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Biochemical evaluation of nutritional status in man[1]

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VARIATIONS IN QUANTITY and composition of the diet are reflected by changes in the concentration of chemical substances in tissues and body fluids, and/or by the appearance or disappearance of specific metabolites. The primary limitations of the biochemical approach to assess nutritional status in man are the degree to which these changes can be measured by existing techniques, and the extent to which the nutritional origin of these changes can be clearly separated from the influence of genetic, environmental, physiologic, pathologic and other factors.

The nutritional spectrum for any single nutrient varies from frank deficiency at one extreme to optimal nutrition in the middle and overnutrition at the other extreme (55). Research on methods of evaluation of nutritional status should be orientated towards the search for 'signposts' that would determine specific nutritional states within this spectrum. At present however very few of these 'signposts' are known. In order to fill the need for practical guidance in the evaluation of biochemical nutritional studies, it is necessary to use methods which give useful information although they do not delineate the entire spectrum.

Most of the biochemical tests now available can be divided into those which measure changes that directly reflect the 'supply of nutrient' and those which detect biochemical changes reflecting metabolic alterations consequent to nutritional effects, i.e. 'biochemical nutritional pathology.' An example of the first is the concentration of plasma ascorbic acid, which is not filling a metabolic function but is merely in transit from one tissue to another. Its lack might or might not indicate the status of intracellular ascorbic acid. Examples of 'biochemical nutritional pathology' are a lowering of the quantity of plasma albumin, or the accumulation of pyruvate in the blood. Plasma albumin, for instance, is not a nutrient, but an internally synthesized metabolite, and its deficiency indicates a metabolic impairment which prevents synthesis from balancing catabolism. In thiamine deficiency, an accumulation of pyruvate means much more than a relatively low level of blood thiamine. As pointed out by Sinclair (81), an individual with a value of 5 mcg of thiamine per 100 ml, shown by a single blood analysis, could not be classed as deficient; in longitudinal studies, however, it became evident that 5 mcg represented a deficiency state for that subject. Accumulation of pyruvate, on the other hand, indicates impairment in the oxidative decarboxylation and points to biochemical thiamine deficiency.

Signs of 'biochemical nutritional pathology' appear when the concentration of an essential nutrient in the tissues decreases to a point where there is interference with metabolism. They may arise from dietary deficiency of the substance, or be due to poor absorption, impaired transport and decreased utilization. The detection of these signs of actual biochemical pathology is of great value and contributes conclusive evidence of functional nutrient deficiency. The practical use of these biochemical measurements for evaluating nutritional status is limited however by homeostasis. It would be impossible to discuss here the variety and complexity of the factors involved, but their net effect is against such biochemical changes. When the capacity of the homeostatic mechanism is overwhelmed by a nutritional deficiency the result is biochemical disease.

Nutrient supplies may be the food, the body reserves, or both, but concerning cell nutrition this distinction is not of practical significance. Indices of 'nutrient supply' usually are measures of the essential dietary constituents as they appear in the body fluids. Their decrease may mean no more than a low intake for a variable period of time. They indicate relative adequacy of intake but not the existence, nature or magnitude of nutritional disease.

Many nutritional conditions considered important by the hospital clinician are not a major concern of public health nutrition workers, because they do not occur frequently. Only nutrient deficiencies known to be the result of inadequate dietary practices in existing population groups will be discussed. Vitamins E and B_6 are included, although of doubtful significance by this criterion. Vitamin B_{12} and biotin are examples of nutrients omitted because it is unlikely that deficiencies of them occur in a significant number of human subjects as the result of an inadequate diet.

PROTEINS

Biochemical measures of protein nutrition may refer to either the relative adequacy of the dietary intake or

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the extent of protein depletion. Bengoa *et al.* (10) stated as recently as 1959 that "in spite of the enormous advances in this field of nutritional biochemistry, there is no new test available which can be used in field surveys as an indicator of protein malnutrition."

For adult population groups consuming a mixed diet and maintained in nitrogen equilibrium, the daily excretion of urinary nitrogen is a good estimate of protein intake. This approach, recommended in 1932 at the International Conference in Berlin (70), was used by De Venanzi in Venezuela (29). He found that in the population group studied, the low excretion of nitrogen in the urine paralleled a low intake of protein in the diet. However, the method has two main objections: first, the extreme difficulty of obtaining accurately timed urine specimens under field conditions, and second, in children, who are not in nitrogen equilibrium, the relationship between nitrogen intake and nitrogen excretion will vary with the degree of nitrogen retention. This in turn will depend largely on the biological value of the dietary protein and the physiological state of the child. A recent report indicates that the ratio of total urine nitrogen to creatinine in periods as short as 4 hours correlates well with the level of dietary nitrogen (69).

There are numerous reports of somewhat elevated total plasma proteins in population groups with inadequate protein intakes. No common pattern of serum protein fractions has been found (6), but it is quite obvious that the simple measurement of serum or plasma total proteins will not detect early stages of protein deficiency. When the deficiency is severe, the marked drop in total protein concentration is mostly due to a lowering of the albumin fraction. As in kwashiorkor this drop serves only as a confirmation of a condition which is already clinically evident.

More labile plasma nitrogen compounds related to protein metabolism would appear to offer a better possibility. Individuals living on chronically suboptimal intakes of protein reduce their 'protein metabolic level' and attain nitrogen equilibrium in the case of adults, or maintain a positive nitrogen balance in growing children, but at a lower plane. The phenomenon is not accompanied by changes in fundamental protein moieties like plasma albumin, until the deficiency is very severe.

Platt measured the urinary excretion of urea of children (66) and lactating women (67) of different nutritional and socio-economic conditions and found the ratio of urea nitrogen to total nitrogen to be markedly lower in the groups with poorer nutrition. We have tested this ratio using a single fasting urine specimen and have confirmed Platt's results for children of both high and low socio-economic groups in Guatemala. This ratio reflects essentially the magnitude of urea excretion, since the amounts of other urinary nitrogenous compounds are not altered as significantly. Therefore, the ratio of urea to creatinine in fasting urine samples also bears a direct relationship to the protein metabolic level of the individual.

This approach seems attractive since it measures a more labile aspect of protein metabolism than the synthesis of components as fundamental as plasma albumin and enzymes. Further studies should be conducted as to the feasibility and reliability of the test in single fasting urine samples under field conditions. Since this test requires that no immediate intake of nitrogen be reflected in the urine, it is recommended that a fasting specimen be collected after emptying the bladder in the morning.

The fasting level of plasma nonprotein nitrogen (NPN), of which urea is the most important fraction, is also influenced by the amount of protein in the diet. If this is large, the NPN will be maintained at a higher level than if the subject is subsisting on a low protein diet (65). This situation of partial protein restriction is not to be confused with starvation, where the plasma levels of NPN tend to increase. De Venanzi (28) determined the concentration of plasma NPN in laborers from Caracas, Venezuela, and found an average value of 23.9 mg/100 ml which is lower than values given in the literature for normal subjects. He attributes this low value to the diets poor in protein consumed by the group studied.

The level of plasma amino acid nitrogen has also been suggested as an indicator of human protein nutrition status (57). When Albanese (2) investigated the relationship of plasma amino acid levels to the dietary protein intake and the nutritional status of an individual, he found good correlation between plasma amino acid levels and the percent weight deficit, as long as the weight deficit did not exceed 30%. As the protein stores of a depleted subject became filled, the fasting plasma amino acid nitrogen increased. Plasma amino acid levels thus appear to be a sensitive measure of protein nutriture.

The finding, that in severe protein depletion not only plasma albumin but also the activity of some plasma enzymes are much reduced, stimulated investigations to determine enzyme plasma levels in population groups with suboptimal protein intakes as judged by dietary and physical examination data. Under field conditions, however, even very different socio-economic groups of children have no differences in serum pseudocholinesterase activity (4). Direct measurement of liver protein and enzymes have also shown patterns of changes associated with severe protein malnutrition (103). However, the effect of milder degrees of deficiency is not known in humans, and liver biopsy obviously cannot be done routinely to detect mild degrees of malnutrition.

It is well known that the sacrifice of skeletal muscle during protein deprivation protects more essential tissues. This occurs early, under conditions of insufficient protein intake. Stearns (87) says that maintenance of skeletal musculature is compatible only with adequate protein intake. The indirect estimation of muscle mass from 24-hour urinary creatinine excretion data has been recommended. Decreases in muscle mass are proportionately larger and their estimation more sensitive than decreases in body weight and body height. The expression 'mg of creatinine in 24-hour per cm body height' is to be

preferred over that of 'per kg of body weight' since variations in fat depots do not affect the former. Since the collection of 24-hour urine specimens is very difficult in the field, the use of carefully timed shorter periods deserves further testing. Examples of studies in which this technique has been successfully employed are those of Stearns (87) in North American children 1–10 years, Standard *et al.* (86) in kwashiorkor, and Arroyave *et al.* (5) in Central American children of different socioeconomic levels.

VITAMIN A

Inadequate dietary vitamin A activity, either as preformed vitamin A in foods of animal origin or as active carotenoids in vegetable foods, results in tissue decreases of this vitamin. Since the liver is the main site of vitamin A storage, its concentration in liver tissue is a direct measurement of vitamin A reserves. In the living human subject this can be determined in liver biopsy specimens, but, as mentioned before, this complicated procedure is impractical for the evaluation of vitamin A nutriture in population groups.

The serum levels of carotene and vitamin A have been used extensively to corroborate the diagnosis of vitamin A deficiency (36). The levels of carotene directly reflect changes in dietary intake although they are not per se indicative of vitamin A nutrition. Subjects receiving only preformed vitamin A in their diets may have adequate vitamin A reserves with practically no carotene in their plasma. However, in populations living on a mixed diet, particularly diets of vegetable origin, a low carotene level is very informative. If accompanied by a low plasma vitamin A level it is conclusive evidence of inadequate vitamin A nutrition.

Vitamin A plasma levels do not necessarily reflect recent intake. Large stores of vitamin A in the liver in well-nourished human adults tend to maintain the plasma level for about 1 year (98), whereas the child requiring an extra amount of this vitamin for growth might exhaust the liver reserves in a shorter time. In infants this seems to occur in 3 to 4 months (16).

While a marked decrease in plasma or serum vitamin A levels is compatible with a diagnosis of vitamin A deficiency, one might encounter occasional febrile conditions, not related to nutrition, which depress the vitamin A plasma concentration (37). Furthermore, the extremely low levels of serum vitamin A found in children with kwashiorkor may be the result of an impairment in blood transport since they may rise upon the recovery of the plasma albumin even with no dietary vitamin A (3). Such severe protein malnutrition cases would be clinically detectable and there is no evidence that milder degrees of protein depletion have an effect.

The ICNND manual for nutrition surveys indicates that serum levels of vitamin A between 20–50 mcg are acceptable, 10–19 mcg are low and below 10 mcg clearly indicate deficiency in young adult men (49). However, the level at which deficiency of vitamin A begins to limit human growth is unknown. Vitamin A values per 100 ml of serum for pre-school age children from lower-income families in Amatitlan, Guatemala, were 11 mcg for the first quartile, 17 mcg for the second, and 22 mcg for the third (9). These biochemical results coincided with critically low intakes, although not a single clear-cut clinical deficiency was encountered in this study.

There is no readily determined biochemical measurement of metabolic derangement due to vitamin A deficiency. Although the regeneration of rhodopsin is known to decrease in rate as a result of severe deficiency of this vitamin (1), its measurement is obviously impractical for survey purposes. Incomplete knowledge of the mechanism of action of vitamin A in metabolism has prevented further advances in biochemical methods for the detection of its deficiency. A new hypothesis for the action of vitamin A has been put forward recently by Dowling and Wald (31), The protein moiety opsin needs to be bound to vitamin A to preserve its integrity, and these authors suggest that this might be true for other protein moieties in the body, such as those of the epithelial cells. Since plasma vitamin A alcohol is bound to the albumin fraction, the decrease in this plasma protein observed in vitamin A deficiency in cattle (33) could be the result of an accelerated catabolism of the albumin moiety when insufficient protecting vitamin is bound to it. Obviously more research is needed to clarify this point, but if the hypothesis is correct it offers a new basis for devising biochemical tests for vitamin A deficiency at the metabolic level.

VITAMIN D

Biochemical changes characteristic of nutritional disease due to deficiency of this factor have been known for some time. Obviously the adequacy of vitamin D nutriture cannot be correlated with dietary intake since body synthesis replaces the dietary need for all or part of this vitamin when sufficient exposure to sunlight takes place. The most specific laboratory measurement for the confirmation of the clinical diagnosis of rickets is the alkaline phosphatase serum level. The activity of this enzyme rises early in the development of the deficiency and tends to be proportional to the severity of the disease. Decrease in the phosphorus concentration of the blood serum is also a constant finding (48, 52) and helpful in diagnosis. Calcium serum levels decrease inconsistently and are unreliable as the only index of rickets (52).

The problem of detecting subclinical deficits of vitamin D is more complicated. The alkaline phosphatase serum activity of groups of children is not a generally accepted criterion. Bessey (11) suggested its measurement as an index of possible value "in determining adequacy of antirachitic agents," and this has been included in many biochemical-nutritional surveys. In general, the results have not been interpretable, mainly because the relationship of the degree of vitamin D deficit to the level of this enzyme in serum has not been established for subclinical deficiency. Since the levels of serum alkaline phosphatase

activity decrease in children with severe protein deficiency (30, 75), a tendency to rise due to vitamin D deficiency might be masked when the two conditions coexist.

In adults, osteomalacia may occur as the result of a deficiency of vitamin D and is manifested biochemically by an increase in the serum alkaline phosphatase and a decrease in the concentration of serum calcium. The increase in alkaline phosphatase is not specific, since it increases in a number of other pathologic conditions (37).

VITAMIN E

In 1954 Bessey (11) commented on the probability that the determination of blood vitamin E would find wide application in evaluating vitamin E nutrition if it were established that man required this vitamin. Two years later, Goldsmith (34) stated that vitamin E had not come of age in human nutrition. Four more years have elapsed and, despite the continuous efforts of some investigators to demonstrate the nutritional importance of vitamin E in human nutrition, Dr. Goldsmith's statement is still applicable.

Vitamin E deficiency has been observed in isolated cases in both adults and children. Useful information on the biochemical alterations which are characteristic of vitamin E deficiency has been derived from the study of these cases. Woodruff reported a case of deficiency due to lack of intestinal absorption in a patient suffering from xanthomatous biliary cirrhosis (106). The serum tocopherol level was 0 and the patient had creatinuria and excreted a pentose compound in the urine. The hydrogen peroxide hemolysis of the red blood cells (RBC) was high. Administration of large doses of vitamin E for several months raised the serum levels to 0.35 mg/100 ml, the creatinuria and pentosuria disappeared and the susceptibility of the RBC to hemolysis decreased. The relationship between vitamin E nutrition and integrity of muscle mass is evidenced by the creatinuria as well as the presence of pentose in the urine; the latter was probably derived from excessive destruction of nucleic acid. At least two of these biochemical changes have been observed in the human subjects of the well-controlled experiments at Elgin State Hospital (44), i.e. increased susceptibility of RBC to hydrogen peroxide hemolysis and lowered vitamin E serum levels. There were no other abnormal findings even after more than 3 years of observation.

Newborn infants have lower levels of plasma tocopherol than adults, but these increase in the breast-fed child (91). Premature infants fed partially skimmed milk are perhaps the nearest to primary deficiency of vitamin E; their low plasma levels at birth decrease even further to values around 0.13 at 2 months, at which time a high RBC susceptibility to hydrogen peroxide hemolysis is found (62).

In the absence of additional clinical or pathologic evidence these alterations are only suggestive of deficiency. Aside from premature infants, evidence of vitamin E deficiency in children, as in adults, is associated only with pathologic conditions causing impaired absorption of fat, such as cystic fibrosis of the pancreas and biliary atresia (63). The very low vitamin E serum levels found in kwashiorkor (75, 92) are probably the result of either malabsorption or impaired transport rather than deficient intake.

It is concluded that the following biochemical observations assist in the diagnosis of vitamin E deficiency: *a*) low vitamin E serum or plasma levels, *b*) increased susceptibility of the RBC to hydrogen peroxide hemolysis, and *c*) excretion in urine of metabolytes indicative of increased muscle destruction, such as creatine and pentoses.

ASCORBIC ACID

The concentration of plasma ascorbic acid decreases rapidly with dietary deprivation of this factor. Crandon *et al.* (26) found that it reached a level of 0 mg/100 ml in about 40 days, but clinical scurvy was not evident until the 143rd day of deprivation. From these results, confirmed by other workers, two practical conclusions may be drawn: *1*) that the ascorbic acid concentration in the blood plasma reflects the recent past intake of the vitamin, and *2*) that its disappearance from the plasma is not diagnostic of scurvy although it is compatible with it. The urinary excretion of ascorbic acid in 24 hours gives an estimate of the prevailing dietary intake. The ratio of ascorbic acid to creatinine in a random single urine specimen, preferably fasting, gives information of similar value (55).

There is strong evidence that the concentration of ascorbic acid in white blood cells (WBC) is a good estimate of its concentration in other tissues and, thereby, of total body ascorbic acid. With a sufficiently high intake, complete tissue saturation is attained. In the adult this intake is about 100 mg daily with a corresponding WBC ascorbic acid content of 20–30 mg/100 ml. At intakes of zero the WBC ascorbic acid of a well-nourished adult decreases to nearly zero in 3 to 5 months (26, 99) and scorbutic lesions soon appear. According to Lowry (55), in the lower range of intake and tissue saturation, the WBC ascorbic acid level is an admirable index of nutritional status. The extent to which the determination of ascorbic acid in WBC may be considered practical depends on the laboratory facilities available, but the assay is more complicated than the measurement of serum ascorbic acid. It has been suggested that ascorbic acid serum levels be determined as a screening test.

Although not applicable to field work, a more direct estimation of tissue ascorbic acid deficit is accomplished by Lowry's method (55) of administering large amounts (500–2000 mg) in divided doses throughout the day to avoid flooding. With suitable correction for metabolic destruction of the administered ascorbic acid, the amount retained should equal the absolute deficit. Load tests have also been employed in which the amount of ascorbic acid excreted in a given time is measured after the ad-

ministration of a dose of the vitamin, either orally or parenterally. A review by Vilter (96) points out that these methods indicate only whether or not the tissues are saturated, but are poor indicators of the degree of unsaturation. "If the load test is conducted with an amount of ascorbic acid much smaller than the tissue deficit, there is no return, or if the dose is given rapidly there may be spillage due to momentary flooding of the blood stream" (55).

Cheraskin *et al.* have evaluated the intradermal test with dichlorophenolindophenol (23) as an indicator of the degree of tissue saturation of ascorbic acid. Although the method is not specific, owing to the large number of other reducing substances present in the skin, it shows some degree of correlation with ascorbic acid nutrition. This fact, plus its great convenience, favors its use as a screening test in nutrition surveys, but the method needs further standardization before it can be recommended for this purpose.

The demonstration of Sealock and Silverstein (78), that the scorbutic guinea pig excretes abnormal tyrosine metabolytes when the amino acid is given, was later extended to premature infants (54) and adults (73) with severe ascorbic acid deficiency. The work of Steel *et al.* (88) showed that human adults maintained for as long as 78 days on a diet containing 7 mg or less of ascorbic acid excreted urine with a higher reducing power, but no increase in tyrosil compounds was noticed.

RIBOFLAVIN

When Burch *et al.* (19) described a micro-fluorometric method for the estimation of free riboflavin, flavine adenine mononucleotide (FMN) and flavine adenine dinucleotide (FAD) in blood, interest was aroused in these measurements as indicators of riboflavin nutritional status.

Despite the large number of workers who have determined blood riboflavin in humans, only a few have reported the relative usefulness of the different forms in which this vitamin occurs. In 1952, Suvarnakich *et al.* (90) studied the variations of free riboflavin and FAD riboflavin in normal subjects and concluded that free riboflavin was too variable to serve as a useful criterion for riboflavin nutriture, and that FAD has more diagnostic value in detecting riboflavin deficiency in view of 'its greater stability.'

The work of Bessey *et al.* (12) is an example of a well-controlled study of the relation between riboflavin intake and blood riboflavin measurements. These authors maintained 10 men on a diet supplying only 0.55 mg of riboflavin/day for 16 months, and 6 more on intakes of 2.55 to 3.55 mg/day. The most consistent and significant difference was in the red blood cell riboflavin content, being 10.0 to 13.1 mcg/100 ml in the deficient group and 20.2 to 27.6 mcg in the supplemented group. All subjects on the restricted intake showed clinical manifestations of ariboflavinosis, 3 of them very severely. Although the plasma levels of the free FMN riboflavin decreased in the deficient group, the variability was very large due to some striking exceptions. The plasma FAD and the white blood cell riboflavin did not decrease significantly. In a second experiment the levels of RBC riboflavin of 8 men were determined periodically during 9 months on 0.5 mg/day of riboflavin intake as compared to 4 control groups with 1.6 and 2.4 mg of daily intake. The RBC riboflavin in the restricted group decreased significantly within 7 to 8 weeks. The authors' conclusion that the RBC riboflavin concentration 'is a reasonably sensitive and practical index' of riboflavin nutritional status is entirely justified. This work is important in the sense that it relates the biochemical findings in the blood to a definite state of deficiency. Many values, 'low,' 'normal' or 'high,' have been reported for groups of population, but their meaning in terms of individual deficiency of riboflavin has not been clearly defined because the point of reference has been the presence or absence of clinical signs which are, to a large extent, non-specific.

Significant attention has been given to the urinary excretion of riboflavin. The direct measurement of the 24-hour urinary excretion of riboflavin has been studied under controlled conditions in terms of daily intake and, in some instances, in relation to the appearance of clinical manifestations of deficiency. There is a general agreement in the figures given by several investigators for excretions corresponding to intakes ranging from 0.5 mg to around 1 mg daily. Within those limits of intake about 9 to 14% of the ingested riboflavin is excreted daily (36). Some disagreement may be explained by the different ways in which diet composition can affect the biosynthetic production of riboflavin by the intestinal flora, since the actual amount of the vitamin entering the circulatory system is the sum of the dietary riboflavin plus that synthesized in the digestive tract.

In well-controlled experiments in human adult subjects, Horwitt *et al.* (45) observed that variations in riboflavin intake from 0.55 mg to 1.1 mg/day produced only a slow proportional increase in urinary excretion. Raising the intake to 1.6 mg daily increased sharply and disproportionately the absolute amount, as well as the percentage of the dose excreted in the urine. Subjects receiving 1.6 mg/day excreted 4 times as much riboflavin as did those receiving 1.1 mg daily. According to these authors this result suggests that an adult male, under the conditions of the experiment (2,200 calories per day), requires between 1.1 and 1.6 mg/day. Williams *et al.* (104) arrived at practically the same conclusion.

Load tests have been extensively used for evaluating riboflavin intake and nutrition. Of the various techniques available, the 4-hour excretion following the parenteral administration of a 1-mg dose of the vitamin is promising (94) and deserves further study.

For field surveys, the determination of riboflavin urinary excretion in a single nontimed specimen, preferentially fasting, may be used. An excretion of 150 mcg/gm creatinine has been estimated by Lowry (55) as indicative of tissue saturation. The *Manual for Nutrition Surveys* of ICNND (49) has set excretions of 80–270

mcg/gm of creatinine as acceptable for young male adults. Lowry (55) states that expressing urinary excretion per gram of creatinine has the advantage of tending to correct for body size. This might be true within narrow limits but does not mean, for instance, that a child's riboflavin excretion calculated per gram of creatinine should approximate that of a mature man. In fact, the Newfoundland study (7) shows evidence of a much larger excretion of riboflavin per gram of creatinine in children than in adults.

The finding is not surprising since children need and usually consume more riboflavin in relation to their muscle mass, while creatinine excretion is proportional to it. Therefore, relationships between riboflavin excretion in the urine and adequacy of intake or deficiency in children must be established *de novo*. This is an open field for nutrition research.

THIAMINE

The thiamine concentration in the plasma, red blood cells and white blood cells of man can be determined at present by using very small quantities of material (18). The plasma levels in man are so low (0.5–1.0 mcg/100 ml plasma) that available methods are not sufficiently sensitive. The red blood cells contain higher levels (6–9 mcg/100 ml) but they do not seem to lose their thiamine even under thiamine restriction compatible with clinical beriberi (20).

The relationship between urinary excretion and level of intake has been more extensively studied for thiamine than for any other vitamin. The 24-hour output, the 1-hour excretion on fasting, and the random specimen thiamine content per gram of creatinine have proved useful. They help to estimate the relative level of intake, particularly when comparing populations or when evaluating the effectiveness of dietary supplementation with thiamine.

Since the requirement for thiamine is dependant on the carbohydrate content of the diet, an increase in the latter without a proportional increase in thiamine intake can aggravate thiamine deficiency despite a dietary improvement. This was recently evidenced in children of a poor orphanage in Guatemala City (INCAP, unpublished data), when a marked improvement of the protein, vitamins and caloric content of their diet resulted in significantly lower urinary thiamine excretion.

Sebrell (79) has reviewed the abundant evidence for an adult thiamine requirement of 0.23 mg/1000 calories as recommended by the NRC. Subjects consuming less of the vitamin excrete very little of it in the urine (36). Unglaub and Goldsmith (94) have stated that if the mean 24-hour excretion is low in a group of individuals, potential or actual thiamine deficiency may be suspected.

A variety of load tests been have applied in the hope that they might measure tissue deficit (94). Although these tests reflect the intake of thiamine they do not identify clinical deficiency. Among the tests which have been proposed, Melnick's parenteral administration of 350 mcg of the vitamin per square meter of body surface, with determination of the thiamine excretion in the subsequent 4 hours (58), offers good possibilities for standardization. Load tests are, however, quite impractical for field studies. Measurement of the excretion of pyrimidine compounds has also been suggested by Pollack *et al.* (68) and Mickelsen *et al.* (59), but practical methods have not been developed.

Thiamine offers one of the best illustrations of biochemical nutritional pathology due to its deficiency. Lack of thiamine interferes with the entrance of pyruvate into the tricarboxylic acid cycle and this keto-acid tends to accumulate in the body fluids. An elevation of pyruvic acid in the blood is observed in thiamine deficiency, but lacks consistency and specificity (47). Stotz and Bessey (89) stated that the ratio of lactate to pyruvate is sufficiently altered in thiamine deficiency to be of diagnostic value. Horwitt (47) found basal blood levels of these compounds of limited value for estimating mild degrees of this deficiency, and developed a test based on the effect of glucose and exercise on the lactic and pyruvic acid blood levels. The results are expressed by an equation which relates the blood glucose, lactic and pyruvic acids, 1 hour after a dose of 1.8 g of glucose/kg body weight, and 5 minutes after completion of a standardized exercise. This is called the 'Carbohydrate Index.' Derangement in carbohydrate metabolism due to thiamine deficiency gives a high carbohydrate index. Figures below 15 are found in non-deficient individuals. This interesting approach to the diagnosis of thiamine deficiency has the disadvantage of being too elaborate for widespread application in field surveys. For the latter, thiamine excretion per gram of creatinine in single random urine specimens is a practical biochemical test which aids in the evaluation of thiamine nutritional status.

NIACIN

This water soluble vitamin differs from the others of the B-complex because an amino acid, tryptophan, serves as its precursor in the body of some animals, including man. According to Horwitt *et al.* (46) approximately 60 mg of tryptophan are equivalent to 1 mg of niacin. Another characteristic of niacin is that it is not excreted in the urine but is metabolized to at least two methylated derivatives, N^1-Methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Methylnicotinamide (pyridone). Goldsmith believes that the pyridone is the most important end-product of niacin metabolism (40).

Values for the concentration of diphosphopyridine nucleotide (DPN) for whole blood, serum and red and white blood cells of well-nourished adults were given by Burch *et al.* (21) using their own micromethod; in general, these confirm previous results with other methods. Morley and Storvick (61) give fasting levels of oxidized pyridine nucleotides in blood, N^1-Me in serum and the urinary excretion of a number of niacin metabolytes in 4 women under controlled dietary intake, supplying 8.7 mg of niacin and 770 mg of tryptophan daily. These data

are useful as a reference point for biochemical studies in states of deficiency or dietary restriction.

Klein *et al.* (52) indicate that the concentration of nicotinic acid in the blood cells of human subjects suffering from pellagra, and of dogs with 'black tongue' was not lowered. Vivian and co-workers (100) studied women on controlled niacin and tryptophan intakes. The lowest intake was 2.5 mg of niacin and 30 mg of tryptophan daily. The tryptophan intake was raised stepwise, and each level maintained for 6 day-periods. On daily intakes of 2.5 mg of niacin and 30 to 190 mg of tryptophan, the blood pyridine nucleotides (PN) decreased from 20 to 40% below control period levels. Increases in blood PN started at 270 mg of tryptophan intake and reached the values of the control 'non restricted' period on an intake of 670 mg. It is apparent that more investigations, under controlled niacin and tryptophan intakes, are required to establish the extent to which the blood levels of niacin, or its derivatives, are useful for the appraisal of the niacin nutriture of humans.

The urinary output of N^1-Me and pyridone decreases when the intake of niacin is restricted (74), an effect which is more marked and rapid on the pyridone (40). Low urinary excretion of niacin metabolytes is not necessarily indicative of clinical pellagra. Excretion levels compatible with 'adequate body stores of niacin' have been suggested by the preliminary studies of Goldsmith *et al.* (39). Their results indicate that on a 'corn diet' supplying 200 mg daily of tryptophan, an intake of 8–10 mg of niacin insures body niacin stores in human adult subjects.

The need for continuing such investigations is obvious. Studies should not consider the niacin content but the 'niacin equivalent' (46) of the experimental diets. The availability of methyl-donor compounds and the efficiency of the methylating process influence the formation of urinary niacin metabolytes independently of the balance between intake and need. Relative niacin nutriture may be appraised in field surveys by measuring the urinary excretion of niacin metabolytes in 24 hours, in a single random specimen in relation to creatinine or, as suggested in the *Manual for Nutrition Surveys* of ICNND (49), in 6 hours in a post-absorptive state. This manual recommends the determination of N^1-Me only, because of the relative complication of the assay for pyridone. The fact that normal persons may occasionally show a very low urinary excretion of niacin metabolytes and that under certain pathologic conditions an excess is excreted (36) makes their measurement unsatisfactory for evaluation of niacin nutrition in individual subjects.

The effect of the ingestion of doses of niacinamide on the urinary output of N^1-Me and pyridone has been explored. The principle that a high retention of the dose means a state of tissue depletion must be accepted with some reserve. As previously mentioned, impairment of methylation would also alter the excretion of these 2 niacin derivatives. This has been pointed out by Ellinger and Coulson (32) and is particularly applicable to tests in which a large dose is administered.

A variety of different conditions have been proposed for niacin load tests. The oral administration of 500 mg of the amide, followed by quantitative estimation of the extra 'trigonelline' plus nicotinic acid bodies, was early suggested (17, 65). Better knowledge of niacin metabolism has since resulted in more refined procedures. Unglaub and Goldsmith (94) favor using a relatively small dose of 10 mg of niacinamide followed by at least a 12-hour urinary collection. Another method proposed by Goldsmith *et al.* (38) is an interesting research tool to discover more about the relationship between the degree of tissue depletion and the urinary levels of niacin metabolytes. In essence, they measured the 24-hour excretion of N^1-Me and pyridone under a controlled diet, supplying 10 mg of niacin and 1 gm of tryptophan. Low excretions were characteristic of deficient patients as compared with normal subjects. An additional 10 mg of niacin to the basal diet resulted in a larger increase in excretion during the following 24 hours in the normal subjects; if a 10 mg supplement was continued for several days, adequately nourished subjects rapidly attained a high plateau in niacin metabolyte excretion while deficient patients required more time to reach maximum excretion.

For field surveys, estimation of niacin metabolytes per gram of creatinine in random urine specimens is recommended, since it contributes information corroborative of suggestive dietary and clinical data.

VITAMIN B_6

The wide and uniform distribution of vitamin B_6 among food stuffs has been pointed out by Snell and Keevil (83). In their opinion, few foods can be considered really poor sources. This fact explains why vitamin B_6 deficiency is not found as a naturally occurring nutritional disease. Nevertheless, interest in this factor has been recently aroused by observations suggesting an increased demand during pregnancy, and by the accidental occurrence of a serious outbreak of vitamin B_6 deficiency in infants fed a newly modified milk preparation, proved *a posteriori*, to be deficient in the vitamin.

Although the requirements have not been definitely set, figures around 0.03 mg/kg body weight/day for adults (105) and between 0.01 and 0.02 mg/kg body weight/day for infants under 6 months are accepted as approximate (42). Experimental observations of vitamin B_6 in man have been carried out, either by the administration of diets deficient in the factor or by the addition of a pyridoxine analogue. Typical of such studies is that of Snyderman *et al.* (84) who observed 2 infants given a diet low in vitamin B_6 for periods of 76 and 130 days. The subjects were mentally defective and the diet was an attempted therapy. Three biochemical features were observed: *a*) disappearance of pyridoxic acid from the urine, *b*) marked reduction of urinary pyridoxine excretion, and *c*) loss of ability to convert tryptophan to niacin.

Observations of adults in whom vitamin B_6 deficiency has been induced, either by the administration of desoxy-

pyridoxine (61, 97) or by a deficient diet (41), lead to the conclusion that the urinary excretion of xanthurenic acid without a tryptophan load is not a reliable index of early deficiency. On the other hand, the work of Greenberg *et al.* (41) shows that in adult subjects, only about 2 weeks on a synthetic diet devoid of B_6 are sufficient to produce a ten-fold increase in the response of xanthurenic acid excretion in 24 hours to a 10 gm dose of DL-tryptophan. The authors consider this "one of the earliest detectable manifestations of pyridoxine deficiency."

Chiancone (24) has defined conditions for a tryptophan load test which can be used as a biochemical index of vitamin B_6 deficiency in an early stage. Tryptophan in a single dose of 100 mg/kg body weight is administered to the subject and the amount of xanthurenic acid excreted during the following 24-hour period, expressed as the percentage of the dose of tryptophan, is called the 'Xanthurenic Acid Index.' Indices not exceeding the value of one are regarded as normal (95). Whether this or other experimental conditions should be chosen is secondary to the necessity of adopting a standardized technique in order to obtain comparable results. It is important to investigate the excretion curve of xanthurenic in detail after the test dose of tryptophan in order to see if shorter collection periods would give information as useful as that obtained with the 24-hour collections. This question is of obvious practical importance.

The work of McGanity *et al.* (56) suggests that the changes in blood urea after a load dose of alanine might be related with vitamin B_6 nutritional status. An abnormal blood urea curve was found in patients with hyperemesis gravidarum after a dose of 30 gm of DL-alanine; the levels of urea rose reaching a maximum at the 6th hour, but remained elevated for 12 hours, while in normal pregnant and non-pregnant women they decreased to the pre-test levels in that time. The administration of pyridoxine to the hyperemesis gravidarum patients corrected the abnormal response to the test.

Vilter *et al.* (97) showed the same type of abnormal response in 2 of 3 subjects receiving desoxypyridoxine to induce pyridoxine deficiency. Abnormal tests coincided with the presence of clinical manifestations of deficiency. The patient who showed no clinical deficiency, although receiving the vitamin antagonist, responded normally to the test at all times. The test, therefore, seems to reflect well-advanced deficiency but, apparently, is of no value for detecting it at an early stage.

In a recent work, Wachstein *et al.* (102) measured the concentration of plasma pyridoxal phosphate in subjects at different time intervals up to 24 hours after an oral dose of 100 mg of pyridoxine hydrochloride and found a lower response in pregnant women during the last trimester of gestation than in normal men and non-pregnant women. This agrees with previous results suggestive of an altered vitamin B_6 metabolism in pregnancy (101) and also illustrates another test, the results of which might be interpreted as reflecting relative tissue saturation in a similar manner to the load tests for other water-soluble vitamins. Since only one sample of blood is necessary 24 hours after the pyridoxine dose, this test may have a practical advantage over the tryptophan load test which requires the collection of 24-hour urine specimens.

Recently, Babcock *et al.* (8) have shown that subclinical deficiency of vitamin B_6 in adults reduces the serum glutamic-oxalacetic transaminase. Measurement of the increase in this serum enzyme following administration of vitamin B_6, "provides an indication that the vitamin B_6 supply had been inadequate for optimum protein metabolism." The test is less sensitive than the xanthurenic acid excretion test after a tryptophan load.

Research is needed on laboratory methods to measure vitamin B_6 as a complex and determine its three biologically active forms, pyridoxine, pyridoxal and pyridoxamine (80, 82). More practical procedures, particularly in micro-scale, applied to blood and other biological materials would facilitate further studies of the nutritional biochemistry of this vitamin.

CALCIUM

Among the biochemical abnormalities which appear as manifestations of severe calcium deficiency is a decrease in the concentration of calcium in serum, which may reach values of less than 4 mEq/liter (35). When this occurs tetany and other clinical signs become obvious so that the biochemical index has only confirmatory diagnostic value. The maintenance of plasma calcium concentration is accomplished through a mechanism in which several factors, each more important than moderate variations in dietary intake, are operating (50). Investigation of the relationship between dietary inadequacy of calcium and its possible biochemical indexes is further hindered by the fact that the optimal intake of this mineral is unknown (43).

IRON

When iron is not available to the body in sufficient quantities, because of either inadequate intake or excessive loss, several types of biochemical alterations have been shown to occur. The best known manifestation of biochemial pathology is an impairment in the biosynthesis of hemoglobin. The clinical entity is, of course, iron deficiency anemia. In the laboratory this is easily detected by determining the concentration of hemoglobin in the whole blood, aided by the simple microscopical study of stained blood smears.

Anemia, however, is an advanced manifestation of lack of iron. In view of the great importance of iron in other fundamental biochemical systems, such as the cellular electron-transport chain, interest is beginning to develop in the study of possible alterations in the efficiency of this and other iron-dependent mechanisms. Evidence from animal studies (13) supported by clinical observations (15) led Beutler to suggest that some of these mechanisms might suffer even before hemoglobin levels become subnormal. Although no practical biochemical method for evaluation of iron deficiency based on these changes

yet exists, such studies are important contributions to the definition of 'adequacy of intake' and 'requirement' for this mineral in terms of biochemical pathology which occurs earlier than blood changes.

Early depletion of iron body reserves may be detected directly, by the estimation of hemosiderin in the bone marrow (72). The relative difficulty of obtaining bone marrow specimens under field conditions, however, makes this method an impractical approach to the evaluation of iron nutrition in population groups.

A lowering of the plasma iron levels is, in general, a manifestation of relative lack of available iron. Although in some specific instances this may be due to pathologic conditions such as infection, the occurrence of generalized reduced plasma iron levels is presumptive evidence of latent deficiency. It may be recommended that in surveys, tests of iron-binding capacity of plasma (71) be made in a selected subsample. Since increased binding capacity is a primary characteristic of uncomplicated iron deficiency (14, 26), it would confirm the nutritional origin of low iron values.

Hypoproteinemia, due to protein malnutrition (kwashiorkor in children), may disguise the signs of iron deficiency. There is a suggestion that this condition produces a decrease in the specific iron-binding B-globulin of plasma, and consequently a reduced iron-binding capacity is found simultaneously with low plasma iron levels (53). In these cases, however, the signs characteristic of a typical iron deficiency anemia appear when therapeutic diets low in iron are given (77).

IODINE

When insufficient iodine is available to the thyroid gland, this endocrine organ undergoes hyperplasia and hypertrophy, which in turn helps to maintain the supply of thyroid hormone of the body within limits compatible with normal metabolism. Decreased iodine availability to the gland may result directly from insufficient iodine intake or indirectly from the presence of goitrogenic factors, usually of food origin (25, 27, 93). The latter interfere with the normal uptake and utilization of iodine by an otherwise normal thyroid, although the actual mechanisms apparently vary with different compounds.

The 24-hour urinary excretion of iodine has been used to estimate the dietary intake. At least, the amounts excreted by persons in goitrous regions are markedly lower than those in nongoitrous areas (27). The work of Stanbury (85) clearly demonstrated an inverse correlation between the uptake of labeled iodine and urinary iodine excreted in euthyroid patients in the endemic area of Mendoza, Argentina.

Although the iodine bound to serum proteins reflects the functional state of the thyroid gland, its value as an indicator is very limited because goiter patients in endemic areas are generally euthyroid. It would be logical to expect that, in the initial stages of the development of goiter, an individual might develop a relative hypothyroidism before compensation occurred. This is suggested by the study of Scrimshaw *et al.* (76) who found that the relatively low-protein-bound iodine values found in a group of Central American children increased with the administration of potassium iodide or iodate simultaneously, with a decrease in the prevalence of goiter. To elucidate the value of this index more studies should be carried out of plasma protein-bound iodine in children living in endemic goiter areas.

The quantitative relationship between iodine intake and excretion and the presence of goiter is influenced by several physiologic, environmental and dietary factors. Research directed towards the study of this relationship should be conducted in different population groups with and without endemic goiter. Methods are needed to assess iodine nutrition at a subclinical level.

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