Assessment of Micronutrient Status in Mothers and Young Infants

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Introduction

Public health decisions are seldom straightforward. An example of the complexities that may surface when a potentially beneficial intervention has been identified, is the recent experience of attempts to introduce flour fortification with folic acid in the UK. Following the definitive Medical Research Council intervention trial [1] more than a decade ago, which demonstrated a massive reduction in the risk of neural tube defect (NTD) recurrence in high-risk mothers by giving folic acid supplements, subsequent studies have confirmed the benefits of folic acid against first, or population-level, occurrence of NTDs [2, 3]. Introduction of flour fortification in North America has resulted in clear, indeed surprisingly large increases in blood folate levels in this population [4], and is reported to have been accompanied by a reduction in NTD incidence [5], although the investigators urge caution because the interpretation is not necessarily straightforward. However, an early adoption of this intervention in the UK now seems unlikely. The most widely quoted concern is the theoretical possibility that raised population folate intakes may mask or exacerbate early stage vitamin B$_{12}$ deficiency, especially in older people [2]. Although there are many more older people in the ‘at-risk’ category for vitamin B$_{12}$ deficiency than there are women ‘at risk’ of NTD occurrence today in the UK, the risk to older people is unquantified and probably quite small. Indeed, many older people with marginal folate status may benefit from folic acid fortification, for instance by a reduction in plasma homocysteine which in turn is associated with vascular disease risk [2]. Another concern regarding the desirability of fortification has arisen from the observation that twin fetus frequencies may be increased by folic acid
supplements, and that this, in turn, may be associated with increased risk of unfavorable pregnancy outcome [6]. Again, the risk is difficult to quantify, and it may be offset by decreased risk resulting from better folate status. When debated in a public forum recently, the concerns that were mainly voiced about fortification included ‘adulteration of natural food’, ‘limiting consumer choice’ and (for the bakers’ representatives) ‘cost of implementation and risk of litigation arising from possible cases of suspected harm’. What had appeared to be a straightforward potential benefit for the consumer has thus proved unexpectedly contentious!

Another observation illustrates what is, I suspect, a typical developing country public health problem. It concerns the ongoing and largely successful intervention program for the elimination of iodine deficiency world-wide. The Gambia, in West Africa, is no more than 30 km wide for most of its 300 km length, and is almost completely surrounded by Senegal. Although iodine deficiency has not, in the past, been highlighted as a major public health problem there, goiters have been recorded especially in the eastern division, which is the furthest from the sea and from foods of marine origin. However, most of the salt that is used for human consumption is imported in donkey-carts from Senegal, by traders who enter at many uncontrolled points along the 600-km-long border. For various reasons, a recent attempt by UNICEF to provide salt iodination equipment for use in Gambian salt production plants was rejected as probably unsustainable and difficult to monitor. Most of the salt produced in the existing small-scale salt production plants near the coast in the west of the country was used locally in animal feed, and it would not have contributed to human iodine intake in the areas where iodine deficiency disorders (IDDs) occurred.

These two recent experiences illustrate the practical problems that can arise in the course of planning public health interventions. My contribution to this workshop will focus principally on the available techniques for the measurement of micronutrient status in mothers and young infants. It is a subject which, I believe, helps to provide the first essential rung of the evidence-base for the estimation of the prevalence of deficiencies, and then later for the monitoring of the efficacy of interventions. Since it is first necessary to define the problem, then to develop the tools for its investigation, and finally to use them to try to solve the practical problems, I hope that my contribution will be relevant to some of these challenges.

**Characteristics, Needs, and Uses for Micronutrient Biochemical Status Indices**

The measurement of specific micronutrient status indices links estimates of intakes of food and supplements (and hence nutrient intakes), and evidence of health status and the outcome of physiological processes,
including those of pregnancy, lactation and early development in infancy. The three parameters (intake, biochemical status and outcome) all represent different facets of an integrated process, and ideally all three should always be measured. In practice, biochemical status is sometimes substituted for intake estimates, either because it is quicker, easier and more reliable to carry out at the logistically demanding fieldwork end of the study, or because it provides information that is better averaged over time, or is physiologically closer to the end-product of functional outcome. The different categories of measurement give complementary viewpoints of the whole integrated process, and a raft of different measurements can often give a more informative picture than a single choice of index can.

The investigation of a new hypothesis linking specific nutrient inadequacies (or inadequate diets) and physiological outcomes often begins with the identification of cross-sectional relationships between biochemical status measures (or diet estimates) and the specified outcomes either within a ‘representative’ population, or by comparison between two or more contrasting population groups [7]. If a significant cross-sectional relationship is observed, then the question of possible causality (i.e., does the nutrient really affect the outcome?) needs to addressed. Unfortunately, there are many dangers of wrong conclusions arising from confounding influences which are often difficult or impossible to separate and correct for, statistically. The next rung of the causality investigation ladder may be a prospective study, where nutritional factors (intake and/or status) are assessed before the key outcomes occur. In practice this can often be made more efficient by collecting and storing the samples or raw data, and then doing the expensive analytical work on only a sub-sample of these, thereby greatly increasing the statistical power. This type of study can eliminate some of the ambiguities of interpretation that arise from cross-sectional studies, especially where the outcome or an underlying condition that influences the eventual outcome may also affect dietary choices and status patterns. However it cannot prevent all types and possibilities of confounding.

The gold standard (or the closest that is available) is the randomized, placebo-controlled, double-blinded intervention trial (RCT), in which randomized, matched groups of subjects are exposed to the alternative nutritional inputs (these may, for instance, be micronutrient supplements), during a defined time period before the physiological result (e.g. pregnancy outcome) is anticipated. If performed properly, this can avoid most of the artifacts that are inherent in the other two approaches. Unfortunately it is also the most logistically difficult and costly, and this has, in the past, resulted in many of the RCTs that are of nutritional interest being under-powered [7, 8]. Often, too few subjects have been used to detect an important but small difference in outcome. Although several small trials can sometimes be ‘combined’ or compared by meta-analysis, this only works well if they have all addressed similar populations and are similar in design. Other practical
problems may arise with RCTs: they may be deemed ethically unacceptable if they deny to any subjects a treatment which is believed to be beneficial (even if this has never been formally proven), and poor compliance may compromise their efficiency and interpretation both by affecting the power of the trial if the drop-out rate is high, and by causing treatment category misclassification if the monitoring process for compliance is imperfect. In trials with micronutrient supplements in Western countries it is, for instance, advisable to monitor and control the purchase of over-the-counter nutrient supplements.

Experimental animal and in vitro studies may be useful in complementing human population studies and RCTs, especially with respect to the elucidation of biological mechanisms and the understanding of nutrient-effector interactions at the cellular and subcellular level. However, species differences in metabolism usually make it impossible to predict human population outcomes with confidence from animal or in vitro studies. This is especially true for the subtle changes in functional status that are typically achievable by nutrient supplementation programs.

Biochemical status index measurements provide an important element of the information which is needed to interpret cross-sectional and prospective nutritional studies, and RCTs. It is clearly important, not only to seek significant correlations or characteristic differences between specific status measurements and specific outcomes, but also to characterize the populations being studied in terms of their underlying nutritional status. Many of the published studies relating micronutrients to pregnancy outcomes or to growth and health performance indicators in infants have been carried out in industrialized countries where any undernutrition is generally mild. Fewer studies have been undertaken in Third World countries where more severe and widespread nutrient deficiencies occur. An important feature, especially of zinc intervention studies for example, is that a significant benefit (e.g. increased growth rate of infants) may be confined to a specific high-risk subgroup of the population. Subgroups that respond significantly to zinc supplements, for instance, are those that have initially low serum zinc levels or those which are small for their age. The remainder of the population is likely to be unresponsive to the intervention. This illustrates the intuitive prediction that the choice of population is critical, and that any generalizations extrapolating from one population or group to another must be made with caution.

In RCTs, biochemical status assays should therefore be used to assess pre-trial status, and to monitor compliance with the intervention, and the adequacy of the biochemical response to it, as a prerequisite for expecting to see a physiological response or a health benefit. The efficacy of an intervention, both at the biochemical and physiological or health level, is likely to vary, not only with the presence or absence of any preexisting nutrient deficiency, but also with the size, frequency and perhaps the composition and mode of delivery of the nutrient dose. Its absorption, tissue distribution and metabolic turnover
also need to be considered, and all of these factors may impinge on the response patterns.

There is a hierarchy of blood-level responses for different micronutrients. At one extreme, plasma concentrations of minerals such as calcium, zinc and copper are homeostatically controlled and vary little if at all with moderate variations in intake over the physiological range. Variations in plasma albumin concentrations account for much of the variance in Ca and Zn, and the acute phase reaction has a major effect on both Zn and Cu. For these micronutrients, biochemical measurements may be useful for risk group selection and in some cases for intervention compliance checks, but their usefulness is limited. Information about status adequacy for these nutrients has come mainly from RCTs, because the question: ‘is a particular population or subgroup adequately supplied?’ – can only be answered by studying functional responses, such as improved growth or reduced susceptibility to environmental insults. For these mineral nutrients, in the UK at least, there is essentially no inter-person correlation between plasma levels and intake estimates in survey samples.

At the other extreme, for a water-soluble vitamin such as vitamin C, whose concentration in plasma is not under strong homeostatic control, plasma levels vary directly with intakes over a wide range. In this case, the plasma levels approach an upper plateau only at high intakes, where a ceiling of plasma concentration is accompanied by an increase in urinary excretion. Vitamin A occupies an intermediate position. When hepatic vitamin A stores are low, plasma retinol concentrations directly correlate with hepatic stores and are a good index of status, but at normal-to-high store levels, the availability of retinol-binding protein becomes the limiting factor, and plasma retinol concentrations no longer reflect the size of the hepatic store. Indeed at very high intakes, a different biochemical index, namely that of the retinyl esters associated with plasma lipoproteins, becomes the preferred index of the risk of toxicity from vitamin A overload.

Implicit in the discussion above, is the conclusion that there exists a continuum of micronutrient status indices, progressing from those which are purely biochemical and which measure the concentrations of specific nutrients in body fluids, through to those which measure much more general physiological outcomes. In between, there exists a raft of indices that are based on specific nutrient-dependent enzyme reactions. These contain a limited degree of ‘functionality’ because they help to link specific nutrients with the functional integrity of specific biochemical pathways. Examples include the erythrocyte enzymes: transketolase measuring thiamin status; glutathione reductase measuring riboflavin status; keto acid aminotransferases for vitamin B6 status; glutathione peroxidase for selenium status, and superoxide dismutase for copper and zinc status. For their activities, all of these enzymes depend on specific micronutrient-derived cofactors. Another status-assessment option is to use selected products or intermediates of specific
micronutrient-dependent enzyme-catalyzed pathways. Some of these are very specific for a particular nutrient, e.g. plasma or urine methylmalonic acid, which is specifically responsive to changes in vitamin B\textsubscript{12} status. Others, like plasma homocysteine, may respond to changes in status of several B vitamins.

Physiological functions may also respond either specifically to a single nutrient, or more broadly to a number of different nutrients and perhaps to other environmental influences. Goiter, for example, represents essentially a nutrient-specific response to functional iodine deficiency. Dark adaptation defects are usually specific for vitamin A deficiency, but can sometimes arise from zinc deficiency in cases where vitamin A utilization is affected by a limited supply of zinc [9]. Other physiological indices such as birth weights, degrees of prematurity, growth rates in infancy, etc., can be modulated by many external influences, both nutritional and non-nutritional; therefore their specificity towards any individual nutrient is low. Nevertheless, because of their considerable physiological importance it may be highly relevant and desirable to include them in population studies and RCTs. This raises another problem. Although one particular nutrient may be both deficient and rate-limiting (e.g. for growth) in a particular individual, other nutrients may be only marginally adequate, and can then become rate-limiting when the most severe deficiency is corrected. In such a situation, a multinutrient supplement is likely to be more effective than a single nutrient. Thus in the context of pregnancy outcomes, a multivitamin supplement given to HIV-infected pregnant women in Tanzania improved birth weights, selected T-cell counts and other functional indices, whereas vitamin A alone did not [10]. This poses the question: is it best to provide a ‘blunderbuss’ multinutrient supplement, on the basis that some of its components will be beneficial and none are likely to be harmful, or is it better (and is it feasible) to identify, and to provide only the most high-risk nutrients? Another practical question is the effectiveness of infrequently provided high-dose supplements, such as those used for vitamin A and iodized oil, which are stored for many weeks or months in the body. This approach is clearly less feasible for those nutrients, like the water-soluble vitamins, whose life-span in the body is much shorter.

Interpretation of nutrient status indices that are measured during pregnancy and in very young infants can be particularly challenging. During the course of pregnancy, there occur changes in blood volume and blood cell mass which not only change during the course of pregnancy, but which also vary in extent between individual mothers. Indeed, some of these variations may help to predict the outcome of the pregnancy. Thus the inter-subject relationship between the lowest hemoglobin concentration that is encountered during pregnancy and the final birth weight is found to be J-shaped. The minimum incidence of low birth weight and of premature labor, and thus arguably the most satisfactory outcome, is associated with a hemoglobin concentration (95–105 g/l) which is low enough to be considered by WHO to
represent anemia [11]. Similar paradoxical relationships may exist for other maternal status indices. We do not yet have a clear answer to the question: how can we recognize moderate nutrient deficiencies in pregnant mothers against the background of variable physiological adaptation, and what are the best criteria for determining the need for intervention? There is, for instance, a continuing controversy about the need for iron supplements for all pregnant women, as distinct from those that are the most anemic. Another paradox concerns calcium. In most women, the demand for calcium secretion in breast milk results in a loss of bone mineral during lactation [12]. This cannot be prevented by dietary calcium supplementation during lactation, but somehow it is efficiently replaced later in life, even in women with very low dietary calcium intakes.

For public health programs, the individual micronutrients that are essential for human health are frequently, for convenience, subdivided into three tiers. The first of these, containing iodine, vitamin A and iron, is considered to be responsible for the largest proportion of the burden of morbidity and mortality that is attributable to micronutrient deficiencies world-wide. As indicated in table 1, the overall percentage prevalence for anemia and vitamin A deficiency in children is as high as ca. 40%, with IDDs being currently estimated at about 10%. The prevalence of each deficiency clearly varies between the continents, and the spectrum of deficiency also varies quite markedly between different regions. The second tier of micronutrients that are important for the world-wide burden of malnutrition includes the others listed in table 2. For this second tier, the prevalence of low intakes and status and the attributable burden of morbidity and mortality are even less well-defined, although individual studies have indicated their importance. Finally, there is a third tier, containing vitamins such as E, K, biotin, pantothenate and several essential mineral elements, which are not considered to be responsible for widespread deficiency disease, but which are nevertheless important for optimum health. For these too, the burden of malnutrition world-wide is poorly defined.

I will next examine the most commonly used micronutrient status indices, and indicate some of the special characteristics of pregnant and lactating women and young infants with respect to their selection and interpretation. My focus will be mainly on Third World countries, where the problems of micronutrient deficiencies are especially common and severe. I shall focus more on the problems of deficiency than on those of overload, since the former is the more frequently encountered public health problem. However, it needs to be borne in mind that very high intakes, particularly of some essential mineral elements such as selenium, do occur naturally in some parts of the world, and must be included within the overall ‘burden of malnutrition’, just as the burgeoning problems of energy overnutrition, with its widespread implications for the risk of degenerative diseases, are also part of that burden.
Table 1. Estimates of global prevalence of iodine and vitamin A deficiency and anemia, by world region

<table>
<thead>
<tr>
<th>Region</th>
<th>Iodine deficiency disorders&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Child vitamin A deficiency&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Anemia&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total population</td>
<td>number affected</td>
<td>% prevalence</td>
</tr>
<tr>
<td>Europe</td>
<td>873</td>
<td>98</td>
<td>11.2</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>482</td>
<td>91</td>
<td>18.9</td>
</tr>
<tr>
<td>Americas</td>
<td>827</td>
<td>41&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Africa</td>
<td>640</td>
<td>73</td>
<td>11.4</td>
</tr>
<tr>
<td>SE Asia</td>
<td>1,535</td>
<td>206</td>
<td>13.4</td>
</tr>
<tr>
<td>W Pacific</td>
<td>1,688</td>
<td>124</td>
<td>8</td>
</tr>
<tr>
<td>Global</td>
<td>6,045</td>
<td>633</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Source: WHO Nutrition for Health and Development Department (Dr. I. Egli, personal communication). Numbers in millions.

<sup>1</sup> Iodine deficiency disorders: preliminary estimates for 2002, all age groups.

<sup>2</sup> Vitamin A deficiency: estimates for preschool children in 1994, based on publication WHO/NHD/95.3.

<sup>3</sup> Anemia (as a proxy for iron deficiency anemia), all age groups for 1990–1995, based on WHO/NHD/01.3.

<sup>4</sup> Data not available.

<sup>5</sup> Data still being checked.
Table 2. Biochemical and functional status indices for commonly measured micronutrients

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Most commonly used biochemical index</th>
<th>Other biochemical indices</th>
<th>Functional status indices</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>Plasma retinol</td>
<td>Relative dose-response test, Plasma provitamin A carotenoids</td>
<td>Dark adaptation, specific ocular pathology, impression cytology</td>
<td>WHO have defined 'population adequacy' based on the prevalence of abnormal results</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Plasma 25-hydroxyvitamin D</td>
<td>(1,25-dihydroxyvitamin D$_1^1$)</td>
<td>Parathyroid hormone$^2$</td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Plasma vitamin C$^3$</td>
<td>Buffy coat vitamin C$^4$</td>
<td>None sufficiently specific$^5$</td>
<td></td>
</tr>
<tr>
<td>Vitamin B$_1$ (thiamin)</td>
<td>Erythrocyte transketolase activation coefficient (AC)$^6$</td>
<td>Plasma, red cell or urine thiamin concentrations</td>
<td>(carbohydrate tolerance test – seldom used today)</td>
<td>Older urine tests for B vitamin status were not sensitive enough</td>
</tr>
<tr>
<td>Vitamin B$_2$ (riboflavin)</td>
<td>Erythrocyte glutathione reductase AC$^6$</td>
<td>Plasma, red cell or urine (ribo)flavin concentrations</td>
<td>(none)</td>
<td>See above</td>
</tr>
<tr>
<td>Niacin</td>
<td>Urine niacin metabolites</td>
<td>Red cell niacin coenzymes</td>
<td>Plasma homocysteine$^8$, lobe numbers in polymorphonuclear leukocyte nuclei</td>
<td>Not frequently performed</td>
</tr>
<tr>
<td>Folate</td>
<td>Serum folate</td>
<td>Red cell folate$^7$</td>
<td>Others, e.g. deoxyuridine suppression test, are research, not population, tools</td>
<td></td>
</tr>
<tr>
<td>Vitamin B$_12$</td>
<td>Serum vitamin B$_{12}$</td>
<td>Holo-transcobalamin etc.</td>
<td>Methylmalonic acid$^8$</td>
<td>Population adequacy based on cretinism, goiter, and low urine iodine levels</td>
</tr>
<tr>
<td>Iodine</td>
<td>Urinary iodide</td>
<td>Serum thyroid-stimulating hormone (TSH) and thyroid hormones</td>
<td>Goiter prevalence, including ultrasound methods</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>Serum ferritin$^9$</td>
<td>Iron percent saturation, serum transferrin receptors, red cell protoporphyrin</td>
<td>Blood hemoglobin$^9$, mean red cell volume etc.</td>
<td>Multiple tests required, since iron deficiency has multiple manifestations and origins</td>
</tr>
<tr>
<td>Calcium</td>
<td>(Plasma calcium)$^{10}$</td>
<td></td>
<td>(Alkaline phosphatase)$^{10}$</td>
<td>No satisfactory indicators</td>
</tr>
<tr>
<td>Zinc</td>
<td>Plasma zinc</td>
<td>White cell zinc$^{11}$</td>
<td>Growth or immune function response to zinc supplements$^{12}$</td>
<td>For zinc (and other elements): a functional intervention response is the best evidence of status</td>
</tr>
</tbody>
</table>

(continued overleaf)
Table 2. (continued)

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Most commonly used biochemical index</th>
<th>Other biochemical indices</th>
<th>Functional status indices</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other trace elements: Se, Cu, etc.</td>
<td>Se: Plasma selenium</td>
<td>Se: Red cell selenium or glutathione peroxidase</td>
<td>Other Se and Cu proteins: are being investigated</td>
<td>Although many other elements can also be measured in plasma, their interpretation is not easy</td>
</tr>
</tbody>
</table>

Serum is equivalent to plasma for many tests. For a few, serum is preferable (because anticoagulants may interfere with assay chemistry), and for others, leakage of nutrients from cells or hemolysis-damage-avoidance may tip the preference towards plasma. For red cell enzyme assays, use an anticoagulant; saline wash the red cells to remove plasma and remove the buffy coat. Finger- or heel-prick blood is acceptable for some assays, but note that the cell counts may be distorted and that trace element assays may suffer contamination.

1. 1,25-Dihydroxyvitamin D is a hormone whose production and concentration is under endocrine control. It is not an indicator of vitamin D supply, but it may provide information about the status of metabolic pathways that involve calcium, phosphorus and vitamin D.

2. Serum parathyroid hormone may increase in functional vitamin D deficiency, but it is not a vitamin D-specific index.

3. Plasma vitamin C assays should include both ascorbic and dehydroascorbic acid; the latter may accumulate during storage. Vitamin C is very readily oxidized and must be stabilized with acid (preferably metaphosphoric) followed by freezing at very low temperature, e.g. −80°C or lower.

4. Buffy coat vitamin C has been used, but the assay is demanding and problematic. Fasting plasma vitamin C is usually a better compromise.

5. There are several (alternative) tests for ‘antioxidant capacity’ in plasma, plus tests for ‘damage markers’ such as malondialdehyde, DNA oxidation products etc., but none is nutrient-specific. They require careful choice and interpretation.

6. The red cell enzyme tests used to assess B vitamin status use the ratio of enzyme activity in the presence of the B vitamin-derived enzyme cofactor (e.g. thiamin pyrophosphate, flavin adenine dinucleotide), and the activity in its absence. Tissue deficiency results in loss of endogenous cofactor and an increase in exogenous cofactor stimulation (= ‘activation coefficient’). In a sense these are functional tests.

7. Red cell folate is considered a better long-term index than serum folate; the two tests are highly correlated between individuals. Red cell folate assay samples require a stabilizer (e.g. ascorbate) for long-term storage.

8. Plasma homocysteine is inversely correlated with and responsive to folate, vitamin B₁₂ and sometimes vitamin B₆; its disposition involves pathways that require these B vitamins. MethyImalonic acid (an intermediate in the catabolism of several aliphatic amino acids) accumulates specifically in vitamin B₁₂ deficiency and is a specific functional index of vitamin B₁₂ status.

9. Although blood hemoglobin measurement is not specific for iron status, it is the most commonly used surrogate test for ‘iron deficiency’, being easy to measure under minimal-facility fieldwork conditions, and able to provide early warning of the need for intervention. However, both anemia and iron deficiency are complex diagnostic problems, requiring several tests to elucidate their etiology.

10. There are no good biochemical indices for calcium status since plasma concentrations are very tightly controlled. Plasma calcium (and alkaline phosphatase) usually only respond to severe deficiency.

11. White cell concentrations of several trace metals are potentially promising indices, but are difficult to measure accurately and meaningfully.

12. For most trace metals, the response of selected functional indices to supplements provides the best evidence of status adequacy.
The Choice of Specific Micronutrient Indices

In table 2 is listed a selection of biochemical and functional status indices for individual micronutrients. Table 3 lists the most commonly quoted cutoff points for interpretation of frequently used biochemical status indices, and table 4 lists some practical problems and the precautions that are critical for the achievement of reliable results. With the exception of urinary iodine and urinary niacin metabolites, all of these biochemical status indices require blood samples. For most of the vitamin assays, the blood samples normally require processing (e.g. serum or plasma separation; washing of red cells, etc.) followed by storage at a low temperature (preferably below \(-25^\circ C\)) and transport as frozen samples to a dedicated analytical laboratory. For trace elements, although stability is less of a problem, environmental contamination is a serious risk, and trace element-free collection and storage containers are advisable. These demanding requirements are, of course, not always easy to fulfil, especially in remote study locations, therefore the assay procedures may need to be tailored to a particular environment and its limited resources. For instance, some progress has recently been made with the development of blood or serum dried spot assay techniques for a few key analytes, notably for serum ferritin [13], transferrin receptors [14], vitamin A [15], folate [16, 17] and thyrotropin and thyroglobulin [18]. Although potentially very useful for screening purposes, some destruction of labile analytes during storage of dried spots seems inevitable, and further development and assessment of this new technique is ongoing.

Fat-Soluble Vitamins and Bone-Related Micronutrients

For vitamin A, serum or plasma retinol is the most widely used biochemical index, especially for population studies. A low concentration implies either low stores, or an acute phase reaction (infection, inflammation) or both. Either of these underlying causes produces a similar short-term result, namely a reduced supply of the vitamin to key vulnerable tissues, although the acute phase effect should later resolve and serum retinol should rise without intervention once the inflammatory reaction has diminished. Since diets in many non-industrialized countries contain little or no animal products and hence little preformed vitamin A, measurement of the pro-vitamin A carotenoid pigments, the carotenes and cryptoxanthins in serum, can provide further information about dietary adequacy. Modern high-performance liquid chromatography-based assays can separate and measure both retinol and the key carotenoid pigments in a single 10- to 15-min separation. Simultaneously low levels of both retinol and the pro-vitamin A carotenoids constitute strong evidence of inadequacy. The relative dose-response test [19] and the modified relative dose-response test [20] are both able to provide a more accurate index of body (mainly hepatic) stores than plasma retinol can, but they are more cumbersome (requiring a 5-hour equilibration period in vivo), so their use is
Table 3. Suggested deficiency ranges\(^1\) for the principal status indices listed in table 2, during pregnancy, lactation, and infancy

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Range</th>
<th>Micronutrient</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma retinol, (\mu)mol/l</td>
<td></td>
<td>ETKAC(^2) (thiamin), ratio</td>
<td></td>
</tr>
<tr>
<td>Severe deficiency</td>
<td>&lt;0.35</td>
<td>Deficient</td>
<td>&gt;1.25</td>
</tr>
<tr>
<td>Moderate deficiency</td>
<td>0.35–0.7</td>
<td>Marginal</td>
<td>1.15–1.25</td>
</tr>
<tr>
<td>Marginal status</td>
<td>0.7–1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma vitamin C, (\mu)mol/l</td>
<td></td>
<td>Serum vitamin B(_{12}), pmol/l</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>&lt;11</td>
<td>Deficient</td>
<td>&lt;110</td>
</tr>
<tr>
<td>Marginal</td>
<td>11–22</td>
<td>Marginal</td>
<td>110–147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGRAC(^3) (riboflavin), ratio</td>
<td></td>
<td>Iron</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>&gt;1.4(^4)</td>
<td>Hemoglobin, g/l</td>
<td></td>
</tr>
<tr>
<td>Marginal</td>
<td>1.3–1.4(^4)</td>
<td>Serum ferritin, (\mu)g/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Iron saturation, %</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transferrin receptors, mg/l</td>
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<td></td>
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<td>Red cell protoporphyrin, (\mu)g/l</td>
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<td>Serum folate, nmol/l</td>
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<tr>
<td>Deficient</td>
<td>&lt;6.8</td>
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<td>Marginal</td>
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<td>Red cell folate, nmol/l</td>
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<td>Deficient</td>
<td>&lt;220</td>
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<td>Marginal</td>
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<tr>
<td>Urinary iodide, (\mu)g/l</td>
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<td>Severe</td>
<td>&lt;20</td>
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<tr>
<td>Moderate</td>
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<tr>
<td>Plasma 25-hydroxyvitamin D, nmol/l</td>
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<tr>
<td>Deficient</td>
<td>&lt;12</td>
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<tr>
<td>Marginal</td>
<td>12–25</td>
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</table>

\(^1\) Summarized from Sauberlich [106]. Where several alternative versions were quoted, only the most commonly used one is given here. Values given are for adults (including pregnant and lactating women) and for children aged over 6 months. Deficiency ranges are not available for younger children. For the iron indices, the categories are separated, with different values: C = children; P = pregnant; L = lactating. The normal or acceptable ranges are implicit once the deficient ranges have been excluded, being: >1.05 \(\mu\)mol/l for plasma retinol; >25 nmol/l for 25(OH)\(\Delta\)D; >22 \(\mu\)mol/l for vitamin C; <1.15 for ETKAC; <1.3 for EGRAC; >147 pmol/l for vitamin B\(_{12}\); >13.4 nmol/l for serum folate; >330 nmol/l for red cell folate; above the stated cutoff values for hemoglobin, ferritin and iron saturation (%); below the stated cutoff values for transferrin receptors and protoporphyrin; >50 \(\mu\)g/l for urinary iodide, and >13 \(\mu\)mol/l for plasma zinc. For some of the indices, extremely high values may be indicative of overload and possible toxicity, which can occur for some fat-soluble vitamins and trace elements at very high intakes.

\(^2\) Erythrocyte transketolase activation coefficient.

\(^3\) Erythrocyte glutathione reductase activation coefficient.

\(^4\) Minor differences in assay methodologies can produce differences in normal ranges.
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Problems and precautions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample collection: blood</td>
<td>Some procedures (e.g. cell counts) must be done on unfrozen, anticoagulated whole blood within 24 h or less, of collection. Others require a specific blood fraction (serum, plasma, washed red cells, buffy coat) which can be stored. Some will need an added stabilizer, e.g. metaphosphoric acid for vitamin C; ascorbic acid for red cell folate. It may be necessary to collect several tubes with different anticoagulants, to avoid assay interference. Trace element assays may demand collection in contamination-free containers with special precautions. Avoid hemolysis (which catalyses oxidation of some sensitive vitamins). A few analytes can, however be measured in extracts of blood or serum spots dried on filter paper, for population screening.</td>
</tr>
<tr>
<td>Sample collection: urine</td>
<td>Urine is commonly used for iodide assays, and for the estimation of sodium and potassium intakes and niacin status. A preservative (e.g. boric acid) may be added to prevent bacterial growth before freezing, provided this does not interfere with the assays.</td>
</tr>
<tr>
<td>Sample collection: breast milk</td>
<td>Useful for some vitamins (e.g. vitamin A) where breast milk levels vary with maternal intake. Concentrations usually vary with stage of feed (fore versus hind milk), with stage of lactation, and sometimes with fat concentration; therefore the collection procedures may need to be controlled. Storage requirements are similar to those of blood, but precautions are needed to ensure homogeneity (fat + whey) after thawing.</td>
</tr>
<tr>
<td>Subdivision and storage</td>
<td>Where several analyte assays are required from each sample, subdivide and store samples in small aliquots to minimize storage freeze-thaw damage. An antioxidant preservative may help to reduce deterioration.</td>
</tr>
<tr>
<td>Selection and validation of assays</td>
<td>Commercial (‘kit’) assays are available for some analytes, but may be designed to detect ‘abnormal’ values for patient screening, but not to provide accurate values within the normal range. For vitamins: avoid heat-, light- and oxygen-catalyzed damage during the assay.</td>
</tr>
<tr>
<td>Quality control and harmonization</td>
<td>Use externally supplied quality control materials with assigned values where available, and in-house ‘drift’ controls similar in composition to the samples assayed (same anticoagulant and storage history). Participate in external quality assurance (‘round robin’) schemes for inter-laboratory harmonization, where available.</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Many micronutrient indices, especially those in serum or plasma, are shifted (some down, some up) by the acute phase reaction associated with infection or inflammation. Therefore, always include an acute phase marker (such as α1-antichymotrypsin or C-reactive protein) as a marker of acute phase status, and interpret accordingly. Some indices are poorly responsive to changes in nutrient intake and body stores, often because homeostatic mechanisms normally maintain them within narrow limits. Changes may only be discernible after severe prolonged deficiency or after very high intakes of the nutrient. Evidence about functional status adequacy may require a multi-pronged approach, including dietary information, biochemical data, medical examination, and a selective intervention.</td>
</tr>
</tbody>
</table>
confined to small-scale intensive studies. The same is true of the conjunctival impression cytology test (which quantitates the stainable goblet cells in epithelial cell samples transferred from the surface of the conjunctiva) [21]. Dark adaptation is a cumbersome functional test if performed by the older ‘classical’ procedures; however simpler and more user-friendly versions based on pupillary and visual threshold testing are being developed [22]. Ocular pathology, e.g. corneal xerosis, Bitot’s spots, keratomalacia, etc., require the screening of many thousands of ‘at-risk’ children in order to obtain statistically meaningful prevalence estimates; therefore records are usually obtained from hospital cases, which can only provide an approximate estimate of prevalence. In addition, the grading and recognition of the key clinical signs is notoriously sensitive to interobserver variation. The available biochemical and physiological indicators for the assessment of vitamin A deficiency have been summarized by the WHO [23].

The most specific and sensitive biochemical index of vitamin D status is the circulating concentration of 25-hydroxyvitamin D (25-OHD), which is the major form of the vitamin in the blood-stream. It reflects the variations in vitamin D supply from the combination of diet and sunlight-catalyzed de novo synthesis of vitamin D in the skin. Until recently, when commercial kit assays became widely available, the plasma assay, usually based on liquid chromatography, was demanding and was performed by only a few laboratories. Even now it is an expensive assay, and investigations of population status are confined mainly to those countries where deficiency is known or suspected. High-risk regions include those parts of the temperate zone of the northern hemisphere where the ultraviolet component of sunlight is attenuated, especially in winter, and plasma 25-OHD falls to low levels in the absence of dietary vitamin D [24]. However, deficiency also occurs in some countries near the equator, where body-covering may be so complete that sunlight exposure is almost totally excluded. Low levels of 25-OHD are usually accompanied by raised levels of parathyroid hormone, which is a useful but less specific status index. In the UK, the highest risk population groups include the mothers and young children of immigrant groups from South Asia (India, Pakistan, Bangladesh) [25–27], and it appears that several components of their diet as well as inadequate sunlight exposure may contribute to this risk. Vitamin D deficiency is probably one of the most common vitamin deficiencies in the UK at the present time.

In some countries in and near the equatorial regions, clinical evidence of rickets is associated not with vitamin D deficiency but with very low calcium intakes. Pettifor [28] has described this condition which is characterized by somewhat reduced plasma calcium and considerably raised alkaline phosphatase levels.

A third bone-related nutrient, which is not included in table 2, but which Dr. Shearer will discuss, is vitamin K. Recent evidence has indicated several potentially important roles with health significance besides its long-established roles in the blood coagulation cascade [29], and indeed, one of the
most promising functional indices of vitamin K status is the extent of carboxylation of osteocalcin, a blood component which is also a marker for bone synthesis. In addition, the occasional occurrence of vitamin K-preventable intracranial hemorrhage poses a problem especially for breast-fed infants. It arises largely because of the very low breast milk content of the vitamin [30]. It is countered by the provision of prophylactic vitamin K, usually in a single large parenteral dose given to newborn babies in most industrialized countries.

Studies of vitamin E have mostly been confined to industrialized countries and, with the exception of preterm infants, to older population groups. Plasma vitamin E concentrations are frequently measured but are difficult to interpret. Vitamin E at alternative sites such as erythrocyte membranes may prove to be more useful [31]. A variety of antioxidant protection models have been explored, but further development is required to make them more biologically meaningful. The erythrocyte hydrogen peroxide-catalyzed hemolysis test, originally developed in the context of studies on severely vitamin E-deficient preterm infants, is rarely used nowadays.

Water-Soluble Vitamins

Although clinical vitamin C deficiency, presenting as overt scurvy, is rarely seen today, the measurement of vitamin C intakes and status has nevertheless remained of interest and priority. This is partly because vitamin C, whose full range of biological functions and health significance is incompletely understood, can nevertheless act as a robust surrogate marker for ‘healthy’ diets, at least in industrialized countries. Measurement of plasma or serum concentrations of vitamin C provides a reasonably reliable index of broad categories of intake, provided that adequate collection and storage precautions are taken. Ideally the samples should be mixed with freshly dissolved metaphosphoric acid and stored at a very low temperature – preferably around −80°C. An index based on the vitamin C content of the buffy coat (mixed white cell) fraction of blood was preferred until recently, but the fieldwork component of this assay (namely the isolation and counting of the white cells and the extraction of their vitamin C) is cumbersome and time-consuming, and the assay also suffers from some problems of interpretation, so that it has now fallen out of favor. There are no very specific and robust functional indices of vitamin C status. Although several laboratories have used the antioxidant properties of vitamin C (and likewise those of vitamin E) to develop in vitro oxidation damage-protection assays, their relevance to the essential in vivo roles of these vitamins still has to be proven. In the authors’ laboratory it has been shown that changes in vitamin C intakes and status in the guinea pig specifically modulates the concentration of a bone-specific collagen cross-link, deoxypyridinoline (alias hydroxylysyl pyridinoline) [32]. However, the usefulness of this approach to provide a functional probe of human vitamin C status has yet to be proven. Our studies of the massive seasonal variation in vitamin C status in rural Gambian pregnant and lactating women are described later.
Overt thiamin (vitamin B₁) deficiency, presenting as classical beriberi, is of sporadic and rather unpredictable occurrence, and is mainly encountered in tropical countries where poor-quality staples, including highly polished rice, form a major part of the diet. There have been a few sporadic outbreaks in The Gambia over the past few decades, but in contrast to most other nutrient deficiencies, the disease appears to have affected mainly adult males [33], rather than the potentially more-vulnerable population groups of pregnant or lactating women and young infants. The manifestations of thiamin deficiency that are associated with alcohol abuse likewise occur sporadically, and possibly have a genetic association [33]. The transketolase test is generally chosen and preferred for its high sensitivity, since the alternative and older urinary thiamin excretion tests for thiamin status fail to distinguish between severe and marginal deficiency.

Overt riboflavin (vitamin B₂) deficiency is likely to be a rather common condition, in many Third World communities where dairy and animal food products are in short supply. It is especially characteristic of late pregnancy, and our studies in The Gambia are described later. Although the main clinical results and manifestations of riboflavin deficiency ('sore mouth', angular stomatitis, cheilosis, tongue lesions) are not life-threatening, there is accumulating evidence that a severe biochemical riboflavin deficiency may affect several key biochemical pathways in human subjects, including those of iron metabolism and certain other B vitamins. The glutathione reductase status test for riboflavin, like the transketolase test for thiamin, is generally the assay of choice because of its high sensitivity and robustness. Modern clinical chemistry analyzers can perform the test quickly and efficiently.

Although overt pellagra (niacin deficiency) seems not to be as common today as it was in the early part of the 20th century, it still occurs in some developing countries, and niacin deficiency might be studied more frequently if simpler and better biochemical tests were available for it. The most commonly used status test, based on the measurement of urinary niacin turnover products, is tedious to perform. Other promising tests, based on red cell pyridine nucleotides, have also failed, so far, to gain popularity, so that reliable information about the prevalence of niacin deficiency worldwide is seriously lacking.

Severe vitamin B₆ deficiency is rarely encountered unless protein intakes are simultaneously high, which probably explains why it appears to be rarely encountered in a Third World setting. There are many alternative status indices proposed for vitamin B₆ which are in occasional use; however those which provide the most comprehensive picture of all the circulating forms are demanding, and functional tests for this vitamin, based on aromatic amino acid turnover, are likewise insufficently user-friendly.

Folate deficiency was originally identified as a characteristic deficiency of late pregnancy in parts of India. It was associated with megaloblastic anemia, and was linked functionally with vitamin B₁₂ at the metabolic level.
Much more recently, the key role of folate in the prevention of NTDs was investigated, and this aspect of its public health significance will be discussed by Dr. Czeizel. Its recently defined role in modulating levels of homocysteine in the bloodstream has raised the possibility that it may play a role in protection against vascular disease risk. Another recent hypothesis is that it may modulate the risk of some types of cancer, possibly through its function in DNA repair. Biochemical folate deficiency tends to accompany pregnancy, and folic acid supplements, together with iron supplements, have been prescribed for pregnant women for many decades. Nevertheless, the benefits of providing folic acid supplements during established pregnancy, as distinct from preventing NTD periconceptionally in a vulnerable subgroup of women of child-bearing age, are contended.

The most commonly used biochemical indices of folate status are serum (or plasma) folate and red cell folate. The latter has the advantage of reflecting a longer period of intakes and body stores. However, the red cell assay may also be more vulnerable to problems of poor assay control [34]. Plasma homocysteine can now be measured by many clinical chemistry laboratories, and is often included as a functional index for folate status and for the other metabolically related B vitamins: principally vitamin B₁₂ and vitamin B₉. Other well-established functional indices of folate status, such as polymorphonuclear leukocyte lobe counts, formiminoglutamic acid excretion and the deoxyuridine suppression test, are only rarely used today. They are not suitable for large population studies.

**Vitamin B₁₂** deficiency has traditionally been associated with pernicious anemia (due to lack of intrinsic factor secretion) and similar gastrointestinal absorption defects, mainly encountered in older people, but only infrequently in the age groups that are the focus of the present workshop. However, the occurrence of suboptimal dietary intakes of vitamin B₁₂ in all age groups, especially in the Third World, is now beginning to command attention and concern [35–38]. Lactating mothers whose intakes of vitamin B₁₂ are very low, secrete breast milk with amounts of the vitamin that are suboptimal for fully breast-feeding infants [35, 36, 39]. Although the sole standard biochemical test for vitamin B₁₂ status has for many years been serum or plasma vitamin B₁₂, other indices are now being considered. Firstly, recent studies of the specific functional status index, methylmalonic acid [40], have indicated that suboptimum functional status may occur in a larger proportion of the population than was previously recognized on the basis of hematological abnormalities, or by neurological damage. Secondly, a more revealing picture of status may now be achieved by including measurements of the apo- and holo-transcobalamins in plasma [41–43], and perhaps by also including apo- and holo-haptocorrins, which, intriguingly, are reported to change progressively during the course of a normal pregnancy [44]. In countries where folic acid fortification of flour is introduced there is likely to arise a need for more rigorous screening of vitamin B₁₂ status in order to counter the concerns.
that folate supplements may mask incipient vitamin $B_{12}$ deficiency and result in irreversible neurological damage.

**Minerals and Trace Elements**

The measurement of *iodine* status is, in practice, most frequently performed by the measurement of the iodide concentration in ‘spot’ or ‘single’ urine samples [45]. This can provide an approximate estimate of iodine intakes on a group basis, since most of the iodine that is ingested is rapidly excreted into the urine. Because of diurnal and day-to-day variation, urinary excretion estimates will only provide accurate results for individuals, if the urine collections are repeated several times, or are extended over several days [46]. The calculation of iodide:creatinine ratios may improve the accuracy, but creatinine is not an ideal denominator [47]. In conjunction with records of goiter prevalence, simple urinary iodide estimates can serve to determine the need for intervention (preferably with iodized salt, or else with iodized edible oil, if salt iodination is not feasible in the short term [48]). For young infants, the measurement of thyroid-stimulating hormone (thyrotropin, TSH) in serum or plasma is a recommended index [45], replacing the urinary iodide assay where children are too young for urine collections to be possible. For older children, a recommended serum index is thyroid-binding globulin [18, 49], and for all population groups, serum measurements of thyroxine ($T_4$) and triiodothyronine ($T_3$) and their ratio can give useful additional information. In some situations, functional iodine deficiency may be caused or exacerbated by the presence of iodine-metabolism inhibitors (goitrogens) in the diet, or by a deficiency of selenium or iron [50, 51]. Both of these elements are essential cofactors for key enzyme-catalyzed reactions of the thyroid hormone synthesis pathway. The assessment of goiter (which is a sensitive functional sign of the status of the thyroid gland) can be performed by simple visual observation, including backwards tilting of the head. This visual examination may be improved by palpation, or by the more quantitatively accurate procedure of ultrasound measurements of thyroid size. Although goiter assessment provides a sensitive and specific indicator of iodine status in a community, it may be slow to respond to iodine supplements, especially in older people; therefore the assessment of the efficacy of IDD prevention measures tends to rely more heavily on the biochemical measures of iodine status and thyroid function.

Pregnant women comprise the most vulnerable population group with respect to iodine deficiency, because of its causative link with cretinism, an irreversible defect resulting from severe iodine deficiency in utero. For this reason, the elimination of cretinism is one of the most important aims and monitored indices of success of community iodine supplementation programs [52]. In addition to preventing cretinism, infant survival is also improved by supplementation, as demonstrated by the use of iodized oil [53]. Iodine deficiency in young children can compromise their mental development and developmental milestones, thereby contributing to the total burden of IDD [52].
Iron deficiency is traditionally and sometimes simplistically associated with anemia. The most common single cause of anemia worldwide is iron deficiency, and anemia (low hemoglobin concentration or low hematocrit) is in practice much simpler to measure than biochemical iron status. However, iron deficiency is not the only possible cause of anemia, and indeed there are a number of different causes of anemia, and several levels of iron deficiency that need to be distinguished from each other. Microcytic anemia (mean cell volumes <80 fl) is consistent with iron deficiency as a possible cause, although thalassemia and anemia of chronic disease can also result in microcytosis. The earliest appearing biochemical evidence of iron depletion is a low level of serum ferritin (<12 μg/l in adults), indicative of depleted tissue iron stores, and accompanied by nearly total depletion of stainable tissue iron (which requires histological examination of biopsy samples and is therefore more invasive to assess). After a more prolonged deficiency, the disappearance of ferritin is followed by: (a) a reduction in the percentage iron saturation of circulating transferrin; (b) a raised concentration of circulating transferrin protein; (c) raised circulating transferrin receptor concentrations, and (d) raised free erythrocyte protoporphyrin in the red cells. Overt anemia, with a reduced hemoglobin concentration is the final step in the depletion sequence. It is advisable to measure at least two, and preferably more, biochemical iron indices, in order to characterize the iron status of a population satisfactorily [54]. Confounding interferences may arise. Serum ferritin is increased independent of tissue iron stores by infection and inflammation (acute phase effect), while the serum iron concentration is reduced in this situation. Therefore, neither of these two indices gives a reliable picture of body iron status if an acute phase reaction (as detected by raised acute phase protein levels) is present. It is highly desirable to monitor acute phase status during population studies by measuring one of the acute phase proteins (e.g. C-reactive protein or α₁-antichymotrypsin). The transferrin receptor index is claimed to be much less affected by this confounding influence [55, 56]. However, this claim has been challenged [57], and its interpretation in the complex situation of a malarial attack is uncertain [58–61]. To screen for poor iron status in pregnancy in the UK, van den Broek et al. [62] have recommended the use of serum ferritin with a high cutoff of 30 μg/l, whereas others have recommended the use of the transferrin receptor index for pregnancy studies [63, 64]. During the cycle of pregnancy and lactation, the highest concentrations of circulating transferrin receptors were recorded in the third trimester of pregnancy, followed by a slow progressive fall postpartum [65].

Iron deficiency and the resulting anemia constitutes one of the commonest nutritional deficiencies worldwide, especially in pregnant women and young infants. It can have deleterious functional consequences, especially for developmental indices in young children [8]. Iron deficiency in pregnancy reduces fetal iron stores [66], but its relation to other aspects of pregnancy
outcome is less well understood [66, 67]. In one study in Niger, iron supplements given during pregnancy significantly increased body length and Apgar scores of newborn infants and also increased their serum ferritin levels 3 months after delivery [68]. However, in some situations there may be a need for caution when deciding whether to provide iron supplements to mothers and infants. In malarious regions, the interaction of certain types of iron supplements with the malarial parasites and host defenses in vivo can sometimes result in deleterious effects on the delicate host-parasite relationship [69]. This is an important unresolved conundrum for future research, since the unequivocal benefits of iron (and other micronutrient) supplements in Third World situations need to be balanced against possible dangers with regard to the exacerbation of infections [70].

**Zinc** deficiency is one of the hardest of the micronutrient deficiencies to recognize and to investigate at the human community level. Zinc has many known (especially catalytic) functions in the body, and a severe absorption defect, and hence tissue deficiency that is of genetic origin, acrodermatitis enteropathica, produces characteristic biochemical effects and pathological lesions. However, the more mild deficiencies that are common in human populations where the staple foods are poor in available zinc and are rich in competing phytates and other metal ion binders, are harder to recognize. Serum or plasma zinc concentrations may be reduced, but not consistently or dramatically, and zinc measurements at other biopsy sites such as hair are often difficult to interpret. There is no really satisfactory biochemical index that correlates well either with recent zinc intakes or with functional adequacy [71, 72]. Therefore, the majority of human population zinc status investigations have needed to focus on physiological outcomes. These have included birth weight and prematurity indices, growth rates of young children, severity and duration of diarrheal disease in children, and other indices of susceptibility to infection (since zinc is required for several key aspects of immune function and infectious disease susceptibility) [9, 73–77]. Zinc interacts with several other key nutrients (e.g. iron, copper, vitamin A) in a complex manner, and the results of recent intervention trials, mainly in developing countries, have indicated that marginal zinc status is common and is potentially deleterious to health during the course of pregnancy and especially in early postnatal life. Our studies in young Gambian children are described later.

Although several other minerals and trace elements are known to be essential dietary components for humans, much less is known about the prevalence of human deficiencies and the need for intervention programs, especially for women during the reproductive cycle and for infants and young children. **Copper** is an important catalytic element, forming the active center of many essential enzymes, yet there are no effective and accepted biochemical status indices, with the possible exception of red cell Cu-Zn superoxide dismutase, which is also affected by zinc deficiency. Very little is known about the effects and the prevalence of marginal copper deficiency in
human populations although there is some evidence for a role in the immune system [78].

**Selenium** deficiency tends to occur in regions where soil selenium concentrations are low, e.g. in parts of China, and suboptimal intakes may be widespread in parts of northern Europe, including the UK. There are several status indices, including plasma and red cell selenium concentrations and glutathione peroxidase (selenoenzyme) activities. However, the bio-efficacy of different dietary selenium sources, and the functional interpretation of selenium status measurements, remains uncertain.

**Magnesium** supplies may be especially critical during pregnancy, but the plasma magnesium concentration (the traditional index) is not very responsive to dietary modulation, and as with several other metallic elements, plasma levels do not necessarily reflect tissue adequacy. Dietary intakes of **potassium** are important for optimum health, and other essential trace elements include **manganese**, **chromium**, and possibly **boron**, **silicon**, **vanadium**, etc. Nutrient balance from varied and high-quality foods is as important as the optimum provision of single ‘key’ nutrients that are associated with common and overt deficiencies.

My final section describes some studies on pregnant and lactating mothers and young children in a rural Gambian community. They help to illustrate typical uses of micronutrient status measurements as adjuncts to a study of nutrition and health, in a developing country setting.

**Micronutrient Status and Requirement Studies in The Gambia**

**Riboflavin**

In rural farming Gambian communities, biochemical riboflavin deficiency is almost universal, and clinical deficiency signs such as sore mouths with cheilosis and atrophic lingual papillae, are frequently encountered. Our studies in Keneba have demonstrated that, during the cycle of pregnancy and lactation, both the frequency of clinical signs, and biochemical riboflavin status as indicated by increased activation coefficients of erythrocyte glutathione reductase, become dramatically worse during the second half of pregnancy. They reach a nadir just before parturition, and then gradually improve during the following 18 months of extended lactation [79]. The improvement during lactation occurs despite a constant daily drain of 0.15–0.2 mg riboflavin into the breast milk every day, which is around one third of the usual dietary intake of the vitamin by adult Gambians. A 2-mg daily riboflavin supplement was, for nearly all the Gambian mothers, sufficient to bring their biochemical indices into the ‘normal’ range, similar to that seen in countries like the UK [80, 81], where dietary riboflavin intakes are typically enhanced by the widespread use of dairy products and other riboflavin-rich foods.
Infants born to riboflavin-deficient mothers in Keneba are biochemically deficient at birth, and although they improve significantly during the subsequent period of exclusive breast-feeding, their status deteriorates again during the period when they are being weaned onto Gambian riboflavin-poor weaning foods [82]. They are therefore at risk of suboptimum function of a range of key flavoenzyme-dependent biochemical pathways, including those of iron, folate and pyridoxine metabolism. Studies of the riboflavin content of breast milk in various communities, including The Gambia [83], have confirmed that breast milk concentrations are lowered in those communities in which the mothers are biochemically deficient, and that they can be restored by maternal supplementation. However, the body seems to limit breast milk riboflavin levels to an upper ceiling or plateau when maternal intakes are high. In this context, Allen [84] has pointed out that micro-nutrients can be classified into two priority categories with respect to the value of supplementation in lactating women who have low intakes. Category 1 contains those micronutrients for which a maternal deficiency results in reduced breast milk concentration, which can be restored by supplements. This comprises vitamins A, B₁, B₂, B₆, B₁₂ and C, and perhaps iodine and selenium. Category 2 contains those micronutrients for which a maternal deficiency usually does not affect the breast milk concentration, and which are unresponsive to maternal supplements. These include vitamin D, folate, iron, calcium, copper, zinc, and some other trace elements [83]. The responsiveness or otherwise of breast milk micronutrient levels to maternal supplements is clearly of importance with regard to the choice of strategies for nutrient delivery to the breast-fed infant, and our studies have shown that a significant benefit can be achieved by enhanced riboflavin supply to Gambian infants in this way.

Studies of Gambian populations [85, 86] and of experimentally riboflavin-deprived rats [87, 88] have supported the hypothesis that riboflavin is needed for optimum functioning of iron-handling pathways in the body, and that, in particular, the restoration of normal iron status through iron supplementation is especially sensitive to moderate degrees of riboflavin deficiency [89]. In lactating Gambian women [86], riboflavin given in conjunction with an iron supplement resulted in both a significant increase in circulating (transferrin-bound) iron and an increase in tissue iron stores as measured by circulating ferritin, whereas iron given alone did not achieve this. Likewise for men and for 4- to 12-year-old Gambian children, an iron-plus-riboflavin supplement, but not iron alone, achieved significant improvements in several iron-dependent indices, including blood hemoglobin, red cell count, mean cell volume and plasma ferritin. In riboflavin-deficient rats, not only was their iron mobilization impaired [87], but in addition poor riboflavin status at a very young age irreversibly altered gut morphology and function [88], implying early-life programming events associated with an inadequate riboflavin supply.
Zinc

The relatively poor bioavailability of zinc in the mainly plant-based diet of Gambian toddlers is a potential risk factor for functional zinc deficiency [90]. A daily zinc supplement failed to enhance the toddlers’ growth significantly in terms of height, weight or upper arm circumference; however it did significantly improve gut permeability by reducing the high level of leakage of the marker sugar, lactulose, from the gut into the bloodstream and thence into the urine [91]. In view of the evidence that zinc supplements have, in other communities, reduced the severity and duration of chronic diarrhea, and in view of the very strong correlation observed between growth faltering and abnormal gut permeability in Gambian children [92], this action of zinc on gut permeability may partly help to explain the benefits of zinc supplements with respect to gut pathology in vulnerable weanling children. In Gambian women, breast milk zinc concentrations were not compromised despite the apparently poor bioavailability of the nutrient in the mothers’ diet [93], therefore fully suckling infants seem likely to be adequately protected. Our studies [91] together with those of Shankar et al. [94] in Papua New Guinea are consistent with there being some benefit from zinc supplements in the reduction of malaria-associated morbidity. This could be an important observation in view of the high prevalence of malaria-associated mortality for young children in this, and many other Third World, communities. In contrast, another recent West African study has reported significant benefits for diarrheal morbidity in young children, but not for morbidity from falciparum malaria [95].

Vitamin A

Our studies of potentially vulnerable groups in The Gambia have shown that carotene-rich food items such as mangoes, red palm oil, squash, etc., exhibit dramatic seasonal variations in their availability [96–99] (fig. 1), and that the prevalence of suboptimal vitamin A status is much higher than it is, for instance, in the UK [96, 100]. Preschool children appear to be an especially vulnerable age group [101], insofar that their plasma vitamin A concentrations fluctuated between apparently adequate in the mango season and much less adequate in the rainy season when dietary sources of the vitamin and its carotenoid precursors are essentially absent. In contrast, adults, including pregnant and lactating women, did not exhibit such large seasonal fluctuations in plasma retinol concentrations [97] despite huge seasonal fluctuations in daily carotene intakes and plasma carotenoid levels (fig. 1). This is presumably because of the increased and prolonged buffering capacity of their larger hepatic vitamin A stores. In marked contrast to the pattern of riboflavin status, plasma retinol (following a marked decline in concentration during pregnancy) was very rapidly restored after parturition, within as little as 6 weeks, and then declined progressively during the subsequent 18 months of lactation [100]. Secretory losses are high during the early part of lactation, and supplements may be beneficial.
A recent countrywide survey of iron, iodine and vitamin A status, performed in November 1999 [102] revealed that about half of the 1- to 5-year-old children, one third of the pregnant women and one sixth of the lactating women had plasma retinol levels of <0.7 μmol/l. However, for about half of the children and for about a quarter of the mothers with low levels, these were associated with considerably raised α1-antichymotrypsin concentrations, i.e. with an acute phase reaction. The survey was carried out during the malaria season.
transmission season, which may explain why many of the subjects were affected by the inflammatory reaction. Clearly the development of a simple and robust acute phase-independent biochemical status index for vitamin A status would be of considerable value in this situation.

_Vitamin C_

Like the carotenoids, vitamin C intake and status in The Gambia consistently exhibit a major seasonal variation in rural Gambians, as studied in pregnant and lactating women and in preschool children [97, 103, 104]. The mango season, which boosts carotenoid intakes, also provides a major boost to vitamin C intakes and status in April–May, with a smaller contribution by citrus fruit in December (fig. 1). Again the nadir of intake and status occurs at the end of the rainy season, extending into the early part of the dry season. Breast milk vitamin C concentrations follow the same seasonal cycle of intakes and blood levels [104], although they are partly physiologically protected, so that the magnitude of the breast milk vitamin C cycle is smaller than it is in blood. The hypothesis that this seasonal cycle in vitamin C might be responsible for a closely parallel seasonal cycle of bleeding gums in children was not upheld, because vitamin C supplements given during the season of low vitamin C intake and the high prevalence of bleeding gums did not significantly improve this pathological sign. However, a dramatic seasonal cycle in life expectancy by season of birth [105] may be partly related to diet, and vitamin C is an excellent seasonal marker, if not a candidate causative factor, in this respect.

_Conclusions_

Our studies in rural Gambian mothers and infants, including status measurements of riboflavin, zinc, vitamins A and C, have illustrated the following characteristics.

(1) Maternal status is poorest for a range of nutrients, just before parturition. However, changes in plasma volume make plasma concentration indices difficult to interpret during pregnancy; hence the need for new status indices that can avoid this problem. Likewise, new indices which are less affected by the acute phase reaction are needed in many Third World situations, where both acute and chronic inflammatory processes are common.

(2) For many nutrients, breast milk levels are at least partly protected and maintained, either at the expense of maternal stores, or as a result of increased absorption or decreased turnover. The best strategies may be food fortification, plant breeding, or food preservation rather than intermittent massive dosing.

(3) Like vitamin A deficiency, the deleterious effects of zinc deficiency seem to be particularly threatening at the weanling and preschool stage of child development, and may contribute to morbidity and mortality, especially from diarrheal disease; carefully focussed intervention is required.
Biochemical status indices are as useful as their ability to predict functional status. In an ideal situation, we would prefer to measure function directly, and to assess the need for interventions and their efficacy principally in terms of functional improvement. We need well-authenticated connections between feasible biochemical index measurements and the key functional outcomes.

We have witnessed a major evolution of focus with respect to functional outcomes for key micronutrients in recent years. The role of vitamin A in reducing mortality from transmissible disease; that of iodine in improving developmental indices in children, and that of folic acid in reducing the incidence of NTDs, are prime examples. The latter paradigm illustrates the fact that within every community, there are individuals, probably genetically determined, who have unusually high requirements for specific micronutrients, as compared with the population average. We need to develop improved strategies to recognize these high-risk individuals, and to intervene appropriately. In some situations, short-term ‘quick-fix’ supplement-based solutions to nutritional problems are necessary. However, in the longer term, the production and distribution of foods that constitute varied and nutritious diets will probably be more beneficial. The human body has evolved to be dependent on, and to take advantage of, many diverse dietary components, whose dietary balance represents the best option for good long-term health and optimum life span.

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Assessment of Micronutrient Status in Mothers and Young Infants

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Assessment of Micronutrient Status in Mothers and Young Infants

Discussion

Dr. Al Frayh: There is no doubt that micronutrient research is not as simple as one would think. If it is not a micronutrient which is deficient in an individual, then this can be replaced by a simple supplement. This is a fact which is known not only by researchers but also clinicians. If you want to overcome a certain deficiency and you are looking at it from a complex angle, do you think that the deficiency in a certain micronutrient is not simply the deficiency in that particular nutrient, but that there are also some other factors to be considered, such as the status of the other micronutrients which may interfere in the absorption, the metabolism and bioavailability of that particular nutrient? On top of that, the health status of the individual, the chronicity of certain diseases may play a role in absorption and metabolism as well as the health status according to ethnicity. Certain ethnic groups may have a problem in absorbing and metabolizing certain micronutrients. This comes from the clinical point of view of one who is not very much involved in micronutrient research. I am just looking at it from a practical point of view. So the question again is, do you agree that a deficiency cannot just be measured by the number of micronutrients which are deficient and can then be treated by a simple supplement of those particular nutrients?

Dr. Bates: Yes indeed, I would certainly agree with all of those points that you have made. Undoubtedly the interaction between nutrients is an extremely important aspect of this whole problem which we have got to look at in detail. It does seem to be possible, in certain areas and in certain situations, to treat a particularly prevalent problem with a single micronutrient supplement. But that is only the tip of the iceberg and we should also be looking at the interactions between the micronutrients and also asking what happens when we overcome one deficiency, are we not unmasking another one. The second point about the interaction with disease, of course it is extremely important and we may find that a particular deficiency is only going to be manifest in the presence of a particular disease problem. The first thing you mentioned was in effect the problem of gene-nutrient interactions, the fact that there may be genetic pools which are particularly susceptible in different parts of the world. I absolutely agree that all of those are very important things which need to be addressed at this workshop.

Dr. Young: I found your studies on seasonal variation and vitamin A, the carotenoids, etc. to be fascinating. In one of your slides you indicated that, in toddlers in the Gambia, plasma retinol levels were substantially lower than those of UK toddlers, and I assume that this would imply a very different liver retinol status. You showed major seasonal variations in plasma carotenoids with little change in plasma retinol levels. I assume that in adults, if the toddlers showed relatively low plasma retinol levels, this would also be reflective possibly of the retinol status of adults, so I was surprised not to see a change in plasma retinol with those rather marked changes in plasma carotenoid levels. So my question is, is the conversion of β-carotene specifically dependent on the adequacy of nutritional status with respect to other micronutrients, such as zinc or iron for example? Perhaps you could tie these observations together a little bit more than you were able to do in your presentation.

Dr. Bates: I think this is very interesting but also a considerable challenge. Just to clarify the situation in the Gambia in adults, those studies were done mostly in lactating women. We find that although the absolute levels of retinol in the plasma are considerably lower than they are in the UK, they nevertheless do not show very much of a seasonal cycle. It is far smaller than the seasonal cycle in the carotenoid intakes and blood levels. So I can only assume that it represents a buffering effect of relatively adequate hepatic stores, although those stores are presumably much lower than they are in the UK or in Western countries. In one study by Nathanail and Powers [1] in children, a bigger seasonal fluctuation was seen in the retinol levels as well as in
carotenoids. So it does look as if children are more vulnerable to the seasonal availability of carotenoid-rich foods. Of course the main result that we have is that their absolute levels are considerably lower than they are in the UK. In terms of what other factors may affect the efficacy of conversion of carotenoids to retinol, I don’t think we already know that. There is some evidence that protein status has an effect on that conversion at least in animal studies, but there is little evidence as to how important it is in humans. In other studies [2], again in animals we have actually shown that vitamin A status per se can affect that conversion rate. But I think we have a great deal to learn still about all the different factors that affect the efficiency of conversion, and of course there has been quite a bit of literature recently in that particular fascinating area.

**Dr. Endres:** You showed us nicely that in the assessment of micronutrient deficiencies there are, in some instances, not only biochemical parameters but also functional parameters and that, for example for vitamin D deficiency, you have a functional parameter like the parathyroid hormone concentration, but for selenium and copper you don’t have functional parameters. What is your opinion in the context of assessment in interventional studies: is it a tremendous disadvantage not to have a functional parameter, or vice versa is it a tremendous advantage in those micronutrients where you have a functional parameter?

**Dr. Bates:** My suggestion would be that the functional parameters do give us an intermediate endpoint to look at between the measurement of their intakes or the blood levels of nutrients, and their effect on critical conditions, on disease and health generally. This can often be extremely useful intermediate evidence and would certainly be useful in intervention studies where the question is whether an intervention is having an effect on eventual long-term outcome in clinical terms. Clearly there have been attempts to use measurements such as parathyroid hormone as a way of studying the adequacy of different intakes and indeed different blood levels of a nutrient such as vitamin D. So I think they are extremely valuable research tools. They have different roles in different types of study and problems.

**Dr. Zlotkin:** I was particularly interested in your Gambian studies on the synergistic interactions between riboflavin and iron or hemoglobin status. Would you talk a bit about the mechanism that you think is responsible for the observations on the impact of riboflavin on hemoglobin or iron status?

**Dr. Bates:** Our assumption is that since there are known to be riboflavin-dependent enzymes that are involved in the mobilization of iron, particularly the FMN-dependent iron mobilization enzymes, this is the most likely area where riboflavin deficiency could be having an effect on the provision of iron for the improved rate of synthesis of hemoglobin. But I would certainly say that the evidence, even from animal studies, is still somewhat preliminary and I think we need many more studies of this type to clarify, first of all, how important B vitamins like riboflavin are in these pathways in human populations, which are the vulnerable groups, and exactly what the mechanisms are. We still only have preliminary evidence in this area.

**Dr. Al Awar:** Regarding the vitamins, vitamins A and D, and regarding what Dr. Al Frayh has said about there being other factors. We know that in the first months of life, especially the first 2 or 3 months, babies have a physiological or functional pancreatic insufficiency, and we know that vitamins A and D are fat-soluble vitamins which need pancreatic enzymes, lipase and colipase, and of course the bile source from the liver. Although these vitamins are present in smaller amounts in human milk, there are facilitating factors. There is much less iron in human milk but lactoferrin facilitates the absorption of these micronutrients. Because in the first months of life these babies have pancreatic insufficiency, I wonder if perhaps the amount of vitamins needed is much higher than that of babies on human milk, and these are the factors
which enable this micronutrient of vitamins, especially the fat-soluble vitamins, to contribute to the deficiency of this type of vitamin in these babies.

Dr. Bates: My only comment is that I am aware of factors, particularly the release of retinol from retinol esters, that can be enhanced by factors present in human milk and I am certainly aware of this being one of the mechanisms whereby human milk is thought to be a better source of retinol for young infants. I am interested in the other areas that you mentioned, but I don’t have any particular comments.

Dr. Verhoef: I have a remark about the previous question on the variability in retinol observed in the Gambian studies. I could well imagine that this is, at least in part, related to seasonal fluctuations in malaria, considering that a large part of the decline in serum retinol in children occurs after the mango season when malaria transmission is common in the Gambia, and that might also perhaps explain why the variation in women would be less because, I suppose, they have more immunity to malaria. Perhaps you could comment on that. Secondly I have another remark about what you said about inflammation as an important contributor to the differences observed in ferritin. We see a lot of inflammatory conditions that are not related to any specific infections that can be found. Which raises the question that with all the changes in iron status observed whether that is due to anemia of chronic disease or not? I think that question is most important because it seems that iron supplementation would not be very helpful in these conditions. A lot of research needs to be carried out to find out whether iron deficiency is the cause of that anemia of chronic disease or whether this perhaps is related to erythropoietin production and action. I would like to ask you if you have any opinion about that, whether you consider anemia of chronic disease to be an important problem in these children?

Dr. Bates: Undoubtedly the seasonal fluctuations in hemoglobin level are much greater in children in the Gambia than they are in adults, and I absolutely agree with you that one likely reason is that the adults have a greater immunological resistance to malaria and have fewer episodes. I think there is a very clear difference between the age groups. And I certainly agree that the anemia of chronic disease is very likely a major factor in the Gambia. It has not been studied in great detail, and I think there certainly is much more that needs to be done. A number of studies have shown that iron supplements can have quite a considerable effect in all the age groups in increasing the hemoglobin levels, so obviously chronic disease is part of the story but not the whole story.

Dr. Al Faouri: Do you have any criteria for giving calcium to lactating mothers, and if you advise giving it to all mothers, do you think it is an innocent procedure with no side effects?

Dr. Bates: There is a considerable program of research going on in the Gambia at this moment and of course all these are the major questions of that research program. As you rightly imply, obviously there may be side effects of even an apparently innocuous nutrient such as calcium, but they have got to be looked at in detail. One of the questions of course might be whether large doses of calcium could interfere with the absorption and utilization of other elements, trace elements in particular. So far I would say that the limited evidence available shows that the long-term effects of calcium on the other trace elements seem to be minimal. Where you can show an effect is perhaps in the early stages of absorption of elements such as iron and zinc, and then the question arises whether those effects do in fact translate to long-term effects on body stores. Clearly this is an extremely important area. Of course we don’t have all the answers as to whether the changes in calcium levels actually translate to an advantage in long-term parameters such as bone health, for instance.
Dr. Abbasy: What is your opinion on the controversies about the supplementation of vitamins and micronutrients to the exclusively breast-fed full-term, preterm and twin babies, and when?

Dr. Bates: I am not sure I can give a very specific answer to that question. Certainly the evidence that we have is that the supplementation of certain nutrients to vulnerable Gambian infants at that stage of life can have measurable advantages, not only in their biochemical indexes but in some of the more important things, one of the examples I gave was gut function. But as you know one always has to balance the advantages against possible harm and also of course against the implications that any program has for the economic ability of the populations to sustain such an intervention. I think we are still at too earlier a stage to say exactly how we can best intervene and how to take a short-term investigation of benefits forward to a population intervention that will eventually produce greater benefits. Clearly those population interventions that are particularly being focused on are with nutrients such as vitamin A and iodine, and to a lesser extent iron. But for the other nutrients I think we still have a long way to go to know what we should be doing and when, and how we should be doing it.

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