

LABORATORY ASSESSMENT OF NUTRITIONAL STATUS

with Emphasis on Immunological Measurements

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The ability to determine nutritional status is becoming more and more important as world population expands and food supply diminishes. Although the field of nutritional metabolism and physiology has achieved a high degree of sophistication, experts continue to be hampered by the lack of a single battery of tests which can be applied to populations throughout the world and in all environmental conditions. The ideal set of determinations would be one where all the tests are easily corrected for confounding variables. Since, however, a standardized human environment does not exist, it will probably never be possible to develop an ideal set of tests for the evaluation of nutritional status. Multiple factors are involved in the production of states of malnutrition; hence, a multidisciplinary approach is essential for the proper evaluation of each malnourished individual. Moreover, he should be compared with standards derived by testing normal members of his own population. This is rarely done. If a person is removed from his environment or if any form of treatment is given prior to testing him, his resulting nutritional "profile" will be biased beyond tolerable limits. Nutrient deficiencies in human populations are almost invariably multiple and they may affect any organ system of the body including the immune system, depending upon the specific deficiencies. Deficiency of one nutrient frequently alters the metabolism of another. The process of adaptation may alter or even obscure the manifestations of deficiencies which are real.

It is possible, despite all the above difficulties, rather accurately to evaluate human nutritional deficiency if one maintains a broad overview of the problems and correlates the results with each other. Tests are available which measure many deficiencies with great accuracy. Appraisal of data by a team of experts: clinicians, biochemists, physiologists, immunologists, epidemiologists, food technologists, sociologists, anthropologists, psychologists, economists, and statisticians - continues to improve our basic understanding of nutritional problems and has led to a greater degree of operational success in alleviating malnutrition. Although nutrient deficiencies are usually multiple, they commonly occur in well-recognized patterns, permitting a certain degree of selectivity in evaluational testing. Advances in the knowledge of nutritional metabolism also facilitates testing for the interactions involved when depletion of a specific nutrient affects metabolic pathways other than its own.

I shall attempt to present a brief survey of

some of the more important biochemical and immunological tests for the assessment of human nutritional status. Unfortunately, space does not permit a commentary upon clinical evaluation, which should always constitute the first approach to the problem. By examining the subject clinically, one can usually get a general impression of the combination of nutrient deficiencies present, and can then pinpoint specific ones through biochemical and other tests. For example, prolonged niacin deficiency may result in the classical clinical stigmata of pellagra; dermatitis, diarrhea, and dementia. Since most low meat diets are also low in all the "B" vitamins, clinical pellagra would alert the examining physician to look for signs and symptoms of deficiencies of the other "B" vitamins, such as the cheilosis, etc. of riboflavin deficiency and the peripheral neuropathy of thiamine deficiency. Deficiencies of all these nutrients could then be assessed biochemically. Populations which consume corn as a staple food frequently display a high incidence of pellagra, as was detected in the Southeastern United States during the early part of this century.

Retardation of growth rate is the most common physical manifestation of malnutrition, and usually reflects combined nutrient deficiencies, protein being most important. Signs of deficiencies of nutrients other than protein often confuse the clinical picture, but it is possible to solve this problem by doing biochemical tests (Tables I and II) for the suspected specific deficiencies. Seven important tests of protein deficiency are described in Table I. Facilities and available personnel are the usual factors in determining which tests are done. Most of these tests have been adapted for field use, so that samples can be rather easily collected and sent to a central laboratory for processing. The comments on each test given in the tables should indicate the feasibility of its performance in a given situation. To obtain an accurate biochemical profile of protein malnutrition, one should perform tests for: (1) plasma proteins (Table I, test 1); (2) basal urea nitrogen excretion, the best of which is urea nitrogen/creatinine (Table I, test 5); (3) body protein mass (Table I, test 6); and (4) the pattern of plasma amino acids (Table I, test 7). It should be stressed that individual results must always be compared with standards generated from the same population, and that the recent past history of the subject must be known, especially with respect to diet. For example, a low blood ascorbic acid level indicates that the tested individual absorbed very

TABLE I.

BIOCHEMICAL EVALUATION OF PROTEIN MALNUTRITION (PM)

some appropriate tests

<u>TEST</u>	<u>NORMAL RANGES</u> for U. S. population	<u>COMMENTS</u>
1. Plasma proteins (total; albumin, alpha, beta, and gamma globulins)	Total 6.5-6.9g/100ml Alb. 3.5-4.2g/100ml Alpha -1 4-7 Alpha -2 9-11 %of pl. Beta 11-15 protein Gamma 12-16	Levels of serum proteins only informative in severe P. M. Thus these tests only detect gross deficiency. Various methods are available for these determinations, some of which require simple apparatus.
2. Hemoglobin and/or Hematocrit	Hb Men 14-15g/100ml Hb Women 11-14g/100ml Hct Men 42-44% Hct Women 38-42%	Although anemia is usually present in moderate or severe PM, its diagnosis does not indicate causation. The anemia associated with protein-calorie malnutrition is of a mixed and complicated etiology, and may involve deficiencies of iron, protein, folic acid, vit. B ₁₂ , vit. E, and even selenium.
3. Urea excretion (urine)	Urea is the principal end product of protein metabolism in mammals. Its excretion is directly related to protein intake, but the amount of urea excreted must be expressed relatively, since its absolute amount in the urine is meaningless. For example, if a single high-protein meal is given to a protein-malnourished subject the night before his timed urine specimen is collected, the absolute amount of urea excreted might well be normal.	
4. $\frac{\text{Urea N}}{\text{Total N}}$ (urine)	Normally, urea comprises 80-90% of total urinary nitrogen. (Total urinary N about 13 g / day; urea N about 11 g / day). However, because even in PM urea N. comprises most of the total urinary N. changes in the numerator are not easily detectable in PM.	
5. $\frac{\text{Urea N}}{\text{creatinine}}$ (urine)	This test is better than 4. because changes in the numerator are more perceptible. Creatinine N comprises a small percentage of total urinary N and tends to remain fixed except in severe stages of muscle catabolism. To use this test, must restrict water intake, because urea clearance is directly related to protein intake only at minimal urine volume. Approximate ratios if water is restricted are: Normal: about 20. PM: 7-9.	
6. Creatinine-height index (C H I)	This is a modification of the creatinine coefficient used in well-nourished populations. The usual creat. coeff. is creat. excreted/kg. body wt. /24 hr., and in "rich" areas is an index of obesity, among other things. In protein malnourished populations, obesity is almost non-existent, and body height is much more meaningful than body weight. The CHI of Dr. Viteri is expressed as follows: $\frac{\text{mg. creat. exc'n. /cm. ht. /24hr.}}{\text{mg. creat. exc'n. /cm. ht. /24hr.}}$ (in person tested) regardless of age	This test is a useful measure of decrease in body protein mass. 8-11 A. M. is best time to collect a 3 hour urine sample for CHI.
7. Plasma aminogram	The ratio of non-essential (NE) to essential (E) plasma amino acids reflects protein nutrition in general, but the subject tested must not have been treated (diet-wise) prior to this determination. In PM this NE/E ratio increases, reflecting greatly reduced blood levels of essential amino acids, especially the ones with branched-chains. Leu, ileu, val, met, thr, and trp are all greatly reduced. The ratio may increase to 6 in kwashiorkor. Normal is 2 or below. Test is done on 200 mcl. of "fingerstick" blood, using paper chromatography, elution of the pink ninhydrin positive spots, and expression of results as optical density ratios.	

little vitamin C during the day before his blood was taken. This finding does not necessarily mean that the subject is actually deficient in vitamin C, because blood levels of ascorbic acid do not reflect tissue concentration. In general, low blood levels of a nutrient do not necessarily indicate deficiency of that nutrient. This is especially true of the vitamins. A summary of some important concepts regarding the assessment of vitamin deficiencies is presented in Table II. Not included in this survey is a summary of methods by which depletion of essential mineral nutrients may be measured, because space does not permit it. While forming only a small portion of the total body weight, minerals are nevertheless vital to metabolism. The animal body requires seven principal minerals: Calcium, magnesium, sodium, potassium, phosphorus, sulfur, and chlorine. These minerals constitute 60 to 80 % of all the inorganic material in the body. Trace quantities of at least five other minerals are essential to human metabolism: iron, copper, zinc, manganese, and iodine. The essentiality of molybdenum and selenium has not yet been established, but they are utilized in trace amounts. Cobalt is an essential nutrient as a constituent of Vitamin B₁₂, but this vitamin must be supplied preformed to monogastric mammals. Tests for mineral deficiencies are mostly indirect measurements; for example hypochromic, microcytic anemia usually indicates iron deficiency (without disclosing the cause of the deficiency), and enlargement of the thyroid (simple goiter) usually signifies iodine deficiency in underdeveloped areas. The seven major minerals function mainly in acid-base regulation, body structure, nerve transmission, and blood clotting; the trace minerals are usually enzyme activators or integral parts of important compounds, such as iron in hemoglobin, zinc in insulin, and iodine in thyroxin.

The importance of the immune response, in general, in protecting against infectious diseases was recognized historically at about the same time as was the importance of essential nutrients and of nutritional deficiency disease. Thus, medical literature during the first half of the twentieth century is replete with reports, largely conflicting, on the effects of nutrient deficiencies on the antibody response, both in experimental animals and man. The classical association of famine with pestilence leads to the assumption that malnutrition reduces resistance to infectious disease. It is not my purpose here to attempt an appraisal of this belief. Suffice it to say that there exists a well-documented interaction between nutrition and infection but that the details of this interaction are unclear. Almost no work has been published on the extent to which human populations actually suffer through failure of humoral antibody response resulting from protein depletion, the most

important global nutrient deficiency. More importantly, the relationship between deficiency of protein and protective antibody production in man has not been studied. A systematic investigation of this problem of world health would be difficult to design because of the multitude of variables involved. Protein deficiency: (1) affects all organ systems and their interrelationships; (2) can rarely be quantitated as to degree; (3) produces sociocultural changes which cannot be measured, such as overcrowding, with its consequent effects on infectious loads, infant weaning time, and altered dietary practices; (4) may have a differential effect on humoral and cellular immunity; and, (5) has a different effect on the immune response depending upon the type of infectious agents. The nature of protective antibodies must be defined for each separate infection; all antigens evoke an antibody response which is heterogeneous. It is therefore difficult to determine which class of induced antibodies are actually protective and which are mere by-products of the heterogeneous antibody response. The antibodies most easily or most frequently measured are not necessarily the ones which are protective. Consequently, in a well-designed experiment concerning a specific infectious agent at least three things would require measurement: (1) the degree of protein depletion, (2) the specific protective antibody titer, and (3) some assay of presence or absence of the infection. To undertake such a study would omit, however, multiple factors concerned with aspects of the mammalian defense mechanism which are unrelated to antibody production, such as tissue integrity, cellular phagocytic activity, endocrine imbalance, and levels of protective enzymes such as lysozymes, the complement system, properdin and interferon, to name a few. In other words, one must be specific about: (1) the nutrient involved, (2) the host, (3) the infectious agent, and (4) the end points to be measured, for (4) varies with (1 - 3).

The foregoing omits from consideration the other major limb of the immune response: cell-mediated immunity (CMI). CMI is responsible for delayed hypersensitivity, allograft rejection, the graft-versus-host reaction, and defense against viruses, protozoa, fungi and some intracellular bacteria. The inclusion of tests of CMI would also be necessary in our hypothetical assessment of the effects of nutrient deficiency on the immune responses.

Thus, attention to proper experimental design is of paramount importance in assessing the effects of malnutrition on bodily metabolism and immunocompetence. A computerized approach in an idealized setting is clearly impossible when dealing with human populations. Therefore, if pragmatism is our objective, we must attempt to collect our data in the laboratory using a convenient mammalian species,

TABLE II: Some Data on Vitamins Important in Nutrition

Vitamin; Notes on Structure, etc.	Sources	Normal Daily Requirements	Important Functions	Lesions of the Deficiency State	Test(s) for Determination of Deficiency and Notes
Thiamine: (B ₁); pyrimidine ring and thiazole. Heat labile and easily oxidized.	Yeast; brans of rice and other cereals; peas; beans; liver; muscle. Poor sources: flour, polished rice, processed cereals, milk.	1 mg. (min.) (0.5 mg./1000 cals.) (age-dependent)	Coenzyme (TPP) for 1. Oxidative decarboxylation 2. Transketolation (inHMP shunt)	Beri-beri Multiple Peripheral neuropathy.	Best is measurement of urinary thiamine excretion per unit time by thiochrome test (blue fluorescence). Normal: 100 mcg./24hr. or more, but must know age and weight of individual, because thiamine excretion falls with age. Very sensitive method (0.05 mcg/100 ml).
Riboflavin; (B ₂); 3-ring structure with ribotyl group. Heat stable.	Meats, liver, germ and brans of cereals, yeast. Exposure to light may destroy it; also pasteurization and U.V. irradiation.	1.5-2.0 mg.	Coenzyme (FAD;FMN) for flavoproteins, dehydrogenase enzymes involved in hydrogen transfer for cellular respiration. Function as hydrogen acceptors in this biologic oxida- tion system.	Most prevalent avitaminosis in U.S.A. Causes cheilosis, sebor- rhea, glossitis; photophobia, circumcorneal vascularization.	General principles similar to those for thiamine. Riboflavin levels in plasma and urine vary widely, depending upon tissue saturation and state of protein metabolism. A brief period of neg. N balance, for ex., mobilizes it from the tissues and actually causes plasma level to increase. Chemical assay based on yellow-green fluorescence of the free, oxidized form. Must use red glassware.
Niacin: nicotinic acid. Pyridine ring. Occurs mostly bound in body, as coenzymes NAD and NADP	Liver, lean meat, yeast, poultry, wheat germ. Poor sources: most fruits and vegetables. Heat stable, but water soluble; thus, may be lost in cooking. Many diets low in quality protein are also low in all "B" vitamins. Corn low in trp., and niacin is bound in corn.	13-21 mg niacin equivalent (50 mg. trp. = 1 mg niacin in man). Pellagra is usually due to a combined deficiency of trp and niacin.	Coenzyme (NAD;NADP) which function, together with FAD and flavopro- teins, in the oxidative reactions described for other basic metabolic reactions such as oxidative decarboxyla- tion fatty acid synthesis and oxidation, and oxid- of org. acids (TCA cycle)	Pellagra. Early symptoms: weakness, lassitude anorexia, indigestion, glossitis especially of tip and margins of tongue. The three "D's" of advanced pellagra are dermatitis, diarrhea and demen- tia. Lesions cannot be correlated with known functions of niacin.	Urinary excretion of N-methyl nicotinamide, a metabolite. Normal daily excretion over 3 mg, but this test is actually inadequate to assess borderline niacin deficiency. More sensitive is measurement of the urinary N-methyl pyridone with subjects on standard diets of 10 mg. niacin and 1000 mg trp., but this test is more difficult than the above. Perhaps best for population groups is a "loading test". 4-6 hrs. after a test dose of 50 mg. of niacinamide, measure timed urinary excretion of N-methyl nicotinamide. Subjects with incipient pellagra excrete only 5% of test dose; normals excrete 25% or more.
Pyridoxine (B₆) Pyridine ring. In body occurs in the active coenzyme form, as pyridoxal phosphate. Heat labile.	Meats, viscera, yeast, wheat germ, whole wheat. Milling of wheat may reduce the content by 90%. Milk rather low.	1-2 mg recommen- ded by NRCFNB, but actual requirement is probably 0.5mg or less.	As coenzyme for trans- aminases, deaminases, decarboxylases, desul- furases, and others. Functions in active transport of amino acids; in synthesis of cysteine from homocystine and serine, of serine from glycine, and of delta amino levulinic acid (heme synthesis). Folate metabolism.	Hypochromic anemia, ? dermatitis, irritability and convulsions, esp. in infants. Peripheral neuritis in patients on isoniazid for T.B. The necessity of B ₆ in synthesis of gamma amino butyrate (GABA), important in brain metabolism, may help explain convulsions in B ₆ deficient infants.	Pyridoxine is necessary in Conversion of tryptophane to niacin in the body. After a tryptophane loading dose, if the subject is B ₆ -deficient, he is unable to convert the tryptophane to niacin, and instead excretes xanthenic acid in the urine. (see Vilter, 1953, J. Lab. and Clin. Med. 42:335). Any significant increase above normal in the excretion of xanthenic acid after a tryptophane load is indicative of B ₆ deficiency.
Pantothenic Acid Pantoic acid and beta alanine. Part of coenzyme A. Fairly heat stable. Easily hydrolyzed in acid and alkali (peptide bond).	Egg yolk, kidney, liver and yeast are excellent. Lean beef, broc- coli, skimmed milk and sweet potatoes are fair sources. Large losses may occur in wheat flour manufacture and cooking of meat, but not of vege- tables. However, the vitamin occurs in all foods.	5-10 mg/ kg. of diet, 20 mg when given as the calcium salt. (Calcium pantothenate).	As part of coenzyme A, it combines with acetate to form "active acetate", with basic functions in carbohydrate, fat, and amino acid metabolism. Also forms acetylcholine. "Active succinate" (succinyl coenzyme A) takes part in heme synthesis Acetyl Co-A helps form cholesterol and thus steroid hormones. Protein- bound pantothenic acid in the cell (acyl carrier pro- tein) is a coenzyme required for fatty acid synthesis.	Spontaneous deficiency is very rare, because of the presence of this vitamin in all foods. However, the "burning feet syndrome" was thought due to panto- thenic acid deficiency in WWII prisoners in the Philippines and Japan, and in mal- nourished south Indian populations.	No chemical methods for its determination are available. Most favored assay techniques are microbiological. An improved technique using <i>Lactobacillus casei</i> is popular. (see Clarke, M. F., 1957. Anal. Chem. 29:135.)
Folic acid; pteroylglutamic acid. A combination of the pteridine nucleus, PABA, and glutamic acid. Heat labile and easily oxidized.	Liver, kidney, yeast, cauliflower, dark green, leafy vegetables. Fair amount in Milk	0.5-1.0 mg	Major one is one- carbon unit metabolism, such as formyl, hydroxy- methyl, and formimino group transfers. Purine synthesis, esp. thymine. Cell mitosis: metaphase to anaphase. (Powerful antagonists are aminopterin, and amethopterin).	Nutritional megaloblastic anemias. "Tropical sprue" Folic acid should not be used to treat pernicious anemia. The hematologic response is not perman- ent, and the neuropathy is unresponsive).	Methods of choice (1) microbiolo gical assay using <i>L. casei</i> on <i>L. fecalis</i> . (2) "FIGLU" excretion test. In the metabolism of histidine, there is a folate-dependent step where form-iminoglutamate is converted to glutamate. When folate-deficient subjects are given a loading dose of histidine, there is increased urinary excretion of formimino- glutamic acid (figlu).

Vitamin: Notes on Structure, etc.	Sources	Normal Daily Requirements	Important Functions	Lesions of the Deficiency State	Test(s) for Determination of Deficiency and Notes
Biotin. Organic ring compound with side groups.	Egg yolk, liver, yeast, seeds. Meats (muscle) rather low, but biotin is present in all foods.	Unknown, but human experimental subjects of biotin de- ficiency recovered on 75-300 mcg. m	As coenzyme in carb- oxylation and decarb- oxylation reactions. Best known reaction is that of CO ₂ transferring agent in conversion of acetyl Co-A to malonyl Co-A (extramitochondrial synthesis of fat).	Spontaneous deficiency almost unknown. Avidin, a protein in egg white, binds biotin, making it unavailable for intestinal absorp- tion. The human experimental deficiency causes dermatitis, anorexia, and myalgia.	No chemical method. Bioassay using chicks. Microbiological assay using <i>L. arabinosus</i> .
B₁₂; cyanocobalamin "Corrin" ring with atypical nucleotide containing cobalt. Heat stable.	All foods of animal origin, especially liver and kidney. Muscle meats, milk products and eggs are rather rich. Not present in plants.	Only 1 mcg needed to treat per- nicious anemia, but man's req't ranges from 0.6 to 2.8 mcg.	1) As coenzyme with FAD and vit. B ₁₂ in methyl group synthesis from reduced folic acid and serine. 2) In conversion of propionate to succinate, which then enters TCA cycle. 3) In gluconeogenesis, fat metab., synthesis of thymine, and protein synthesis.	Pernicious Anemia. True dietary deficiency very rare. In most cases, there is an intestinal absorptive defect, notably in pernicious anemia, where "intrinsic factor", a gastric mucoprotein, is lacking.	Two types of tests are available: 1) Micro- biological assay, using <i>L. leishmanii</i> or <i>Euglena gracilis</i> (see Hutner, et al. 1949, Proc. Soc. Exp. Biol. and Med. 70:118). 2) the "Shilling test", another "loading test". Give 0.5 mcg. B ₁₂ labeled with cobalt-60 p.o., with or without intrinsic factor. With- out intrinsic factor, 80-90% of the radio- activity appears in feces (with intrinsic factor, only 20-25%). If a flushing parenteral dose of 1mg of "cold" B ₁₂ is given 1 hr. after the oral dose, the amount of radioactivity flushed out in urine may be used as a measure of B ₁₂ absorption. (see Shilling, 1953, J. Lab. and Clin. Med. 42:946). For a double isotope procedure for rapid estimation of B ₁₂ absorption, see Katz et al. 1963, J. Lab. and Clin. Med. 61:266.
A Beta ionone ring with side group Easily oxidized. Fat soluble. A ₁ - retinal; A ₂ - 3 dehydro retinal.	Liver, milk butter. Only animal sources contain pre- formed vit. A. Yellow and green vegetables con- tain carotenes.	5000 I. U. (One I. U. - 0.3 mcg of vit. A alcohol) A ₂ is only 40% as potent as A ₁ .	1. Dim light vision 2. Maintenance of epithelia and mucous membranes 3. In bone formation. 4. In synthesis of mucopolysaccha- rides (PAPS) 5. ? in adrenal steroid synthesis	1. Nyctalopia 2. Xerophthalmia and keratomalacia 3. Keratinization of epith. tissues and folliculitis 4. Large, soft, spongy bones; failure of skeletal growth.	Carr-Price reaction, in which a blue color is produced when solution of antimony trichloride in chloroform is added. Serum determination is good measure of vit. A status, because serum level does not change after ingestion (not true for serum carotenes). Carotenes not absorbed as well as vit. A, but beta carotene can produce two molecules of vit. A after absorption. In deficiency, dry keratinized epithelial tissues are more susceptible to invasion by infectious organisms.
D A group of sterols, chiefly of animal origin, which vary in potency. D ₂ and D ₃ most important Fat soluble	Fish liver, oils, and viscera. Provit. D ₂ (ergo- sterol) in yeast. Mammals can synthesize pro- vit. D ₃ from cholesterol, which is then activated in skin by U. V. light.	400-800 I. U. Req't very low or nil if there is exposure to sunlight. Ergosterol is activated to D ₂ (calciferol) by U. V. in skin.	1. Increases calcium and phosphate ab- sorption from gut. 2. With PTH, in- creases resorption of bone minerals. 3. Promotes ossi- fication of cartilage 4. Increases serum citrate level 5. Activates alk. phosphatases.	Rickets. In children, bones become soft and bowed, with en- larged ends, and their mineral content decreases. Adult rickets (osteomalacia) is rare.	No good test for practical clinical use. The only test suitable for survey work is measurement of plasma alkaline phosphatase, which increases in Vit. D deficiency before clinical rickets occurs. Must always compare with normal children of same age group. A bioassay and a spectrophotometric assay are available but difficult.
E Alpha tocopherol; chroman ring with side chain Fat soluble. Easily oxidized.	Milk, eggs, muscle meats, green plants, cereals, wheat germ, corn and soy bean oil.	30 mg of the acetate. (large amounts of unsaturated fat in diet in- crease require- ment because the fats oxidize vit. E).	Not well understood. There is evidence that it acts as a cofactor in the electron transfer system between cytochromes b and c.	May rarely cause anemia in children. (Acts with selenium to prevent liver necrosis in protein- deficient rats).	Thin layer chromatography followed by spectro- photometric assay can be used and is quite sensitive. For measuring vit. E content of foods, a bioassay using pregnant rats is done. The fetuses are resorbed on E-deficient rations.
C Ascorbic Acid; lactone ring Water soluble. Easily oxidized.	Citrus fruits and most other fruits, most vegetables, esp. salad greens. Easily destroyed by cooking.	70 mg is re- commended but probably too high. 10 mg a day prevents scurvy in man.	Biochemical functions still not known. Helps maintain normal inter- cellular material of cartilage, dentine, and bone. Probably has several other functions.	Scurvy. In children, the joints of growing bones are affected, especially the costochondral junc- tions. Subperiosteal hemorrhage may cause severe pain. In adults, gum and skin changes.	Chemical test based on dye reduction by the reduced form of ascorbic acid. Has been adapted to the microdetermination of blood ascorbic acid. Only 0.01 ml. of serum needed. However, plasma levels are good indicators of recent intake but poor for tissue concentration. Leukocyte content of vit. C is best assay, but difficult. Best way to assess status is to give an oral dose of ascorbic acid (15 mg/kg body wt.) and measure plasma level 4 hrs. later. Depleted subjects show little or no increase.

extrapolate our results to human populations, and then attempt to test our conclusions on the latter.

Briefly described in Table III are some valuable tests of immunologic capacity. They are outlined and described in the table. The format for the table derives from the basic dual nature of the immune system: humoral and cellular. Each of these major limbs has an afferent and efferent part, and each limb involves cells which are usually morphologically indistinguishable but functionally heterogeneous. The general model, much of which is based upon experimental verification, is as follows: I. Lymphoid cells controlled by the bursa of Fabricius or its mammalian equivalent make humoral antibodies, some of which are "protective". The antibodies fall into 5 categories of immunoglobulins: IgG, IgM, IgA, IgD, and IgE. IgG, IgM, IgA may be primarily "protective" against disease, depending upon the antigen (infectious agent). In the primary response IgM is mainly produced; in the secondary response, IgG predominates. These responses may be altered differentially by nutrient deprivation, again depending upon the antigenic stimulus. II. Thymic-dependent lymphoid cells participate in CMI, which is responsible for delayed hypersensitivity, allograft rejection, and the graft-versus-host reaction, and which restricts intracellular parasitism (mycobacteria, viruses, fungi and protozoa) and maintains surveillance for neoplastic cells. Usually more than one cell type participates in the process of CMI (cell "cooperation"). Lymphoid cells involved in the immunologic recognition and response to altered or foreign cells appear to release a variety of soluble factors which would tend in nonspecific ways to destroy such abnormal cells (lymphotoxin) and render surrounding tissues resistant to further viral infection (interferon). Additionally, factors may be released which encourage the influx of granulocytes (leucotactic factor) and macrophages (migration inhibitory factor) and recruit lymphoid cells by specific (transfer factor) and nonspecific means (blastogenic factor) to augment the total response. The relationship between nutrient depletion and CMI has only recently begun to be explored. In some human benign and malignant lymphoproliferative diseases probably caused by oncogenic viruses: Burkitt's lymphoma, carcinoma of the posterior nasal space, infectious mononucleosis, and sarcoidosis, CMI is impaired. This impairment of cellular immune defense results in unrestricted proliferation of the virus-infected cells, predisposing to increased production of virus and presentation of its antigens to the antibody-forming apparatus of the host, with consequent increases in humoral antibody production. The end result is a sort of "antibody escape", where the diseased

individual manifests CMI poorly, but may have inordinately high titers of antibody against the virus. Another interesting example of this escape mechanism occurs in patients with a mycobacterial disease: the lepromatous form of leprosy. Some of these individuals have poor CMI and high levels of circulating antimycobacterial antibodies. In the case of leprosy, it is likely that the insufficiency of CMI is a congenital defect, allowing *Mycobacterium leprae* to establish itself inside cells and produce slowly progressive leprosy, where levels of antimycobacterial antibodies have little protective effect against the disease.

The few recent studies attempting to demonstrate alteration of CMI by malnutrition indicate that, for example, protein malnutrition impairs the delayed skin hypersensitivity reaction to tuberculin. Delayed hypersensitivity probably plays a role in resistance to tuberculosis, and most authorities agree that protein deficiency impairs resistance to tuberculosis. On the other hand, some animal studies indicate that protein deficiency enhances cell-mediated immunity with respect to defense against certain intracellular organisms. For example, the proportion of mice surviving infection with swine influenza virus is greater in protein-deficient mice than in normally-fed mice. My own experimental results indicate that moderate protein depletion, while decreasing humoral response in general (mice, rats) may enhance cell-mediated responsiveness, but the level and duration of protein depletion is quite important, as is the age of the animal. Humoral responsiveness is attenuated in states of moderate protein depletion; as the depletion worsens, CMI is affected. I have found that chronic protein depletion (8% casein diets) in inbred mice leads to a higher survival rate after infection with pseudorabies virus than does a diet normal in protein (27% casein). The enhancement of CMI in depleted mice of the same inbred strain was further demonstrated by more rapid rejection of skin allografts and the greater *in vitro* blastoid response to phytohemagglutinin of spleen lymphocytes from depleted mice. Experiments concerning the humoral limb of the immune system gave a different kind of result. Whereas protein deficiency depresses the primary antibody response to sheep erythrocytes, it has no effect on either the primary or secondary agglutinating antibody response to *Brucella abortus*. Thus, chronic protein depletion in a particular strain of inbred mouse seems to have a disjunctive effect on humoral and cell-mediated immune responses, and a differential effect on the humoral antibody response, depending upon the type of antigen used to elicit it.

TABLE III. ASSAY OF IMMUNOLOGIC COMPETENCE IN MAN

some appropriate tests

HUMORAL IMMUNITY

<u>TEST</u>	<u>Material and Methods</u>	<u>Interpretation and Comments</u>
1. Immunoglobulins G, M, and A assays. (serum conc. of D and E are too low to be easily detected by this method).	Less than 1 ml. of serum. For each immunoglobulin, 0.01 ml of serum is required. Single radial immunodiffusion technique (Mancini).	The Mancini method (Mancini, G., et. al. 1965. Immunochem. 2:235) assumes a linear relationship between the area of antigen-antibody precipitate and the concentration of protein to be measured. The antigen diffuses into agar containing specific antiserum. Standard sera, with known amounts of immunoglobulin in question, must be used for reference. See also Rowe, D.S. 1969. Bull. Wld. Hlth. Org. 40 :613. (Many other types of tests are available).
2. Complement assay.	Less than 1 ml. of serum, fresh, or frozen at -70°C.	The many proteins of the complement system usually function, after an antigen-antibody reaction has occurred, to destroy foreign cells, notably bacteria. Complement assays require skill and specialized equipment, and should not be attempted in the field.
3. Immunoglobulin E assay (Reagin; skin-sensitizing antibody).	1. Prausnitz-Kustner (PK) test (in vivo; qualitative) 2. The red cell linked antigen-antiglobulin reaction (in vitro; see Coombs et. al. 1968. Lancet i, 1115).	These globulins mediate immediate type hypersensitivity, and include the reagins, which have high affinity for skin, leukocytes, and other cells. They cause anaphylactoid reactions in man, which release histamine, serotonin, "slow reacting substance", and kinins. Anaphylactoid reactions may be mild (mucosal surface involvement, as in asthma), or severe (systemic, as in penicillin anaphylaxis). IgE may represent a major defense in both the upper and lower respiratory tracts.
4. Isohemagglutinin assay (for anti-A and anti B titers)	May be done on fresh (fingerstick) or stored blood. Needed are: at least 0.4 ml. of subject's serum, and normal types A ₁ and B red cells. Reference anti-A and -B antisera are commercially available.	Four major blood group phenotypes exist in man; A, B, O (H), and AB. These are antigens which are a structural part of the surface membranes of red blood cells. Most persons with type A, B, or O blood have serum antibodies (isohemagglutinins) against the A or B antigen not part of their own cells. "Natural antibodies", cross-reacting with A or B antigens, cause titers of the A or B isohemagglutinins to vary widely in different individuals.
5. Circulating antibody assay after antigenic stimulation		These tests measure the person's ability to produce antibodies to specific antigens, and range widely in sensitivity depending upon the method used.
A. Typhoid-paratyphoid vaccine or plain typhoid (S. typhosa, heat-phenol or acetone-killed) vaccine both used.	Commercial vaccine is injected usually subcutaneously, according to manufacturer's directions. Pre-and post-inoculation Salmonella serum antibody titers are determined. (Many bacterial, viral, or other types of antigens may be used, depending upon relevance to prevailing conditions).	These assays are a measure of the ability of the tested subject to respond to antigenic stimulation by producing specific antibodies. They are tests of the immunocompetence of the humoral immune response, without regard to the biological function (if any) of the induced antibodies. Naturally acquired infectious disease almost always results in better immune protection against a reinfection with the same organism than does passive immunization. For example, in the case of S. typhosa, either infection or vaccination causes rises in at least 3 specific IgM antibodies, directed against the H, O, and Vi Salmonella antigens. However, vaccination induces mainly anti-H antibodies, while the natural disease, typhoid fever, may cause greater increases in anti-O and anti-Vi antibodies, generally considered most important prophylactically.
B. Shick test for diphtheria antitoxin. Done either before or after antigenic stimulation.	0.1 ml diphtheria toxin intradermally. Use heat-inactivated toxin as control. For prior immunization, use commercial D-T vaccine.	This is a highly qualitative test to answer the question whether the subject has already, or is able, to produce antitoxin to diphtheria toxin. A positive skin reaction (induration and erythema), beginning at 24-48 hours, and persisting for 7 days, indicates absence of circulating antitoxin (hence, susceptibility to diphtheria). If Shick-positive subjects are then immunized with diphtheria-tetanus vaccine, and remain Shick positive, a very poor humoral antibody-producing capacity is present.

TABLE III

(continued)

CELLULAR IMMUNITY

<p>1. Skin tests A. Delayed hypersensitivity (D. H.) to "natural" antigens</p>	<p>Tuberculin (P. P. D.), histoplasmin, coccidioidin, mumps virus (inactivated), staphylococcal antigen, Candida antigen, and Trichophyton antigen have all been used.</p>	<p>These tests of cell-mediated immunity (CMI) are all similar in that they inquire into the competence of the thymic-dependent limb of the immune response. The presence of induration and/or erythema (depending upon the test) at the site of injection, usually after 48 hours, indicates: (1) that CMI is at least partially operative, and (2) that the tested subject has prior sensitization to the test antigen. Antigens should be chosen with consideration of (2).</p>
<p>B. Contact sensitization to artificial antigens</p>	<p>Dinitrochlorobenzene (DNCEB), oxazolone (a synthetic penicillin-like substance), or picrylated albumin.</p>	<p>Similar to the above skin tests in detecting CMI, but probably more potent elicitors of it (see text). Since these chemicals are not antigens which usually occur in nature, the subject is first sensitized to them with a stronger solution than when challenging the patient 2-3 weeks later. Interpretation of all skin tests requires experience and the use of standardized techniques.</p>
<p>2. In vitro tests A. Lymphocyte "transformation" by mitogens. Non-specific (e. g. phytohemagglutinin) or specific (e. g. C. albicans) mitogens may be used.</p>	<p>The subject's blood lymphocytes, obtained by standard methods from heparinized blood; culture media; de-complemented serum; the mitogen to be used for lymphocyte stimulation.</p>	<p>Special laboratory apparatus and conditions are required for these tests; consequently the necessary materials should be assembled in a central place. In the presence of mitogens, small lymphocytes transform into large, metabolically-active (blastoid) cells, and proliferate. These events may be monitored either morphologically (% blast transformation) or biochemically with tritiated thymidine, which is incorporated into the DNA of dividing lymphocytes. The latter technique gives a more quantitative estimate of CMI, which, if impaired, is reflected in decreased transformability of the subject's lymphocytes <u>in vitro</u>.</p>
<p>3. Notes on other tests for CMI: Clinical examination of the patient and of his blood, bone marrow, and lymphoid tissue should yield valuable information on the extent to which his thymic-dependent immune system is active. Other <u>in vitro</u> tests are also available.</p>		

In Table III I have listed the immunological tests which are practicable for general use, where the resources of a research laboratory may not always be available. It is evident that measurements within both limbs of the immune system are necessary, and that tests should be selected on the basis of the type of information needed and the prevailing conditions.

Immunoglobulin assays are numerous and vary widely in sensitivity. The most sensitive methods (bactericidal and virus neutralization tests) can detect as little as 10^{-5} mg of antibody nitrogen per ml. of test material. Less sensitive are precipitin tests and immunoelectrophoresis. The radial immunodiffusion method of Mancini is intermediate in sensitivity between these two extremes and has been adapted for field use. Materials are available commercially to permit the expeditious performance of these measurements. For example, using this technique, Egyptian workers found that serum concentrations of immunoglobulins G, M, and A were all below normal in babies with clinical kwashiorkor under the age of 7 months. IgM concentration continued to be very low in kwashiorkor victims as old as 2 years, and 6 months after cure of the disease IgM levels remained low. IgA concentration was low before age 7 months, but in infants 1-2 years old the levels were thrice normal. The pattern for IgG was similar to that of IgA, but IgG concentrations in the older babies were only moderately elevated. (Aref, G. H., et. al; in press). I emphasize this work for two reasons: (1) current immunologic methodology was used to study the breakdown of "natural" immunoglobulins in a malnourished population before and after dietary treatment; and (2) the immunologic implications of the results are important, especially with respect to IgM. For several reasons, IgM may be the most suitable immunoglobulin candidate for combating bacterial infections in the systemic circulation (see Altemeier, W. A., et. al., J. Immun., 103: 924, 1969). The Egyptian work indicates that protein-calorie malnutrition in infancy may suppress IgM synthesis for a prolonged period, perhaps permanently. The production of IgM represents, both phylogenetically and ontogenetically, the most primitive and basic humoral antibody response. Clearly, further investigation should be done of the effect of malnutrition on the production of IgM directed toward specific antigenic stimuli in malnourished subjects.

The low IgA levels in early life followed after 1-2 years by elevations three times normal, found in the Egyptian study, also raises an interesting point. IgA is synthesized by plasma cells in mucous epithelial surfaces of the respiratory tract and intestine and in nearly all excretory glands. Secreted onto mucous

epithelial surfaces and also into the bloodstream, IgA serves as the first line of immunological defense against invasion by microorganisms, and is especially important in preventing gastrointestinal infections and infections of the secretory glands. Infants in poorly-developed areas invariably have high infectious loads of gastrointestinal microorganisms, frequently succumbing to diarrheal disease during the first years of life, especially after weaning. (IgA is present in breast milk, although withdrawal of this source of IgA is not the most likely principal cause of weaning diarrhea. Substitution of post-weaning feeding formulas of far less nutritional value than breast milk is undoubtedly the major cause of these diarrheas). It has recently been found that antigenic stimulation via the gut not only leads to production of IgA in bowel secretions, but also to the selective production of antibodies of IgA type in the circulation. Conceivably, IgA might combine with antigen and make it a less effective stimulator to the IgM- or IgG-producing cells. This mechanism might explain the shift from low to high IgA levels in very young and in older infants, respectively, found in the Egyptian study. It also might, in part, account for the continued low levels of circulating IgM in these infants.

This leads to the mention of a subject important in malnourished populations, that of antigenic competition, defined as follows: when two or more antigens are administered to a subject, the response to each individual antigen may be less than the expected response if a single antigen were given by itself. Antigenic competition probably represents a competition for commitment of individual cells to the production of a specific antibody. Individual cells usually produce only one kind of antibody upon stimulation by several antigens. This type of competition for antibody-producing cells by multiple antigens may play a role in the poor immune resistance of malnourished populations, where the infectious loads (exposure to many different pathogens) in early life are enormous. Such children usually have higher than normal IgG levels, as illustrated in the case of the Egyptian study, where infants 1-2 years old had high levels. These antibody levels are high because of the hypersensitized state of the individual, but this does not mean that his humoral defense mechanisms for specific infections are more active. They are probably less able to cope with specific "superinfections" than in the case of normal children. For example, Gambian children repeatedly infected with malaria in early life, have higher gamma globulin levels than their malaria-free counterparts, and much of this gamma globulin is undoubtedly specific immunoglobulin directed toward malaria and other endemic infectious

diseases. However, when given tetanus toxoid, an antigen unrelated to the malaria plasmodium, the malaria-infected children responded very poorly in producing tetanus antitoxin (McGregor I. A. and Barr, M. 1962. Trans. Royal Soc. Trop. Med. Hyg. 56:364). This specific example of antigenic competition is doubtless one of the many which are occurring throughout the less developed world, and illustrates the fact that malnourished populations cope immunologically with their infections progressively less efficiently as the infectious load increases.

Concerning tests for CMI (Table III), the ones available can be done *in vivo* (skin tests), or *in vitro* (cellular studies). CMI is much more difficult to quantitate than is humoral immunity. Depending upon antigenic strength, a wide range of cellular immune responsiveness can be detected with skin tests, but only qualitatively. Patients with lepromatous leprosy and depressed CMI often cannot be sensitized to dinitrochlorobenzene, but can be induced to develop skin sensitivity reactions to the more powerful antigen; hemocyanin. Thus, the degree of depression of CMI could probably be semi-quantitatively assessed if a battery of sensitizing antigens of increasing antigenic strength were developed for this purpose, but the investigator would still be faced with many problems of interpretation of results, such as antigenic cross-reactivity, the subjects' pre-exposure to antigens, etc.

IgE, or skin-sensitizing reagents, are immunoglobulins responsible for immediate hypersensitivity reactions (15 minutes to about 6 hours after exposure to the antigen), as opposed to reactants which determine delayed hypersensitivity (D. H.), where IgE does not participate. D. H. manifests itself 24-48 hours or more after antigenic challenge. Some tests for D. H. are outlined in Table III, under the heading cellular immunity. Some cellular studies for CMI require the procurement of stained blood, bone marrow, or solid lymphoid tissues, so that the presence and morphology of the cell types participating in CMI may be assessed. The range of tests available for the *in vitro* study of CMI is expanding rapidly, but because the performance of the more complex tests usually requires special skills and apparatus, strictly field use of them is currently impractical.

At the outset, I implied that world food supply was decreasing with respect to world population. Very recently, I have read that during the past 2-3 years, world food production per capita has increased. These statements, superficially, appear to contradict each other. The real question is probably disguised in the statements themselves, and cannot be answered in such simple terms. The answer has to do with the quality-food imbalances that

exist today on our planet, and with the myriad forces which have created that imbalance. In 1964, president Mohammed Ayub Khan made the following statement: "In ten years' time, human beings will eat human beings in Pakistan".

Nutrient deficiency, especially that of protein in the less-developed world, has produced generations of people who are incompetent physically, mentally, and perhaps immunologically. If, indeed, the world's quality foods were more evenly distributed, it would not be necessary, except from a basic standpoint, to study the interactions of malnutrition and immunocompetence, and president Khan, in 3 years' time, would have to "eat his words". In the meantime, it will become necessary to explore, in greater depth, the effects of malnutrition on immunologic performance.

We are now in a position to consider priorities and feasibilities in attempting to assess nutritional status. Our current degree of sophistication in nutritional metabolism and in immunobiology has created new problems, primarily logistic and technical. An analogy with the field of cancer might be drawn. We no longer think of cancer as a single type of deviation from the normal, with a single etiology. We think of cancer as a variety of different diseases of different etiology, and our diagnosis must be quite specific for proper treatment to be applied. In much the same way, we cannot regard malnutrition as a single entity, but as many different diseases leading to many types of dislocations in bodily homeostasis, depending upon the etiology of the disease. More than any other biological abnormality, malnutrition is culture-dependent. Consequently, in order to cope with problems of human malnutrition, we must thoroughly understand the culture in which they develop and then apply our methods of diagnosis and treatment.