

## **Guidelines on the investigation and diagnosis of cobalamin and folate deficiencies**

### **A PUBLICATION OF THE BRITISH COMMITTEE FOR STANDARDS IN HAEMATOLOGY**

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The investigation of a patient for cobalamin (cbl) or folate deficiency involves the demonstration of tissue deficiency, delineation of the particular deficiency and establishment of the cause of the deficiency. Tests should be undertaken with a clear objective, which is usually to determine whether clinical or laboratory features are due to deficiency of either vitamin or, less frequently, to exclude deficiency as their cause. At one time deficiency was synonymous with macrocytic anaemia but it is now recognized that many patients with pernicious anaemia (PA), the best example of these deficiencies, may present without either anaemia or macrocytosis which are late signs of the disease process. In most cases, however, the marrow will show megaloblastic change.

#### **Indications for investigation**

The tests need to be restricted to avoid unnecessary investigation of unaffected individuals but be applied broadly enough to include all patients with a clinical state or laboratory abnormality which may be associated with vitamin deficiency. The clinical indications are of prime importance since routine screening tests, such as the blood count, are not always abnormal. The same criteria apply to both sexes and to all age groups, including pre-term infants and children.

#### **(1) CLINICAL CRITERIA**

The clinical features may indicate which vitamin deficiency is the more likely although in most circumstances both will need to be assessed.

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(a) Gastrointestinal disease including glossitis, abnormalities of taste, previous surgery or radiotherapy to the stomach or small bowel, malabsorption or unexplained diarrhoea. In infants, recurrent vomiting with failure to thrive.

(b) Neurological disease including peripheral neuropathy, evidence of possible demyelinating disease of the spinal cord and visual loss.

(c) Psychiatric disorders including dementia, mental impairment with decreased initiative and concentration, confusion and alterations in mood, especially depression.

(d) Malnutrition including growth impairment in children and those on restricted diets (e.g., vegans).

(e) Alcohol abuse which may be combined with dietary neglect and malnutrition.

(f) Auto-immune disease of the thyroid, adrenal and parathyroid glands. Hypogammaglobulinaemia.

(g) Family history of pernicious anaemia (PA) or of inherited disorders of cobalamin and folate metabolism. The family history should include non-immediate relatives.

(h) Infertility, when anatomical causes have been excluded.

(i) Haematological disease known to be associated with vitamin deficiency—chronic haemolytic anaemias, myelofibrosis, myelomatosis.

(j) Drug therapy known to interfere with vitamin absorption or metabolism including nitrous oxide, phenytoin and other anticonvulsants, dihydrofolate reductase inhibitors, e.g., methotrexate, trimethoprim and pyrimethamine.

(k) In infants, a possibility of inherited metabolic disease.

**The initial investigation of possible vitamin deficiency in these groups of patients should always include a blood count and blood film examination**

## (2) LABORATORY CRITERIA

(a) Macrocytosis is the most common reason for initiating investigation although vitamin deficiency is not the most common cause of a macrocytosis. Macrocytosis without known cause warrants a blood film examination.

(b) Blood film abnormalities. The morphological features of vitamin deficiency, oval macrocytes and neutrophil hypersegmentation, are not always present nor easy to detect. Vitamin deficiency may present without macrocytosis, particularly when there is concomitant iron deficiency or thalassaemia trait. There may be a dimorphic red cell population and macrocytosis may only become manifest when iron deficiency has been corrected. Neutrophil hypersegmentation is not specific for vitamin deficiency, being seen in renal failure, iron deficiency, myelodysplasia and as a congenital abnormality. In addition, it is not present in every patient with vitamin deficiency; its absence is especially to be noted in patients who are seriously ill and neutropenic.

(c) Unexplained anaemia always demands a blood film examination. The features mentioned above (2b) would be indications for further investigation.

(d) Cytopenias. Severe megaloblastic anaemia, for whatever reason, may be associated with thrombocytopenia or neutropenia. Patients with acute folate

deficiency may present with thrombocytopenia or pancytopenia. This may arise in late pregnancy or the puerperium, precipitated by an intercurrent infection, in critically ill patients and in patients with borderline folate stores treated with antifolate drugs. Failure of the platelet count to recover as expected following bone marrow transplantation may also be due to unsuspected vitamin deficiency. Neutropenia can be a dominant feature of cbl disorders in the neonate.

## Investigations

*Essential investigations* are a blood count and blood film examination, serum cbl, serum and red cell folate assays and assessment of the response to treatment. Bone marrow aspiration is always of value.

*Subsidiary investigations* which may be particularly useful in specific circumstances include the deoxyuridine suppression test (DUST), estimation of methylmalonic acid (MMA) in serum or urine and of homocysteine (Hcy) in serum and assay of cbl and folate co-enzymes.

### (1) BONE MARROW EXAMINATION

Bone marrow examination is always of value to confirm tissue deficiency or to indicate an alternative diagnosis. It is essential when the results of other tests are equivocal, e.g., borderline MCV or assays. The recognition of mild megaloblastic change is critically dependent on having well spread films with good quality fixation and staining. Megaloblastic change may be completely or partially masked by concomitant iron deficiency.

### (2) THE ASSAY OF COBALAMIN AND FOLATE

The assay of the vitamins in blood is the current routine procedure for determining the patient's vitamin status (Dawson, Hoffbrand & Worwood 1991). Microbiological assays remain the yardstick against which alternative methods should be compared. Although perhaps more technically demanding they are particularly suitable for the economic processing of large numbers of samples especially by automated and microplate techniques (O'Broin & Kelleher 1992). The use of beta-lactamase (Kelleher, Scott & O'Broin 1990) overcomes some of the difficulties created by antibiotic therapy but not those due to anti-folates.

Radio-dilution assays (RDAs) are in common use and commercial kits have made the assays available to all laboratories with access to the appropriate equipment. They are generally satisfactory. Radio-immune and ELISA methods have been described but have not yet been subjected to an assessment which would allow recommendation for routine use at present. Failure to follow the manufacturer's instructions, when a laboratory uses a commercial kit, may absolve the manufacturer of any product liability.

Whichever assay is used the haematologist should follow the Code of Practice of the Royal College of Pathologists (1989) and the British Haematology

Laboratory Management Practice of the BCSH (1994). The accuracy, precision, compatibility with other procedures, acceptability by the user and the cost of the chosen method should be known. External quality assessment schemes give some guidance to the relative technical performance of the methods in current use. The accuracy of the assays can also be checked by means of cbl standards. The National Institute of Biological Standards and Control (NIBSC), currently hold and will make available the WHO first International standard for serum vitamin B<sub>12</sub>. A secondary UK cbl standard is available from the same source. In regard to accuracy the overall median or mean of cbl assays from a large number of participants in external quality assessment schemes is close to the true result. This is not the case with folate assays; kit results vary, particularly for red cell assays, although most give results of clinical value when compared against the appropriate reference interval for the particular kit.

Variations in the preparation and storage of the haemolysate for red cell folate assay are the main causes of different values obtained by different kits (Gilois *et al.* 1990). It is important that any kit user follows the manufacturer's instructions exactly and is satisfied that the reference interval used is appropriate for that technique.

The precision of most RDAs at normal levels is good and the need for assay in duplicate should be considered. The Code of Practice (Royal College of Pathologists 1989) indicates that singleton assays are permissible provided that some samples are done in duplicate to check on current precision. The in-batch precision quoted by most manufacturers is based, however, upon the repeated assay of samples at only a few concentrations. This does not guarantee that a similar precision is attainable for samples tested in duplicate over a wide range of concentrations. The duplicate results from a number of batches should be reviewed to see whether any fall outside a decision level, e.g., a coefficient of variation of 10% at the lower end of the reference interval, before contemplating the introduction of singleton assays.

To ensure satisfactory quality a certain minimal number of control samples should be included—samples of known analyte concentration at a normal level, at the lower end of the reference interval and at a subnormal level; and three or more samples from the previous batch which should, with the standards, always be done in duplicate.

Samples giving results within 20% ( $\pm$ ) of the lower end of the reference interval, an 'indeterminate' level in regard to decision making, should be repeated in the next batch.

The frequency of assay depends upon the number for suitable batching but should be at least weekly. Although some reagents in a kit may be stored after opening it is generally better not to do this. A laboratory having few requests should consider sending specimens elsewhere. Such a practice could result in some delay in reporting, although this is unlikely to be clinically important.

Performance in NEQAS is deemed satisfactory when results fall within 20% of the method mean. The participant needs to check whether he is following the method protocol exactly when results fall regularly outside this limit.

A laboratory may, when it differs from others using the same system, consider itself to be 'right' and the rest 'wrong' but in this situation its clinical interpretation of the results must be correct.

Kits do not differ greatly in regard to their cost. The larger ones are cheaper per patient sample and dual systems, measuring serum cbl and serum folate at the same time, are even more cost effective. The most useful pair of assays, the serum cbl and the red cell folate, cannot be carried out by a dual system.

#### *Interpretation of assays—serum cobalamin*

A result below the lower limit of the reference interval may be due to deficiency, alterations in cbl metabolism or be normal. As reference intervals are usually based upon 2 SD spread around the mean or median of the population there will inevitably be some normal persons with a serum cbl outside the reference interval.

The serum level in deficiency is related to the degree of tissue depletion with the lowest levels in the most severely affected. Very low levels are seen in some inherited disorders and in PA, although PA may present at any reduced level of serum cbl. The clinical significance of a mildly reduced serum cbl may be difficult to determine but, if the assay request is based upon sound clinical or laboratory grounds, any low level cannot be ignored and investigation to find a cause should be pursued.

Patients with cbl deficiency may occasionally present with a normal serum cbl. This may occur in the presence of liver disease, myeloproliferative disorders, after nitrous oxide anaesthesia, with a marked neutrophil leucocytosis and most importantly, in transcobalamin II (TCII) deficiency and in some other inherited disorders of cbl metabolism.

Disturbances in cbl metabolism, with a reduced serum cbl not associated with tissue cbl deficiency, occur commonly in folate deficiency and pregnancy and in myelomatosis and transcobalamin (TCI) deficiency.

#### *Interpretation of assays—serum and red cell folate*

A low serum folate may indicate deficiency, although a subnormal level is common in hospitalized patients due to negative folate balance. The lower the level the more likely there is to be deficiency. A low level, without deficiency, may occur occasionally in cbl deficiency and with recent ethanol ingestion. Antibiotic therapy is a common cause of falsely low levels with microbiological assays.

A low red cell folate usually indicates tissue deficiency and is of greater significance than a low serum level in the diagnosis of depletion. A low red cell folate without deficiency is common in cbl deficiency. A normal level in the presence of deficiency can occur with acute folate deficiency, in patients with a reticulocytosis and following a blood transfusion.

In a small proportion of patients with vitamin deficiency all assay results are low. This may be due to combined deficiency but more commonly to the

metabolic effect of a lack of one vitamin upon the other. The degree of change of each assay result usually indicates the primary defect. With cbl deficiency the serum and red cell folate are not as low as in folate deficiency and with folate deficiency the cbl level is not as affected as the folate levels and infrequently reaches the very low levels which may be seen in PA.

### (3) RESPONSE TO TREATMENT

Response to treatment not only confirms the diagnosis but failure to respond, partially or completely, may indicate the presence of complicating pathology, e.g., infection, hypothyroidism, iron deficiency or myelodysplasia. For this reason only one haematinic should be given at any one time, except when the patient is seriously ill and the results of serum assays or a DUST are not available.

In severely anaemic patients the clinical and reticulocyte responses confirm vitamin deficiency. Clinical response in the more difficult case is often subjective and significant changes in the red cell count (an increase of 6% or more) and MCV (a decrease of at least 5 fl) together with correction of neutrophil hypersegmentation is required. Such red cell changes may not occur if the initial blood count is normal or if the clinical picture is complicated by iron deficiency or thalassaemia trait.

Additional tests such as the DUST, estimation of cbl and folate metabolites or TCII saturation, may be performed to confirm or indicate the likelihood of vitamin deficiency. The results may be abnormal when serum assays are normal. Assays of cbl and folate coenzymes, fibroblast culture and transcobalamin (TC) estimation are required in the investigation of the rare inherited disorders of cbl and folate metabolism.

### (4) THE DEOXYURIDINE SUPPRESSION TEST

This test is a sensitive indicator that megaloblastosis is the result of impaired thymidine synthesis due to deficiency or metabolic inactivation of either cbl or folate (Wickramasinghe & Matthews 1988). It is of use in confirming the presence of mild deficiency when morphological changes are equivocal, although the test is normal in a small proportion of patients with vitamin deficiency. The degree of abnormality correlates inversely with the patient's red cell count.

The addition of either folic acid or 5-formyltetrahydrofolic acid corrects the defect. The addition of cbls is less uniform; they sometimes fail to correct in cbl deficiency and may correct in folate deficiency.

The DUST should be carried out in a laboratory with experience in the technique and one which has determined its own reference range because minor technical variations can affect the result. It should be carried out on marrow and not on peripheral blood lymphocytes. The test should be set up as soon as possible after bone marrow aspiration; even a short delay may lead to a reduction towards normal in an abnormal DUST value. It is an expensive and time consuming test.

### *Interpretation of results*

Despite variations in technique, most laboratories report normal results to be suppression of the incorporation of tritiated thymidine to less than 10% on the addition of deoxyuridine. The significance of correction with added vitamins depends upon the precision of the technique used; usually a correction of 15% or more of the initial DUST value is significant.

#### (5) METHYLMALONIC ACID AND HOMOCYSTEINE

The serum levels of two metabolites, MMA and Hcy, are increased in patients with cbl deficiency, while in patients with folate deficiency Hcy alone is elevated. In cbl deficiency serum Hcy rises before MMA and provides a more sensitive though less specific index of deficiency. The serum level of MMA may rise dramatically in cbl deficiency and, when measured by a sensitive technique, is now considered by many to be a more reliable indicator of cbl status than the serum cbl assay.

Measurement of urinary MMA in a random sample is a useful screening test. Estimation of 24 h urinary excretion of MMA (Norman, Martelo & Denton 1982) is more sensitive and may be as valuable as a serum assay and possibly of greater specificity, since the serum MMA fluctuates in patients with renal failure. However, urinary MMA also rises in Fanconi's syndrome and other amino acidurias and an advantage of serum assays over urinary assays is that serum cbl and MMA, together with other metabolites if appropriate, can be measured on the one sample.

The preferred method depends on the purpose of the assay, whether for diagnosis or for monitoring progress. Cost and availability of equipment will also influence choice. Capillary gas chromatography-mass spectrometry (GC-MS) permits a definite identification of compounds but is an expensive technique unlikely to be available in any but specialist units. Thin layer chromatography (TLC) is excellent for urinary MMA screening, is inexpensive yet sensitive and should be readily available to the haematologist in any district general hospital. A recommended method is given in the appendix.

#### *Reference intervals (for most methods)*

Serum MMA, 100–750 nmol/l; Urine MMA, 1.0–4.0 nmol/l (5 µg/mg creatinine);  
Serum Hcy, 6–29 µmol/l.

#### (6) TRANSCOBALAMIN II SATURATION

The saturation of TCII with cbl decreases early in cbl deficiency. A cbl assay method of good sensitivity is required because of the small amounts of cbl which may be bound by TCII. As a diagnostic tool this estimation is open to more error than a single cbl assay.

### (7) COBALAMIN CO-ENZYMES

When inherited disorders are under consideration assay of cbl co-enzymes is essential. A specific radioisotopic assay for plasma adenosylcbl has been described which requires high specific activity  $^{57}\text{Co}$ -adenosylcbl. TLC coupled with a microbiological plate assay (bioautography) has the advantage of estimating methylcbl at the same time. This method may also be used to confirm normal synthesis of cbl co-enzymes by detecting the urinary excretion of both methylcbl and adenosylcbl following treatment with hydroxocobalamin in neonates and adults.

### (8) FOLATE CO-ENZYMES

Folate species can be measured semiquantitatively by bioautography or by differential microbiological assays, methods rarely applied today. 5-methyl tetrahydrofolic acid in plasma and red cells can be measured fluorometrically or electrochemically after separation by high performance liquid chromatography, an estimation of value in the investigation of inherited disorders of folate metabolism when used in conjunction with measurement of total folate.

### (9) FIBROBLAST STUDIES

Fibroblasts should be cultured in the investigation of any case in which a genetic error of cbl or folate metabolism is suspected. A sterile full depth skin biopsy should be taken into a rich culture medium for urgent transport to an appropriate centre for specific enzyme and co-enzymes analysis. Fibroblast complementation is required to establish the exact nature of the metabolic error.

### (10) INTERPRETATION OF RESULTS

The interpretation of each investigation has been considered individually above. In practice the results of all investigations should be considered together and in the light of the clinical picture. In this way allowance is made for the relative significance and specificity of each finding, the quality control of each test is enhanced and there are potential benefits for both clinical and laboratory audit.

### (11) MEGALOBLASTIC ANAEMIA IN INFANCY

Severe megaloblastic anaemia in infancy demands urgent investigation and treatment (Cooper, Rosenblatt & Whitehead 1992). Prior to treatment a blood count, blood film examination, serum cbl and serum and red cell folate assays should be performed in the mother and child, and the child's serum should be stored for later estimation of TCII if appropriate. A 24 h collection of urine for MMA, Hcy and protein should be completed and marrow aspirated to confirm the diagnosis, with a DUST if possible (Chanarin 1983).

## Investigation of the cause of cobalamin deficiency

PA is the most important disease to diagnose or to exclude. The demonstration of serum intrinsic factor antibodies (IFAs) or malabsorption of free cbl, improved or corrected by added intrinsic factor (IF), is required to make the diagnosis.

### (1) INTRINSIC FACTOR ANTIBODIES

The presence of IFAs in the serum of a patient with evidence of cbl deficiency is indicative of PA despite their occasional occurrence in other auto-immune disorders (e.g., thyroid disorders, diabetes mellitus, myasthenia gravis). They are found in 50–75% of patients with PA, the incidence depending upon the sensitivity of the test used and the duration of the disease. They are present in 90% of children with the juvenile form but are absent in the congenital type of the disease. Their detection obviates the need for an absorption test in nearly all patients.

Two types of IFA have been detected in the sera of patients with PA; type I prevents the attachment of cbl to IF and type II prevents the attachment of IF or the IF-cbl complex to ileal receptors.

The standard tests depend upon the inhibition by type I antibody of the attachment of isotope-labelled cbl to IF. The sensitivity of the technique depends upon the relative antigen and antibody concentrations and is limited by the effect of TCs in the test and control sera. It can be improved by prior treatment of the sera to remove TCII (Nimo & Carmel 1987). Free cbl in the serum following a recent injection can give false positive results with some techniques and in general should be avoided. A method using <sup>125</sup>I-labelled IF (Conn 1986) is more sensitive than the standard test. An advantage of it and of ELISA methods is that they detect both types of antibody.

### (2) COBALAMIN ABSORPTION

Cbl absorption is initially assessed using free cbl, repeating an abnormal test with added IF. Normal absorption of free cbl does not exclude cbl deficiency due to malabsorption and, if dietary intake is satisfactory, a food cbl absorption test should be considered.

It may be reasonable to delay testing until the patient has been treated since cbl and folate deficiency can affect the bowel mucosa. Correction of the bowel lesion may take up to two months. However, if the patient is in hospital it can be an advantage to proceed with the test during admission. Absorption tests involving the use of radioactive isotopes must not be carried out on women who are, or might be, pregnant.

Free cbl absorption can be assessed by a number of procedures:

#### *The Schilling test*

This is the most commonly used and the indirect one recommended by ICSH (1981). The urinary excretion of an oral dose of radioactive cbl is determined.

Points to note are:

(a) A preliminary 12 h collection is advocated by ICSH so that other isotopes which the patient may have received can be detected and allowed for. This is unnecessary if the management of the patient is precisely known.

(b) A standard oral dose of radioactive cbl should be used in adults. The conventional dose of labelled cyanocbl is 1  $\mu\text{g}$ . For children 0.5 to 1.0  $\mu\text{g}$  is generally used.

(c) The most usual cause of incorrect results is incomplete urine collection. This produces falsely low results. Because the test requires a cooperative and understanding patient it is preferable for outpatients to attend the haematology department or a day ward so that the procedure may be explained by someone familiar with it.

(d) For patients with renal failure the urine collection should be extended to 48 h and a second 'flushing' injection given at 24 h. An alternative test, such as the plasma uptake test, is preferable.

(e) The 'flushing' subcutaneous or intramuscular injection can be hydroxocbl and this can be given at the same time as the test dose is drunk.

(f) Patients on cbl therapy can have the test carried out 24 h or more after the last injection.

(g) Another test can be carried out 24 h or more after the previous one.

(h) The IF should be mixed vigorously with the aqueous cbl just before administration.

A 'dual' test, the simultaneous administration of  $^{58}\text{Co}$ -cbl and  $^{57}\text{Co}$ -cbl bound to IF, has the advantage that the ratio of the excretions shows the effect of IF on cbl absorption regardless of the adequacy of urine collection. An abnormal result indicates a deficiency of IF. However, partly because there is interchange of the isotopes bound to IF, dual tests cannot be relied upon to give a true estimate of the absorption of free cbl.

### *Interpretation of results*

Normal > 10% of 1  $\mu\text{g}$  dose excreted in urine (> 11% when the flushing injection is hydroxocbl). In PA or intestinal disease usually < 5%. The addition of IF in PA should correct the result, or at least improve the excretion by > 25% of the original value. In atrophic gastritis with a low serum cbl often 5–9% but may be normal.

Other tests of the absorption of free cbl are:

(a) The whole body retention test which is the ICSH recommended direct method (ISCH 1981). It is sensitive and reproducible and the best absorption test if the equipment is available.

(b) The plasma uptake test (McIntyre & Wagner 1966) which should be performed with the Schilling test. The result is helpful when there is doubt about the completeness of the urine collection.

(c) The spot-faeces test (Hjelt *et al.* 1977) which overcomes the inconvenience of total stool collection.

An abnormal test by any method should be repeated with added IF. Failure to correct with IF may be due to ileal disease or resection, bacterial overgrowth (repeat after a 7-day course of an oral broad-spectrum antibiotic, e.g., oxytetracycline), pancreatic exocrine insufficiency (repeat with pancreatic enzymes), drugs (para-amino salicylic acid, colchicine, neomycin, anticonvulsants, potassium salts, metformin, phenformin), Zollinger-Ellison syndrome (repeat with bicarbonate), TCII deficiency and inactive or inadequate amounts of IF (check activity of IF in vitro, repeat with increased amount of IF).

#### *Food cobalamin absorption test*

This test should be considered when the absorption of free cbl is normal and the dietary intake of cbl is satisfactory (Doscherholmen, Silvius & McMahon 1983).

The doses may be made individually or in batches and stored at  $-20^{\circ}\text{C}$ . A single dose is prepared by taking 50 g dried powdered egg yolk and reconstituting it with water, adding a 1  $\mu\text{g}$  radioactive dose of cbl, mixing well and cooking this as scrambled egg. This is eaten with tea and one or two rounds of buttered toast. Absorption has been determined from urinary excretion (as in the Schilling test), faecal excretion and whole body counting. Chicken serum, the more commonly used binder in the UK, is no longer available commercially for human administration.

#### *Interpretation of results*

Normal 4–7% of 1  $\mu\text{g}$  dose excreted in urine usually  $< 2\%$  when gastric atrophy or resection is associated with cbl deficiency. Although low results have been described in a variety of conditions, and rarely without cause, defective food-cbl absorption, with normal free cbl absorption, relates closely to reduced gastric acid secretion.

### (3) TESTS OF GASTRIC FUNCTION

Other tests connected with cbl absorption are:

(a) *Assay of intrinsic factor*. This is a direct measurement of the defect in PA and is of value when other investigations have yielded equivocal results or there is a possibility of both a gastric and an intestinal lesion.

(b) *Parietal cell antibodies*. The frequency of these in elderly individuals is such that their presence is of limited value in the differential diagnosis of cbl deficiency. Since they are found in about 90% of patients with PA their absence would weigh against this diagnosis.

(c) Marked changes in the serum gastrin and in the ratio of serum pepsinogen I to II are not specific for PA but at least direction attention to a gastric lesion as a probable factor in cbl deficiency.

**(4) COBALAMIN INTAKE**

This can be assessed by a questionnaire from the hospital dietitian and by the patient recording his/her food intake over a period of at least one week. Particular importance should be given to milk intake. When the precise intake of cbl is in doubt the patient should be referred to the dietitian. The FAO/WHO have revised downward the recommended minimum level of cbl intake to 1  $\mu\text{g}$  daily for adults.

**(5) RESPONSE TO TREATMENT**

Patients with a negative IFA in whom an absorption test is not practicable cannot be given an absolute diagnosis. A response to oral cbl, 50  $\mu\text{g}$  daily, including correction of the serum cbl, over a two month period, indicates normal absorption of free cbl. If the dietary intake is satisfactory a presumptive diagnosis of food-cbl malabsorption can be made.

**(6) TRANSCOBALAMIN II DEFICIENCY**

The diagnosis of this rare, congenital deficiency requires measurement of this individual binder. This may be done by saturating the serum with  $^{57}\text{Co}$ -cbl and separating the binders on a Sephracryl or Sephadex column.

When estimating TCs collect blood (10 ml) into tubes containing 10 mg  $\text{Na}_2\text{EDTA}$  + 20 mg  $\text{NaF}$  to prevent release of R binders from granulocytes.

**Investigation of the cause of folate deficiency****FOLATE INTAKE**

Although there are many causes for folate deficiency malnutrition is nearly always a significant, if not the only, factor. When malnutrition has been determined to be responsible for the deficiency the reason for this should be sought. The common causes are ignorance, apathy and poverty.

**(2) FOLATE ABSORPTION**

The appropriate tests will depend upon the patient's history and clinical features, but would be expected to include tests of cbl and iron status. A cbl absorption test is usually required and, rarely, a folic acid absorption test in the neonate for congenital folate malabsorption. Radiological and special isotopic tests are often required.

It can be difficult to decide if a patient without intestinal symptoms should be investigated for malabsorption. Gluten enteropathy cannot be excluded without biopsy, may first present at any age and only come under consideration when malnutrition is superimposed. The increased incidence of malignancy in untreated gluten enteropathy indicates that exclusion of this is necessary in most patients, except perhaps the elderly and those with increased folate requirements—

although it is not uncommon for folate deficiency in pregnancy to be the first indication of an underlying enteropathy.

The decision to investigate the malabsorption will also depend upon the severity of malnutrition in the individual and in the local population.

### (3) RESPONSE TO TREATMENT

The same principles apply as for cbl therapy. Folate deficiency is a common cause of a low serum cbl. If the serum cbl, serum folate and red cell folate assays are all low, but the relative levels favour folate deficiency (see interpretation of assays), and a test to demonstrate metabolic cbl deficiency is not available it is recommended that the folate deficiency is treated, provided that the patient has no neuropathy and the serum cbl is repeated in four weeks' time. A low serum cbl secondary to folate deficiency will rise to normal in this period and the former low level will require no further investigation. The patient will need to be investigated for cbl deficiency if the serum cbl remains low.

### Costings

Refer to British Haematology Laboratory Management Practice for calculation of the cost of tests. This is a retrospective assessment. A laboratory considering setting up a particular test will obtain some idea of the potential cost from another laboratory, although the figures are affected by volume of work, staff mix, type of equipment etc. The following are costs recorded in some hospitals in 1992.

	£
Serum cbl assay	7
Serum folate assay	7
Serum cbl and folate dual assay	7
Red cell folate assay	7
Urinary MMA, TLC	5
Serum MMA (HPLC)	From 12 to 15
Serum MMA (GC-MS)	16
Cbl-coenzymes (TLC and bioautography)	25
Fibroblast culture and cbl-coenzyme analysis	120
IFA	5
Schilling test, each part	From 27 to 45

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## APPENDIX

### SCREENING TEST FOR METHYLMALONIC ACID IN URINE (BHATT, GREEN & LINNELL 1982)

*Principle:* MMA is separated from other substances by TLC on cellulose followed by staining with a diazo reagent.

*Samples:* The urine may be from a random sample. It can be stored at  $-20^{\circ}\text{C}$  until testing or merthiolate can be used as preservative.

*Standards:* Dilute a stock solution of 100 mg MMA (Sigma Chemical Co., Poole, Dorset, UK) per 100 ml in 0.5 mol/l HCl to give standards of 50, 100 and 500 ng/ $\mu\text{l}$ . The stock solution is kept at  $4^{\circ}\text{C}$ .

*Diazo reagent:* Dissolve one gram tetrazotized o-dianisidine-zinc complex (Sigma) in 12 ml  $\text{H}_2\text{O}$ , mix thoroughly with 3 ml glacial acetic acid and make up to 45 ml with methanol. Prepare in a fume cupboard and use immediately.

*Chromatography:* Apply samples in 1 and 5  $\mu\text{l}$  and standards in 1.0  $\mu\text{l}$  aliquots by syringe 1.5 cm from the longer edge of a  $20 \times 10$  cm aluminium-backed cellulose thin layer plate (Merck 5552, Poole, Dorset, UK). Keep the diameter of the point of application as small as possible. Develop by ascending chromatography in n-butanol/acetic acid/water (5:1:1, v/v). At  $18^{\circ}\text{C}$ , the solvent rises 8 cm in about 50 min, when the plate is removed, oven-dried at  $50^{\circ}\text{C}$ , for 3 min and cooled in contact with a metal surface. Spray for 10 s from a distance of 50 cm with the diazo reagent and reheat at  $100^{\circ}\text{C}$  for 2 min.

*Reading the plate:* Only creatinine, malonic acid and authentic MMA are visible. Amounts of MMA greater than 100 mg are present as bright magenta spots within 5 min. Lesser amounts can be compared visually after leaving the plate face down on the bench overnight.

*Interpretation:* A 5  $\mu\text{l}$  spot as large as the 50  $\mu\text{l}$  standard is equivalent to 10 mg/l, approximately twice the upper limit of the reference interval. If the spot is of this size or greater, carry out full assay, relating excretion to that of creatinine.