B₁₂ Binding proteins

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A reliable sensitive method for measuring vitamin B₁₂ in the circulation was first achieved at Hammersmith in 1950.¹ During the subsequent decade the vitamin B₁₂ status was assessed of patients with a wide variety of conditions.² This culminated in the determination of a unique function for the ileum by Booth and Mollin: that of the absorption of vitamin B₁₂.³ During the course of these early studies greatly increased concentrations of vitamin B₁₂ were found in the serum of patients with very high leucocyte counts – for example, in chronic myeloid leukaemia and other myeloproliferative disorders and in occasional patients with non-leukaemic leucocytosis.⁴ It was shown that circulating B₁₂ is nearly all protein bound and that raised values were associated with an increase in the serum capacity for binding the vitamin.

High values for circulating vitamin B₁₂ were also described in patients with liver cell damage including those with infective hepatitis, hepatic tumours, congestive cardiac failure and abscesses involving the liver or the subhepatic space. Booth recognised the value of these observations in assessing the patients with pyrexias of unknown cause and disturbed liver function.⁵

It had become clear by now that vitamin B₁₂ required specific transport proteins for absorption from the intestine and transport in the body (Figure). The proteins fall into three groups (Tables I, II).

Intrinsic factor and the intestinal passage of vitamin B₁₂

Intrinsic factor (IF) was first postulated in the classical work of Castle (1930) and isolated and characterised by Gräsbeck and his coworkers.⁶ It is a glycoprotein of molecular weight around 60 000, consisting of two polypeptide chains, each binding one molecule of IF. The complex is very resistant to proteolytic digestion and quite specific in its binding properties (much more so than the other B₁₂ binding proteins). Intrinsic factor is essential for the absorption of B₁₂ from the gastrointestinal tract, and it is made and secreted by gastric parietal cells in an amount which greatly exceeds physiological requirements (the mean concentration of 1 µg/ml provides a binding capacity of more than 50 times requirements). The process of producing and secreting intrinsic factor is established early in prenatal life and in atrophic gastritis it persists long after the capacity to produce gastric acid has been reduced to very low concentrations.

A normal daily diet contains 5–15 µg vitamin B₁₂ and the bile delivers a further 5 µg per day into the duodenum. After total gastrectomy lack of intrinsic factor leads to signs of B₁₂ deficiency

within one to four years whereas in those taking a vegan diet (which contains no B₁₂) body stores persist for 10–15 years. Thus B₁₂ status is in part maintained by an effective enterohepatic circulation.

Vitamin B₁₂ in food is bound to peptides and other compounds. Its release from food may start in the mouth but occurs primarily in the stomach and duodenum. Studies *in vitro* suggest that a low pH favours the release of vitamin B₁₂. Curiously, however, in the gastrointestinal tract B₁₂ is not bound initially to IF. R proteins in saliva and gastric juice (*vide infra*) have a greater affinity for the vitamin.⁷ R proteins transfer B₁₂ to IF only after their degradation by pancreatic proteases (Figure). Failure to degrade the R protein-IF complex seems to explain the malabsorption shown in patients with pancreatic insufficiency. This defect is corrected by the administration of either pancreatic enzymes or by cobinamide. Cobinamide is an analogue of B₁₂ capable of displacing the vitamin from the R protein but not capable of binding to IF. It is suggested that normally unwanted and potentially harmful analogues of cobalamin in ingested animal tissues and those produced by intestinal bacteria are bound to R proteins. This prevents their absorption from the upper gastrointestinal tract. They are released by proteolysis in the small intestine along with vitamin B₁₂. The B₁₂ binds specifically to IF rendering it available for absorption whereas cobalamin analogues in the gut and from bile are excreted in faeces. Nevertheless the role of biliary and pancreatic secretions in modulating the absorption of vitamin B₁₂ remains poorly understood.^{8,9} Deficiencies of either bile or pancreatic enzymes are associated with malabsorption of crystalline vitamin B₁₂ but rarely if ever with clinical vitamin B₁₂ deficiency.

Abnormalities of intrinsic factor (Table III) and the subsequent fate of B₁₂-IF complex is discussed in this volume by Schjomsby.⁹

Transcobalamins

In plasma, B₁₂ is bound to two main classes of proteins one with alpha-beta and the other with

TABLE I Sources of B₁₂ binding proteins

Protein	Alternative names	Source
Intrinsic Factors	IF	Gastric Juice
Transcobalamin II	TCII Transcobalamin β-globulin binder	Liver (Other tissues – probably many)
R proteins	R-binder TCI TCI-III Transcortin Cobalophilin Leucocyte binder Salivary binder	Granulocytes Myeloid cells Salivary gland (Other secretory cells in the gastrointestinal mucosa)

Transcobalamin II (TCII) and the transport of vitamin B₁₂

TCII is a plasma protein with β electrophoretic mobility which has a clearcut role in the physiology of vitamin B₁₂. It picks up absorbed vitamin B₁₂, directs it to specific receptors on cell membranes and facilitates its transport into cells. Thus its role in the circulation is closely analogous to that of IF in the gut. It is probable that all tissues require TCII for the adequate uptake of vitamin B₁₂ and this has been shown in several *in vitro* models (including reticulocytes, lymphocytes, fibroblasts, homogenised brain and placenta). Nevertheless brain and liver may have an alternative means of taking up vitamin B₁₂ because although patients with congenital TCII deficiency have severe megaloblastic anaemia they rarely develop neurological deficits; nor do they excrete abnormal amounts of methylmalonic acid or homocystine.

Normal serum contains 20–40 μg (0.7–1.5 nM)/l TCII capable of binding 600–1300 ng vitamin B₁₂. Normally less than 10% of the binding capacity is used. TCII carries B₁₂ both as adenosyl-cobalamin and as methyl-cobalamin (B₁₂ bound to circulating R proteins is primarily in the methyl form). A reduction in the circulating concentration of holo-TCII may be the earliest sign of vitamin B₁₂ deficiency.¹² This potentially important observation awaits confirmation.

In animals TCII appears to be made primarily in the liver. In man reticulo-endothelial cells (as in Gaucher's disease), lymphocytes (as in autoimmune disease) and intestinal cells, along with other tissues, may have the capacity to make the protein. It has a molecular weight of about 40 000. Unlike the other binders it is not a glycoprotein and it is immunologically quite distinct. The NH₂-terminal 19 amino acids have been determined but the elucidation of the molecular structure awaits the development of further monoclonal antibodies to key sites of the structure.¹³

Genetically TCII has been linked to the P blood group system and assigned to chromosome 22. It is polymorphic with an autosomal codominant pattern of inheritance. There are two common codominant alleles, at least three less common forms and an allele which is silent. Racial phenotypic differences are apparent and these show some variation in B₁₂ binding capacity.¹⁴ These genetic differences are important in relating changes in the concentration of B₁₂-binding proteins to tissue pathology.¹⁵

TCII delivers vitamin B₁₂ to tissues where it binds to cell membranes. Adenosyl-B₁₂ is bound more readily than other forms. With reticulocytes transfer into the cell occurs within minutes but in other tissues transfer may take somewhat longer. Receptor binding of TCII-B₁₂ increases as cells move from a resting state to active division.¹⁶ In the liver TCII-B₁₂ bound to the plasma membrane undergoes pinocytosis and is then transferred to lysosomes where it is released by proteolysis. The liver acts as the main store of vitamin B₁₂ (Figure).

Congenital deficiency of TCII is associated with severe megaloblastic anaemia, mouth ulceration, and recurrent infection associated with

hypogammaglobulinaemia and possibly a killing defect of granulocytes.¹⁷ Curiously the condition develops during infancy indicating that the fetus *in utero* has an alternative mechanism for delivering B₁₂ to its tissues. Neurological defects occur only if the diagnosis is long delayed (with the anaemia corrected by the administration of folic acid). Patients with TCII deficiency have low normal values for the circulating vitamin (bound to R proteins) but respond to the administration of large doses of B₁₂. This is also true of patients with apparently normal TCII binding but a failure of tissue uptake.

High values for circulating TCII may occur in active liver disease (alcoholism, acute viral hepatitis, metastatic cancer); in lymphoproliferative disorders (including lymphoma, multiple myeloma and autoimmune disease) and in disorders of the reticulo-endothelial system – for example, Gaucher's disease (Table IV). The association of increased values for circulating TCII with inflammatory and neoplastic disorders suggest that the protein may be released from the liver as an acute phase reactant.

The assessment of the patient with high values for TCII-B₁₂ may be complicated by the findings of immunoglobulin-TCII complexes. These are found most commonly after treatment of pernicious anaemia with B₁₂ by injection. But complex formation may also occur spontaneously in patients with hypergammaglobulinaemia and the amount formed appears to parallel the severity of the pathological process.¹¹

R proteins (R binders; TC1–TC3; cobalophilin; haptocorrin)

R Binders of vitamin B₁₂ have alpha-beta electrophoretic mobility and are found in many body fluids (Table II). Their function is still not wholly clear. The binding occurs over a wide range of pH (1–12) and once bound the vitamin is unavailable to bacteria. This function may be analogous to that postulated for the iron binding protein lactoferrin.¹⁸ In the gut, B₁₂ bound to R-proteins in ingested milk and in secreted bile may be released from its binding by proteolysis and so made available for IF-mediated absorption in the ileum. In the circulation R-proteins appear to have a specific transport function. They provide a mechanism for the re-use of B₁₂ released from tissues and they deliver unwanted B₁₂-analogues to the liver for clearance into bile. Despite these seemingly important functions

TABLE IV Conditions with raised B₁₂-binding proteins (levels of circulating B₁₂ often high)

Rises in	Common	Other
R proteins		
TCI	Chronic myeloid leukaemia* Eosinophilic leukaemia Polycythaemia†	Carcinomatosis Juvenile hepatoma
TCIII		Leukocytosis (Inflammatory disease‡)
Transcobalamin TCII	Gaucher's disease Myeloma Monocytic leukaemia	Liver disease Lymphoma (Cancer) Auto-immune conditions‡

*Good marker of early relapse; †Not raised in secondary erythrocytosis; ‡May be useful in following progress of condition.

congenital R-protein deficiency appears not to carry important clinical implications.²²

The measured content of R-binders in the blood depends on how the sample is obtained and processed because of the *in vitro* release of TCIII from cells (*vide infra*). This can be minimised by immediate centrifugation and separation of EDTA-anticoagulated plasma in the cold. About 80% of circulating vitamin B₁₂ is carried on R-proteins and the measured concentration of the vitamin is not affected by *in vitro* release of binding proteins. Normal plasma contains about 25 µg total R binders per litre most of which are holo-proteins. The molecular weight is about 60 000 and each molecule is capable of binding more than one molecule of B₁₂ or analogue. Unlike TCII the molecule does not shrink when it binds B₁₂.

In the circulation R-proteins appear to be derived primarily from granulocytes. In body fluids R-proteins come from glandular tissue especially lacrimal, salivary, mammary and placental and exist largely as unbound apo-proteins. R-protein has also been demonstrated in the epithelial cells of the biliary tree, small intestine and colon.¹⁹ Gastric juice contains the R proteins of swallowed saliva, some derived from granulocytes and possibly some produced locally. Bile is the richest source of cobalamins in body fluids. Nearly all is in the form of holo-protein representing cobalamins being excreted by the liver bound to R protein. Despite the wide range of proteins produced by a variety of tissues they are determined by a single genetic locus with apparently only two alleles.²⁰ Congenital deficiency of R binder leads to low levels of circulating B₁₂ but no clinical disturbance of B₁₂ metabolism.

The structure of R proteins have been examined in considerable detail. Those in the plasma may be split into overlapping fractions by iso-electric focussing.²¹ This observation has led to considerable confusion. A separate entity called TCIII was characterised by what appeared to be distinct physio-chemical properties. In contrast with TCI, TCIII binds weakly to DEAE-cellulose, is iso-electric above pH 3.35, and is cleared from the circulation very rapidly by the liver. Nevertheless it shares immunological identity, a common polypeptide backbone and common amino acid sequences with the other R proteins. Differences are limited to the content of carbohydrate (fucose) and sialic acid, and most authorities now agree that there are not two distinct classes of R proteins but a variable spectrum depending on the source of the R binder and the mode of separation. Nevertheless the variation in the R binding molecular structure may have physiological significance. The more basic moiety (TCIII) carries little or no endogenous B₁₂, predominates within cells and secretions, and is rapidly cleared from the circulation (which may be responsible for the apparent lack of binding). The more acidic binders (TCI) carry much endogenous B₁₂, predominate in the plasma, and are cleared slowly by the liver. Be that as it may R proteins bind many B₁₂-analogues and provide the body with a system for clearing B₁₂ compounds released from rapidly turning over tissues, pus cells and

necrotic debris. These are delivered to the liver where they are taken up by a system for dealing with asialoglycoproteins, rapidly cleared by hepatocytes and excreted in bile (Figure). Endogenous B₁₂ analogues are not bound by IF and so they escape reabsorption in the ileum. Whether or not such analogues may be harmful in man remains unresolved.²¹

Circulating R proteins are usually markedly raised in patients with chronic myeloid leukaemia, polycythaemia vera and some solid tumours (especially hepatocellular carcinoma) (Table IV). This is almost certainly mainly the result of increased synthesis of the protein but because of changes in molecular structure the half-life of cobalophilin is often much prolonged.¹⁰ Be that as it may, it has become clear that in the plasma of normal subjects R proteins are derived primarily from granulocytes in which their subcellular localisation in specific granules is separate from the disposition of vitamin B₁₂.²³ The release of R proteins from granulocytes appear to explain the raised levels which are found in non-leukaemic leukocytosis (Table III).

Intracellular cobalamin binding protein (ICB)

An intracellular binding protein exists which is immunologically distinct from the R proteins and from TCII.²⁴ It appears to be necessary for the retention of B₁₂ in cells and for the conversion of B₁₂ to metabolically active coenzyme forms such as methylcobalamin and 5'-deoxyadenosyl cobalamin. Most intra-cellular vitamin B₁₂ is bound to apo-enzymes and some appears to exist free in the cytoplasm.²⁵

Clinical value of measuring the B₁₂ binding proteins

The B₁₂ binding proteins are not used in the routine investigation of patients with haematological, inflammatory or neoplastic disorders. The careful control needed in the taking of blood specimens and in laboratory methodology have inhibited development. Most reported studies have been based on the capacity of plasma to bind labelled cyanocobalamin which gives a measure of unsaturated binding capacity (UBBC) and not of circulating holo-proteins. Nevertheless studies of B₁₂ binding proteins are necessary to evaluate patients with unusual forms of B₁₂ responsive megaloblastosis especially in those patients with congenital disorders; may be helpful in the elucidation of myeloproliferative pathology; and can be used as a marker of inflammation in patients with hepatic or intestinal disease.

Investigation is indicated in the megaloblastic anaemias of infancy and in patients in whom there is a chance finding of raised values for circulating B₁₂ without obvious explanation. In patients with an 'acute phase' response TCII concentrations are often raised but B₁₂ concentrations are normal unless the liver is directly involved. In this situation a raised concentration for serum B₁₂ may be a helpful diagnostic pointer.⁴

Low serum B₁₂ concentrations without evidence of B₁₂ deficiency occur during pregnancy

and occasionally in patients with multiple myeloma and associated disorders. In clinical practice, however, a means of determining the significance of a border line value for circulating B₁₂ is the most urgent issue especially in view of recent reports of B₁₂-responsive neuropsychiatric disorders with normal or near normal values for serum B₁₂ and without overt evidence of megaloblastosis.²⁶ The recent report of reduced holo-TCII as the earliest manifestation of B₁₂ deficiency in the serum and thus a clinically useful test is potentially important.¹²

Thus it seems that Dr Beck's statement in a recent editorial is justified: 'To the stalwart little band of investigators of vitamin B₁₂ there is comfort in knowing that the stream of important scientific problems will never end'.²⁶ Studies of these problems have already yielded two Nobel prizes (Minot, Murphy and Whipple for the first successful treatment of pernicious anaemia and Dorothy Hodgkin for unravelling the structure of vitamin B₁₂) and B₁₂ binding proteins may well provide more surprises as we learn about the evolutionary development of B₁₂ metabolism.

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