Current knowledge concerning optimum nutritional status of riboflavin, niacin and pyridoxine

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The term 'optimum nutrition' has evolved from a perceived need to base recommendations for nutrient intakes firmly in the context of function. It follows that 'optimum nutritional status' for individual nutrients should be defined in terms of biochemical or physiological markers having some functional value but also showing an appropriate relationship to nutrient intake. The present short review considers the current position regarding such markers for riboflavin, pyridoxine and niacin. It is concluded that whilst there are several biochemical measures which respond to changes in intake of each of these vitamins, no single measure is wholly satisfactory as a marker of optimum status.

Riboflavin status: Niacin status: Pyridoxine status: Markers of nutrient status

The objective is to consider how best to express optimum nutritional status in terms of biochemical or physiological markers, having, if possible, some functional meaning.

The questions that need to be addressed for each vitamin are:

(1) what are the current biochemical and/or physiological markers of nutrient status?
(2) is there a well-defined relationship between the marker and nutrient intake?
(3) to what extent can the biochemical marker be said to reflect some specific function in a target tissue?
(4) are there any promising developments that may help our search for functional markers for these vitamins?

Riboflavin

What are the current biochemical and/or physiological markers of nutrient status?

This vitamin is consumed in the diet as free riboflavin and two phosphorylated forms, FMN and FAD. Absorption occurs as riboflavin, followed by intracellular phosphorylation in the enterocytes. Phosphorylation and dephosphorylation are features of intracellular metabolism, and all three forms are found intracellularly. The most common vitamer in tissues and in the circulation is FAD. The tissue riboflavin pool is probably best evaluated by measuring blood FAD by HPLC using fluorescence detection (Florida *et al*. 1985). An alternative approach, but with obvious practical limitations, is the measurement of urinary riboflavin, which is a simple reflection of an excess of current intake over tissue requirements (Zempleni *et al*. 1996). This latter approach has provided useful information regarding the relationship between riboflavin intake and tissue saturation (Horwitt, 1986), of value in early studies of requirements for this vitamin. The method generally preferred for the estimation of riboflavin status is the stimulation of FAD-dependent erythrocyte GSH reductase (*EC* 1.6.4.2) *in vitro*, which relies on an associated oxidation of NADPH which can be readily monitored spectrophotometrically (Bates *et al*. 1986). Results are expressed as an activation coefficient (EGRAC).

Is there a known relationship between the marker and nutrient intake?

Plasma or erythrocyte FAD. Reasonable correlations have been demonstrated between plasma or erythrocyte flavin concentrations and other biochemical markers of riboflavin status in human subjects, although there is relatively little information regarding the tissue response to graded intakes of riboflavin. Plasma and urine riboflavin concentrations increased progressively in low-birth-weight infants during 4 weeks of enteral feeding (Porcelli *et al*. 1996), and erythrocyte riboflavin concentrations are reported to increase as quickly as EGRAC in response to

riboflavin supplementation (Beutler, 1969). A recent study of the pharmacokinetics of flavin uptake in human subjects indicated an upper limit of absorption from a single dose of 27 mg and demonstrated relatively modest changes in plasma riboflavin or flavocoenzymes following oral administration (Zempleni *et al*. 1996).

Animal studies are supportive of a relationship between dietary riboflavin and tissue flavin concentrations (Williams *et al*. 1996*a*), but erythrocyte flavins have received little attention.

Urinary riboflavin. The earliest attempts to associate dietary riboflavin intake with a biochemical marker utilized the urinary excretion of riboflavin (Horwitt *et al*. 1950). Balance studies in human subjects show clearly that as riboflavin intake increases there is a progressive rise in urinary excretion of riboflavin, which increases sharply at intakes of approximately 1·0 mg/d (Horwitt, 1986). This intake has been interpreted as reflecting tissue saturation. Although this approach has practical limitations for large population studies, because it provides information concerning tissue 'stores', it can arguably be useful as a marker of optimum nutritional status. This approach is less useful as a marker of riboflavin status at lower intakes.

Erythrocyte GSH reductase activation coefficient. Numerous studies have demonstrated a relationship between riboflavin intake and the extent to which erythrocyte GSH reductase can be stimulated by FAD in vitro (Bates et al. 1982, 1989; Boisvert et al. 1993). EGRAC is most sensitive to changes in riboflavin intake up to levels of intake approaching tissue saturation of this vitamin (about 1·0 mg/d on the basis of urinary excretion studies). However, some studies have highlighted differences in the relationship between intake and EGRAC values among different population groups (Bates et al. 1982; Belko et al. 1982). The measurement can broadly discriminate between levels of intake observed in an apparently-healthy population. Further increases in intake elicit no further fall in EGRAC, which limits its usefulness as a potential marker for optimum nutritional status.

To what extent can the biochemical marker be said to reflect some specific function?

Although EGRAC is sometimes referred to as a functional test of riboflavin status, it does not fulfill the most rigorous definition of this term. Over a broad range of EGRAC values in human subjects there is no well characterized change in any physiological function, or, indeed, altered flux through a metabolic pathway. Effects on work performance, neuromuscular coordination, and the handling of Fe have been observed in human subjects with EGRAC values of 1·7 and higher, and there is an element of riboflavin responsiveness of these variables, supportive of a cause–effect relationship (Bates *et al*. 1982, 1994; Prasad *et al*. 1990; Fairweather-Tait *et al*. 1992). Studies in animals confirm a dose–response relationship between riboflavin intake, EGRAC value and Fe handling (Powers *et al*. 1991) and have also identified impaired fatty acid oxidation, which is exacerbated by starvation-induced flux through fatty acid oxidation pathways (Bates, 1990; White, 1996) in animals fed on diets low in riboflavin. EGRAC values were not always reported, but the nature of the dietary restriction is strongly suggestive of severe deficiency. So far, attempts to reproduce effects of low riboflavin intakes on fatty acid oxidation in human subjects have not been successful (Bates *et al*. 1991). Dietary riboflavin restriction around weaning disturbs normal gastrointestinal development in rats (Powers, 1995; Williams *et al*. 1996*b*; Yates & Powers, 1999); there may be a human corollary which could explain effects of dietary riboflavin restriction on growth.

In the UK population the evidence is that the elderly remain vulnerable to poor riboflavin status, expressed biochemically (Bailey *et al*. 1997). Of particular relevance to this group of the population may be the relationship that has been reported in several studies between dietary riboflavin, riboflavin status and lens opacity (Leske *et al*. 1995).

Current developments

A method has been proposed for the measurement of plasma riboflavin by titration of plasma against riboflavin-binding apoprotein; it is not yet clear whether this approach will offer any particular advantage over current methods of assessment (Kodentsova *et al*. 1995).

Thus, although low intakes of riboflavin will disturb a number of distinct physiological or biochemical processes, the effects are not sufficiently sensitive to riboflavin intake to be useful as functional markers of status. However, they may be useful to interpret EGRAC values.

Niacin

What are the current biochemical and/or physiological markers of nutrient status?

Niacin is a generic term for nicotinic acid and nicotinamide, both of which are substrates for the synthesis of nicotinamide nucleotide coenzymes, NAD and the phosphorylated derivative NADP. The vitamin is found in the tissues predominantly as the nicotinamide nucleotides. Biochemical assessment of niacin status is complicated by the fact that tryptophan is a precursor of niacin, and the availability of this amino acid will influence dietary niacin requirements. The concentration of erythrocyte and plasma NAD and NADP, and the urinary excretion of niacin metabolites have been explored as markers of niacin status, with variable results (Vivian *et al*. 1958; Nakagawa *et al*. 1969; Srikantia *et al*. 1986). More recently, the activity of nicotinate-nucleotide: pyrophosphate phosphoribosyltransferase (*EC* 2.4.2.11; NNPPRT) in erythrocytes has received attention (Fu *et al*. 1989).

Is there a known relationship between the marker and nutrient intake?

Erythrocyte nucleotides. Fu *et al*. (1959) have shown very clearly that the erythrocyte concentration of NAD is sensitive to relatively short-term changes in niacin intake in young men. NADP on the other hand showed no significant response to differences in niacin intake over the range of 6·1–32 niacin equivalents. This relative insensitivity of erythrocyte NADP to niacin intake has also been reported in

other studies in human subjects (Gross *et al.* 1960). NAD : NADP in erythrocytes or whole blood may be a useful variable when there are disturbances to haemoglobin synthesis or erythrocyte production. If total pyridine nucleotides in blood are measured it is to be expected that concentrations would fall as niacin intakes fall, although this decrease would be due almost entirely to changes in NAD.

Erythrocyte nicotinate-nucleotide: pyrophosphate phosphoribosyltransferase activity. The activity of NNPPRT in erythrocytes has been explored as a potential marker of niacin status. This enzyme catalyses the conversion of nicotinic acid to nicotinic mononucleotide. Although enzyme activity has been shown to increase in response to niacin depletion in pigs (Arienti *et al.* 1982), such an effect was not seen in rats (Shibata & Matsuo, 1989), and activity was not found to be responsive to changes in niacin intakes in human subjects over a short period (Gross *et al.* 1960).

Urinary metabolites. In an experimental situation the 24 h urinary excretion of N′ methylnicotinamide, which is the main urinary metabolite of this vitamin, is seen to closely mirror changes in the dietary intake of niacin (Sauberlich, 1984). The lack of any apparent lag stage between changes in intake and changes in urinary excretion of this metabolite suggests that the latter may not be a sensitive reflection of tissue levels. Other metabolites of niacin are excreted in the urine, and a few studies have explored their relationship with niacin intakes. Two such metabolites are N′ methyl-2-pyridone-5-carboxamide and N′ methyl-4-pyridone-3-carboxamide. The correlation between niacin intakes in subjects on a self-selected diet and the urinary excretion of these two metabolites, expressed either as creatinine-corrected concentration or 24 h excretion, was similar for these two metabolites and better than that for N′ methylnicotinamide (Shibata & Matsuo, 1989). There have been significant developments in the analytical methods available for measuring these urinary metabolites, which makes them especially attractive as markers of niacin status.

To what extent can the biochemical marker be said to reflect some specific function?

In the absence of functional variables specifically responsive to niacin intake, niacin status is currently synonymous with the concentration of the vitamin or metabolites in the plasma or erythrocyte or urine. There has been some interest recently in the possibility that low intakes of niacin equivalents may limit DNA repair following oxidative damage, through an effect mediated by NAD-dependent poly(ADP-ribosyl)ation (Rawling *et al.* 1996). This finding may have implications for carcinogenesis, and for this reason could attract future attention.

Pyridoxine

What are the current biochemical and/or physiological markers of nutrient status?

The term vitamin B_6 encompasses three pyridines: pyridoxine (also called pyridoxol), pyridoxal and pyridoxamine, and their 5′-phosphorylated derivatives. The main form of the vitamin is pyridoxal phosphate (PLP) which once formed intracellularly is bound to enzymes for which it has cofactor activity. PLP is also the main extracellular form of the vitamin, being transported in plasma bound to albumin. PLP which is not enzyme-bound is oxidized to pyridoxic acid, which is excreted in the urine.

Widely-used techniques in current use for evaluating vitamin B_6 status include the measurement of circulating PLP (Lumeng *et al.* 1978), urinary pyridoxic acid (Brown *et al.* 1975; Leklem, 1990), and the activation coefficient for the erythrocyte enzyme aspartate aminotransferase (*EC* 2.6.1.1; Brown *et al.* 1975; Fidanza, 1991).

Is there a known relationship between the marker and nutrient intake?

Plasma pyridoxal phosphate. Studies in weanling rats have shown that plasma PLP levels increase progressively with increasing intake of vitamin B_6, in a similar fashion to muscle, which represents the largest site of tissue vitamin B_6 (Lumeng *et al.* 1978). In contrast, other tissues appear to be saturable with PLP. In another rat study (Takami *et al.* 1968) plasma PLP was closely correlated with muscle glycogen phosphorylase (*EC* 2.4.1.1), which lends some functional significance to the measurement of the plasma vitamer.

Other studies in human subjects and animals are supportive of PLP in plasma as having diagnostic value for vitamin B_6 status (Brown *et al.* 1975; Sampson & O'Connor, 1989). In rats, plasma levels of PLP are more responsive to dietary vitamin B_6 depletion than tissue levels, except for erythrocyte PLP, which also responds quickly to depletion. Plasma PLP levels achieved in adults at intakes of vitamin B_6 between 0·8 and 10 mg/d give a reasonably consistent picture: plasma PLP of above 25 nmol/l is achieved at intakes greater than 1·0 mg/d. When intakes rise to 10 mg/d plasma PLP rises to 200 nmol/l (Leklem, 1990).

The question is: are there any measurable benefits associated with this higher circulating level, and are there functional limitations imposed by levels less than 25 nmol/l?

Urinary 4-pyridoxic acid. The amount of 4-pyridoxic acid excreted in the urine daily is a reflection of the irreversible oxidation of pyridoxal (from PLP) taking place in tissues. The daily excretion of 4-pyridoxic acid responds rapidly to dietary depletion and repletion (Brown *et al.* 1975; Leklem, 1990); in subjects among whom daily intakes do not fluctuate significantly, urinary excretion of 4-pyridoxic acid offers a useful indicator of the adequacy of vitamin B_6 status. This marker is not sensitive to changes in vitamin intake when intake is low. Urinary 4-pyridoxic acid can be measured in a single fasting urine sample and expressed relative to urinary creatinine. However, current reference ranges say nothing about the functional significance of abnormally low excretion rates. There is current interest in the potential of plasma pyridoxic acid as a marker of vitamin B_6 status.

Erythrocyte transaminase activity. The activities of erythrocyte transaminases are PLP-dependent; this measurement provides the basis for the indirect measure of vitamin B_6 status, and at the same time is considered to offer a more functional approach to assessment than levels of circulating

vitamers. The activity of either alanine (*EC* 2.6.1.2) or aspartate aminotransferase is measured in the absence and the presence of added PLP in an erythrocyte haemolysate; the resulting ratio of the activities *in vitro* is expressed as an activation coefficient, which increases with decreasing saturation of the endogenous enzyme. There does not seem to be a consensus regarding which of these two enzymes offers the more sensitive index of vitamin B_6 status, or the speed with which the enzymes respond to changes in vitamin B_6 intake (Brown *et al.* 1975; Fidanza, 1991).

To what extent can the biochemical marker be said to reflect some specific function?

Reference ranges can be determined for a population group under study, using particular assay conditions, but there has not been any convincing demonstration of consistent functional abnormality (in a target tissue) associated with enzyme activation values falling outside the reference range. In fact none of the methods currently in use for the assessment of vitamin B_6 status satisfy the criteria for a functional marker in human subjects. Several authors call for the assessment of vitamin B_6 status using more than one biochemical variable; there is reported to be some lack of concordance between certain indices, presumably because each may be influenced differently by factors other than vitamin B_6 intake (Leklem, 1990; Bender, 1993; Kang-Yoon *et al.* 1995).

Some growth advantages of maternal supplements of vitamin B_6 (pyridoxamine hydrochloride) were indicated in a small study of newborn infants (Kang-Yoon *et al.* 1995). In this study plasma and erythrocyte PLP concentrations and the activation coefficient for alanine aminotransferase correlated with calculated daily intakes of vitamin B_6.

Other potential markers. Ornithine decarboxylase (*EC* 4.1.1.58) activity in selected tissues has received some attention as a potential functional indicator of vitamin B_6 status, but so far there is no evidence that it is sensitive to vitamin B_6 status (Sampson *et al.* 1995)

Homocysteine. There is current interest in the factors which determine plasma levels of homocysteine, as moderate elevations of this amino acid are associated with an increased risk of cardiovascular disease (Stampfer *et al.* 1992). As the metabolism of intracellular homocysteine is either by methylation (folate and vitamin B_{12}-dependent) or by trans-sulfuration (vitamin B_6-dependent), studies have focused on the homocysteine-lowering potential of these vitamins. The current impression is that folic acid status is a major dietary determinant of plasma total homocysteine, and the latter is amenable to folate supplementation (Dudman *et al.* 1993; Ubbink *et al.* 1994). Vitamin B_6 status does not appear to have a strong independent effect on plasma homocysteine (Verhoef *et al.* 1996). Plasma total homocysteine does not appear therefore to offer a specific functional marker of vitamin B_6 status.

Conclusions

There are several biochemical measures which respond to changes in dietary intake of riboflavin, niacin or pyridoxine, and each has some value as a marker of vitamin status.

However, markers for a single vitamin differ in terms of specificity and their sensitivity at low or high intakes. Thus, there is no single marker that is wholly satisfactory as a marker of a specific vitamin status. For a measure to be useful as a marker of optimum status it should be particularly sensitive to changes in vitamin intake at the upper end of the physiological intake range. Thus, urinary metabolites would be expected to have the greatest potential. Practical limitations, as well as interference with the relationship between intake and excretion by other factors, such as infection and exercise, have to be taken into account, and generally measurements of urinary metabolites have not been the methods of choice in recent studies of vitamin status. The so-called functional erythrocyte enzyme tests of riboflavin and pyridoxine status are not really satisfactory as markers of optimum nutrition as they are least sensitive at higher intakes. For riboflavin, at least, functional significance can be attached to enzyme activation coefficient values outside the normal range.

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