functional measures of vitamin A status include testing for night blindness or poor dark adaptation, and conjunctival impression cytology.[48]

Vitamin E

The method of choice for assessing vitamin E status is determination of plasma tocopherol. The preferred measure is the ratio of vitamin E to total plasma lipids because tocopherols are transported with the plasma lipoproteins. A ratio of less than 0.8 mg vitamin E per gram total lipid or less than 12 μmol/L (0.5 mg/dL) of plasma vitamin E is considered to represent deficient status.[33,47] The many HPLC techniques that allow specific determination of tocopherols as well as other fat-soluble vitamins have largely replaced older spectrophotometric and fluorometric methods for determination of plasma vitamin E.[14,17,31,49] Vitamin E is functionally active in erythrocytes and platelets, but it has not been shown that tocopherol assay of these samples provides a significantly better measure of vitamin E status than assay of plasma.

Several vitamin E assessment methods based on the oxidative instability of lipid-rich membranes during vitamin E deficiency are available.[31] The most popular among these is the erythrocyte fragility test in which hemolysis of greater than 20% of red cells after exposure to hydrogen peroxide is considered

to represent vitamin E deficiency. Such tests are basically measures of membrane stability and may be affected by factors other than tocopherol status, such as membrane composition and other antioxidant or prooxidant compounds.

Vitamin D

Determination of serum 25-hydroxyvitamin D (25-OH-D) is the most useful measure for assessing vitamin D status.[15] Because its circulating half-life is about 3 weeks, the serum level of this vitamer represents vitamin D nutriture from diet and photoformation over a period of weeks to months. Measurements of the separate forms, 25-OH-D_2 (ergocalciferol) and 25-OH-D_3 (cholecalciferol), does not seem to provide any advantage over assay for total 25-OH-D. Serum values of 25-OH-D below 25 nmol/L (10 ng/mL) are considered to represent deficient vitamin D status, whereas values above 375 nmol/L usually reflect vitamin D toxicity with attendant hypercalcemia and hyperphosphatemia.[15] Serum 25-OH-D can be determined by competitive protein-binding techniques applied to lipid extracts. Specificity can be increased by the use of chromatographic techniques before metabolite determination.

The physiologically active form of vitamin D, 1α,25-dihydroxyvitamin D, 1,25$(OH)_2$D, has a serum half-life of 4 to 6 hours and a concentration several orders of magnitude below that of 25-OH-D. This metabolite's dynamic response to vitamin D intake and metabolism makes it a poor marker of body vitamin D status, but the assay is useful for evaluation of patients with a variety of acquired or inborn disorders that affect vitamin D metabolism.[15] The metabolite can be determined in serum by cartridge chromatography of a lipid extract followed by a competitive protein-binding assay. Assays for both 25-OH-D and 1,25$(OH)_2$D are available from commercial diagnostic laboratories and as commercial assay kits.

Vitamin K

The term *vitamin K* refers to certain naturally occurring hydroquinone compounds that are required for γ-carboxylation of protein-bound glutamate residues in blood clotting and several other body proteins.[45] Two biologically active forms are phylloquinone from green plants and menaquinones, synthesized by bacteria. Direct assay of vitamin K compounds in blood has been difficult because of the molecular similarity of forms (K_1, K_2, K_3) and low circulating concentrations. Hence, status has traditionally been assessed by clotting function tests, such as prothrombin time, and the normalization of clotting activity after the administration of vitamin K. Clotting function tests are insensitive and nonspecific measures of vitamin K status however. Improved HPLC techniques have allowed direct quantitation of phylloquinone in plasma and the observation that most individuals have postabsorption values of less than 2 nmol/L (0.9 ng/mL).[45] The relation of plasma phylloquinone to vitamin K intake and status is currently not well defined, however. Immunochemical determination of an undercarboxylated form of prothrombin, des-γ-carboxy prothrombin, in plasma has been suggested as a sensitive marker of vitamin K deficiency.[45] Direct measures of vitamin K forms in blood are expected to replace clotting assays as analytic methodology advances and as the relationships between circulating vitamer levels and body status and intakes are established.

TRACE METALS

Zinc

The essentiality of zinc for growth and well-being of both plants and animals is well established. The metabolic functions of zinc are based largely on its presence as an essential component of over 300 metalloenzymes involved in virtually all aspects of metabolism. Important zinc-containing enzymes in humans include carbonic anhydrase, alkaline phosphatase, RNA and DNA polymerases, thymidine kinase, carboxypeptidases, and alcohol dehydrogenase. For detailed information on the metabolic interactions of zinc refer to recent reviews.[7,35]

Useful tests for assessing zinc nutriture include determinations of the zinc contents of plasma or serum, blood cells, urine, and saliva; the measurement of activities of zinc-containing enzymes; and the assessment of taste acuity. Although these indices have been shown to decrease with zinc deficiency, *no single test has been proven to be a definitive indicator of zinc status*. Test results must be interpreted with caution because they may be confounded by clinical conditions unrelated to the patient's zinc status per se.

The accepted reference range for zinc in plasma is 10.7 to 18.4 μmol/L (70–120 μg/dL). Fasting morning values below 10.7 μmol/L suggest marginal or deficient zinc status. Serum zinc values are generally 5% to 15% higher than plasma. Other conditions that depress plasma zinc include nonfasting states, infection, inflammation, administration of steroids, pregnancy, and hypoalbuminemic conditions, such as hepatic cirrhosis and malnutrition.

Decreased urinary zinc excretion usually accompanies human zinc deficiency. The reference range for urinary zinc excretion is 2.3 to 15.3 μmol/d (0.15–1.00 mg/d). Some conditions associated with zinc deficiency, such as hepatic cirrhosis, high alcohol intake, sickle cell anemia, postsurgical periods, and total parenteral nutrition, often give rise to increased urinary zinc excretion.

The determination of plasma or serum zinc concentrations by atomic absorption spectrophotometry (AAS) is the simplest and, analytically, most reliable test for the routine assessment of zinc nutriture. Colorimetric methods are available for those laboratories without an AAS instrument and produce results comparable to those obtained by AAS.[22] An AAS method using fivefold diluted plasma and standards in 5% glycerol matrix requires 0.5 mL of a specimen and is recommended for plasma or serum zinc determination.[43] Hemolysis must be avoided during sample acquisition and preparation because erythrocytes contain at least ten times more zinc than plasma. Plasma specimens may be stored in the refrigerator for several days but should be frozen at –20°C for long-term storage.

Urine specimens should be collected acidified (10 mL of concentrated HCl per 24-hour collection) to preserve the sample and to maintain metal solubility. Urinary zinc can be determined by direct aspiration of the acidified urine into the AAS flame. A sample background correction mode should be used because of the high salt content of the urine.

Copper

The major functions of copper metalloproteins involve oxidation-reduction reactions. Most known copper-containing enzymes bind and react directly with

molecular oxygen. Reviews that detail the biochemical and clinical aspects of human copper nutrition are available.[8,32]

Reference ranges for serum copper are age and sex dependent. They are higher during pregnancy, 18.9 to 47.2 μmol/L (120–300 μg/dL), in children 6 to 12 years, 12.6 to 29.9 μmol/L (80–190 μg/dL), and in women 12.6 to 24.4 μmol/L (80–155 μg/dL) than in men, 11.0 to 22.0 μmol/L (70–140 μg/dL) and in infants, 3.1 to 11.0 μmol/L (20–70 μg/dL). Lower concentrations may be indicative of severely depleted copper stores. Circulating copper levels, however, are sensitive to factors not directly related to copper nutriture. Plasma copper concentration has a diurnal variation, with peak values found in the morning. Pregnancy, birth control pills, and infections or inflammatory conditions increase plasma or serum copper, whereas corticosteroids and corticotropin tend to lower serum copper. Thus, conditions that change serum copper levels may obscure changes in copper status.

Ceruloplasmin, a copper-containing protein, accounts for over 95% of the copper found in plasma. Thus, it is sensitive to the same factors that affect plasma copper. Enzymatically measured ceruloplasmin has been shown to be a sensitive indicator of copper status in several species of animals. It was also significantly depressed in men and women fed diets low in copper. The reference range of 200 to 600 mg/L varies slightly depending on the method used and parallels the reference ranges for copper noted above.

Measurement of the enzyme activities of the cuproenzymes erythrocyte superoxide dismutase and cytochrome c oxidase in platelets or leukocytes can also provide useful information in assessing copper status. The activities of both of these enzymes are significantly reduced in copper deficiency and were lower in some adults fed diets low in copper. Nevertheless, both of these procedures are tedious and are method and technique sensitive.

AAS after direct dilution is the method of choice for determining plasma or serum copper. For laboratories without an AAS instrument, colorimetric methods are available, some of which have been adapted for the centrifugal analyzer.[24] Serum ceruloplasmin can be determined by a colorimetric oxidase reaction or by radial immunodiffusion.

Selenium

Selenium helps defend the body against oxidant stress and is involved in the metabolism of thyroid hormones. The existence of a number of selenoproteins has been demonstrated. Glutathione peroxidase and thyroxine 5'-deiodinase, however, are the only characterized selenoproteins with clearly defined functions. Detailed information on the metabolic interactions of selenium has been summarized.[28]

The determinations of urinary and blood selenium are useful measures of human selenium status. Plasma or serum selenium concentration may be more sensitive to selenium status than whole-blood concentration. Hair selenium concentrations were found to correlate with blood concentrations and were useful in assessing the risk of selenium deficiency in China; however, the test is not useful in populations in which the hair is exogenously contaminated with spoat selenium, such as from antidandruff shampoos. The measurement of plasma or erythrocyte glutathione peroxidase activity is useful primarily for confirming frank spoat selenium deficiency.

Selenium concentrations in specimens from healthy adults in the United States range from 0.74 to 2.97 μmol/L (58–234 ng/mL) in whole blood, 0.58 to

1.82 μmol/L (46–143 ng/mL) in serum or plasma, 2.5 to 17.8 nmol/g (0.2–1.4 μg/g) in hair, and 0.1 to 2.0 μmol/L (7–160 μg/L) excretion in urine.[47] Blood and tissue selenium levels vary with the selenium status of the particular geographic area. Low blood-selenium concentrations have been observed in patients with gastrointestinal cancer, during pregnancy, and with protein-calorie malnutrition, whereas high values have been reported in patients with reticuloendothelial neoplasia.

Recommended methods for determining selenium in biologic specimens are flameless AAS, with either deuterium arc or Zeeman effect background correction,[30] and spectrofluorometry.

Other Trace Metals—Chromium, Manganese, and Molybdenum

Chromium

The clinical significance of chromium rests primarily on its relation to glucose metabolism. Evidence for human chromium deficiency is mostly indirect and based on the improvement of insulin-resistant glucose intolerance after supplementation with chromium. Deficiencies of chromium have been documented in two cases of total parenteral feeding. Reviews covering the biochemical and clinical aspects of chromium nutrition are available.[44]

No laboratory tests that reliably define body chromium have been established because the determination of the very low chromium concentrations in tissues and in fluids is most difficult. AAS with a graphite furnace and Zeeman background correction is the preferred method for estimating chromium in biologic material.[50]

Manganese

This metal is associated with the formation of connective and bony tissue, with growth and reproductive functions, and with carbohydrate and lipid metabolism. Manganese is accepted as essential for humans mainly on the basis of its role in manganese-dependent enzymes and on the production of manganese deficiency in experimental animals.[23]

As with chromium, laboratory tests that reliably assess body manganese have not been established. The most commonly used index is either serum or whole-blood manganese concentrations. Acceptable adult reference ranges for manganese in blood are 7.0 to 20.0 nmol/L (0.4–1.1 ng/mL) for serum or plasma and 140 to 220 nmol/L (7.7–12.1 ng/mL) for whole blood. Flameless AAS with Zeeman-effect background correction is recommended for manganese determinations because of the low levels of manganese in blood and urine specimens.[2]

Molybdenum

The essentiality of molybdenum in animals and humans is based on its incorporation into xanthine oxidase, aldehyde oxidase, and sulfite oxidase. No well-defined cases of human molybdenum deficiency have been reported. Current methods for molybdenum determination in biologic specimens include emission spectroscopy, neutron activation analysis, and AAS.

SUMMARY

The choice of nutritional assessment tests must be made with care, as the available tests may reflect recent dietary intakes or body tissue stores to different degrees. The interpretation of nutritional assessment tests is complicated by the dynamic nature and broad range of nutrient intake and metabolism and by a relative lack of age- and sex-specific reference ranges. The latter problem can be minimized by the use of function-based methods, such as the measurement of vitamin-dependent red cell enzyme activities to assess B vitamin status; however, few suitable function-based methods are now available, and more research in this area is needed. Because calibration standards and quality control materials may not be readily available, laboratory analysts planning nutritional assessment testing must be especially careful in establishing the credibility of assays and the quality control program.

Recent advances in analytic methodology include the specificity offered by HPLC techniques and methodologies that provide simultaneous determination of more than one nutrient (or form of nutrient) in a single procedure. Examples of the latter include HPLC methods that provide B vitamer or fat-soluble vitamin profiles, and emission spectrometry techniques that provide trace metal profiles. Further work in providing useful nutritional assessment profiles and convenient automated methods is needed.

References

1. Bailey LB: Folate status assessment. J Nutr 120:1508, 1990
2. Baruthio F, Guillard O, Arnaud J, et al: Determination of manganese in biological materials by electrothermal atomic absorption spectrometry: A review. Clin Chem 34:227, 1988
3. Bettendorff L, Grandfils C, de Rycker C, et al: Determination of thiamine and its phosphate esters in human blood serum at femtomole levels. J Chromatog 382:297, 1986
4. Bötticher B, Bötticher D: A new HPLC-method for the simultaneous determination of B_1-, B_2-, and B_6-vitamers in serum and whole blood. Int J Vitam Nutr Res 57:273, 1987
5. Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990
6. Buttery JE, Pannall PR: Kinetic transketolase assay: Use of whole-blood hemolysate as the sample. Clin Chem 31:1086, 1985
7. Cousins RJ, Hempe JM: Zinc. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 251
8. Danks DM: Copper deficiency in humans. Ann Rev Nutr 8:235, 1988
9. Driskell JA, McChrisley B: Plasma B-6 vitamer and plasma and urinary concentrations in young women as determined using high performance liquid chromatography. Biomed Chromatogr 5:198, 1991
10. Edwards P, Liu PKS, Rose GA: A simple liquid-chromatographic method for measuring vitamin B_6 compounds in plasma. Clin Chem 35:241, 1989
11. Fu-Liu CS, Fujitaki C, Lewis JS: Riboflavin status: Dietary intake, urinary excretion, and erythrocyte glutathione reductase coefficient activity of female university students. Nutr Res 6:601, 1986
12. Herbert V: Making sense of laboratory tests of folate status: Folate requirements to sustain normality. Am J Hematol 26:199, 1987
13. Herbert V: The 1986 Herman Award Lecture. Nutrition science as a continually unfolding story: The folate and vitamin B_{12} paradigm. Am J Clin Nutr 46:387, 1987

14. Hess D, Keller HE, Oberlin B, et al: Simultaneous determination of retinol, tocopherols, carotenes, and lycopene in plasma by means of high performance liquid chromatography on reversed phase. Int J Vitam Nutr Res 61:232, 1991
15. Holick MF: The use and interpretation of assays for vitamin D and its metabolites. J Nutr 120(11S):1464, 1990
16. Horwitt MK: Reply to letter by Bates. Am J Clin Nutr 46:123, 1987
17. Ito Y, Ochiai, J Sasaki R: Serum concentrations of carotenoids, retinol, and alpha-tocopherol in healthy persons determined by high-performance liquid chromatography. Clin Chim Acta 194:131, 1990
18. Jacob RA: Assessment of human vitamin C status. J Nutr 120(11S):1480, 1990
19. Jacob RA, Skala JH, Omaye ST: Biochemical methods for assessing vitamin C status of the individual. *In* Livingston GE (ed): Nutritional Status Assessment of the Individual. Trumbull, CT, Food & Nutrition, 1989, p 323
20. Jacob RA, Swendseid ME: Niacin. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 163
21. Jacob RA, Swenseid ME, McKee RW, et al: Biochemical markers for assessment of niacin status in young men: Urinary and blood levels of niacin metabolites. J Nutr 119:591, 1989
22. Johnson DJ, Djuh YY, Bruton J, et al: Improved colorimetric determination of serum zinc. Clin Chem 23:1321, 1977
23. Keen CL, Zidenberg-Cherr S: Manganese. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 279
24. Kossman KT: Copper in serum measured with the Cobas-Bio centrifugal analyzer. Clin Chem 29:578, 1983
25. Lambert WE, Cammaert PM, De Leenheer AP: Liquid-chromatographic measurement of riboflavin in serum and urine with isoriboflavin as internal standard. Clin Chem 31:1371, 1985
26. Lee W, Davis KA, Rettmer RL, et al: Ascorbic acid status: Biochemical and clinical considerations. Am J Clin Nutr 48:286, 1988
27. Leklem JE: Vitamin B-6: A status report. J Nutr 120(11S):1503, 1990
28. Levander OA, Burk RF: Selenium. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 268
29. Livingston GE (ed): Nutritional Status Assessment of the Individual. Trumbull, CT, Food & Nutrition, 1989
30. Morisi G, Patriarca M, Menotti A: Improved determination of selenium in serum by Zeeman atomic absorption spectrometry. Clin Chem 34:127, 1988
31. Morrow FD, Sahyoun N, Jacob RA, et al: Clinical assessment of the nutritional status of adults. *In* Linder MC (ed): Nutritional Biochemistry and Metabolism with Clinical Applications, ed 2. New York, Elsevier, 1991, p 391
32. O'Dell BL: Copper. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 261
33. Olson JA: Assessment of vitamins A, D, E, and K. *In* Livingston GE (ed): Nutritional Status Assessment of the Individual. Trumbull, CT, Food & Nutrition, 1989, p 275
34. Olson JA (ed): Nutrition Monitoring and Nutrition Status Assessment (Conference Proceedings). J Nutr 120 (11S): 1990
35. Prasad AS: Clinical manifestations of zinc deficiency. Ann Rev Nutr 5:341, 1985
36. Puxty JAH, Haskew AE, Ratcliffe JG, et al: Changes in erythrocyte transketolase activity and the thiamine pyrophosphate effect during storage of blood. Ann Clin Biochem 22:423, 1985
37. Sampson DA, O'Connor DK: Analysis of B_6 vitamers and pyridoxic acid in plasma, tissues, and urine using high performance liquid chromatography. Nutr Res 9:259, 1989
38. Sander S, Hahn A, Stein J, et al: Comparative studies on the high-performance liquid chromatographic determination of thiamin and its phosphate esters with chloroethylthiamine as an internal standard using pre-column and post-column derivatization procedures. J Chromatogr 558:115, 1991

39. Sauberlich HE, Dowdy RP, Skala JH: Laboratory Tests for the Assessment of Nutritional Status. Boca Raton FL, CRC Press, 1974
40. Shils ME, Olson JA, Shike M (eds): Modern Nutrition in Health and Disease, ed 8. Philadelphia, Lea & Febiger, 1993
41. Skala JH: Biochemical criteria for evaluating vitamin B_6 nutritional status. *In* Livingston GE (ed): Nutritional Status Assessment of the Individual. Trumbull, CT, Food & Nutrition, 1989, p 313
42. Smeets EHJ, Muller H, DeWael J: A NADH-dependent transketolase assay in erythrocyte hemolysates. Clin Chim Acta 33:379, 1971
43. Smith JC Jr, Butrimovitz GP, Purdy WC: Direct measurement of zinc in plasma by atomic absorption spectroscopy. Clin Chem 25:1487, 1979
44. Stoeker BJ: Chromium. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 287
45. Suttie JW: Vitamin K. *In* Brown ML (ed): Present Knowledge in Nutrition, ed 6. Washington DC, International Life Sciences Institute, Nutrition Foundation, 1990, p 122
46. Takeuchi T, Nishino K, Itokawa Y: Improved determination of transketolase activity in erythrocytes. Clin Chem 30:658, 1984
47. Tietz NW (ed): Clinical Guide to Laboratory Tests, ed 2. Philadelphia, WB Saunders, 1990
48. Underwood, BA: Methods for assessment of vitamin A status. J Nutr 120(11S):1459, 1990
49. Vanvliet T, Vanschaik F, Vanschoonhoven J, et al: Determination of several retinoids, carotenoids, and E vitamers by high-performance liquid chromatography—application to plasma and tissues of rats fed a diet rich in either beta-carotene or canthaxanthin. J Chromatogr 553:179, 1991
50. Veillon C, Patterson KY, Bryden NA: Determination of chromium in human serum by electrothermal atomic absorption spectrometry. Anal Chim Acta 164:67, 1984
51. Vuilleumier JP, Keller HE, Keck E: Clinical chemical methods for the routine assessment of the vitamin status in human populations. 3. The apoenzyme stimulation tests for vitamin B_1, vitamin B_2, and vitamin B_6 adapted to the Cobas-Bio analyzer. Int J Vitam Nutr Res 60:126, 1990
52. Waldenlind L: An improved method for the determination of endogenous thiamin and its phosphate esters in biological material. Nutr Metab 23:38, 1979

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