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ABSORPTION AND TRANSPORT OF COBALAMIN (VITAMIN B_{12})

Bellur Seetharam and David H. Alpers

Division of Gastroenterology, Washington University School of Medicine, St. Louis, Missouri 63110

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I. PERSPECTIVES AND SUMMARY

This review apprises the reader of recent advances in cobalamin absorption and transport since 1970. This field has been of interest to gastroenterologists, hematologists, and biochemists as well as nutritionists. Clinical condi-

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tions leading to cobalamin deficiency present especially to the first two of these specialties. Many recent advances involve biochemical definition of the specific transport proteins themselves. Full understanding of the multiple clinical conditions associated with cobalamin deficiency requires some knowledge of these proteins. We do not discuss every paper in the field, especially the early literature (see 47, 61, 150).

Despite rapid strides in understanding the mechanism of absorption and transport of cobalamin both in prokaryotic and eukaryotic cells, the complete sequence of events during cellular absorption at the plasma membrane level and events in the cells that mediate intracellular movement of cobalamin have not been elucidated completely. Here we emphasize the current state of knowledge about cobalamin absorption and transport in man and other mammalian systems, particularly the physiological role and biochemical nature of proteins involved (intrinsic factor, intrinsic factor-cobalamin receptor, transcobalamins).

II. INTRODUCTION

A. Historical Background

The saga of vitamin B_{12} began more than 150 years ago. Between 1824 and 1900 Combe (32) and Addison (1) described "idiopathic anemia," later characterized by Fenwick (52) as the inability (caused by infection or toxins) of acidified scrapings of diseased mucosa to digest hard-boiled eggs. Studies in the early 1900s by Howard Whipple (167), Minot (112), Whipple & Robscheit-Robbins (168), Cohn et al (31) and others lead to the concept that pernicious anemia was due to a nutritional deficiency resulting in decreased red blood cells. Cohn and his associates later prepared a liver extract, administration of which elevated levels of reticulocytes in anemic patients.

In the late 1920s Castle (27) and his associates demonstrated the presence in normal human gastric juice of an "intrinsic factor" that readily combined with an "extrinsic factor" contained in animal protein and resulted in the absorption of an "antipernicious principle." Further identification and isolation of the "antipernicious principle" from liver extracts was a slow process until 1948, when Randolph West (166) proved the clinical activity of crystalline cobalamin isolated by Rickes et al (130a). The discovery by Shorb (147) and Hutner et al (88) that *Lactobacillus lactis* and *Euglena gracilis* required cobalamin for their growth helped to establish a reliable microbial assay for cobalamin, which tool greatly facilitated the purification and isolation of cobalamin.

B. Types of Vitamin B_{12} (Cobalamin, cbl)

Cobalamin is a complex water-soluble molecule (mol wt 1357) essential to the health of all higher animals and some microorganisms. Its structure contains four reduced pyrrol rings linked together and designated "corrin" because they are the core of the molecule. All compounds containing this ring are called corrinoids. The prefix "cob" designates the presence of a cobalt atom. The term cobalamin now denotes those cobamides that play a role in human and mammalian metabolism, the name vitamin B_{12} those corrinoids demonstrating activity in microorganisms and/or mammals. The chemist sometimes uses the term vitamin B_{12} to refer only to cobalamin (Figure 1) where the x group is cyano, or cyanocobalamin. Above the corrin in the cobalamin structure other axial ligands coordinating the central cobalt can be found, such as OH (hydroxy cobalamin), H_2O (aquacobalamin), CH_3 (methylcobalamin), or 5'-deoxyadenosyl (adenosyl cobalamin). The planar tetrapyrrol corrin ring is substituted with acetamide (CH_2CONH_2) and propionamide ($CH_2CH_2CONH_2$) residues at the R and R' positions, respectively. Below the corrin, 1-α-D ribofuranosyl-5-6 dimethyl benzimidazoyl-3-phosphate is present and is linked to the rest of the

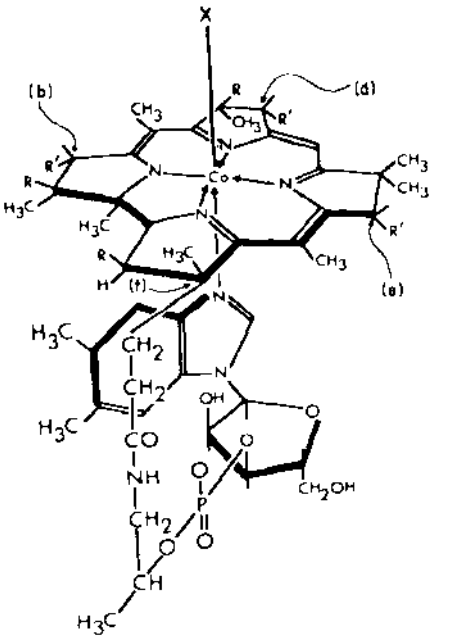

Figure 1 Cobalamin structure. X denotes the beta substituent. R and R' and proprionamide side chains located on the periphery of the corrin ring.

molecule at two points: (*a*) a phosphodiester linkage to a 1 amino-2 propanol substituent on the propionamide (f), and (*b*) through a coordination linkage to the central cobalt via one of its nitrogens.

The naturally occurring vitamins in man are methyl and adenosyl cobalamins. Since the most stable form chemically is cyanocobalamin, it is the one available for medical use. It must be converted in the body to one of the naturally occurring forms. Compounds lacking the nucleotide moiety are called cobinamides. So far all cobinamides found to be active in human metabolism (cobalamins) have included the nucleotide moiety. Analogs of cobalamins (designated vitamin B_{12} analogs) that are used by bacteria may lack either the ribosyl or amino propanol groups or may have substitutions in these structures. Details of the chemistry, structure, and synthesis of all forms of vitamin B_{12} have been reviewed elsewhere (84, 150).

C. Biological Reactions Involving Cobalamin

In all eukaryotic cells intracellular cobalamin exists mainly in two forms: (*a*) methyl cobalamin found principally in the cytoplasm, and (*b*) adenosyl cobalamin found mainly in mitochondria. These two are cofactors for enzymatic reactions.

The only known reaction mediated by methyl cobalamin in mammalian systems involves the biosynthesis of methionine. In this reaction a methyl group is transferred from a donor N^5-methyl tetrahydrofolate to an acceptor homocysteine, giving rise to methionine. The enzyme concerned is 5-methyl tetrahydrofolate homocysteine methyl transferase. Anaerobic bacteria and acetate-producing bacteria can utilize methyl cobalamin to produce methane and acetate.

Adenosyl cobalamin on the other hand is responsible for two types of reactions: (*a*) intramolecular rearrangements mediated by mutases, and (*b*) formation of aldehydes catalyzed by dehydrases. In mammalian systems methylmalonyl coenzyme A mutase converts methylmalonyl coenzyme A to succinyl coenzyme A, and leucine 2,3-amino mutase converts leucine to beta-leucine (126a). In anaerobic bacteria glutamate mutase converts L glutamate to beta-methyl aspartate. The dehydrases on the other hand, are mostly present in aerobic bacteria and utilize adenosyl cobalamin to convert ethyleneglycol and glycerol to acetaldehyde and glyceraldehyde.

Another reaction in bacteria (e.g. lactobacilli) utilizing adenosyl cobalamin involves the conversion of ribonucleotides to 2' deoxyribonucleotides. This reaction is mediated by the enzyme ribonucleotide reductase and is important in the formation of deoxyribonucleotides.

The interrelationship between cobalamin coenzymes and 5-methyltetrahydrofolate in the formation of methionine is of vital importance. During the formation of methionine from homocysteine, 5-methyltetrahydrofolate

is converted to tetrahydrofolate. In the absence of cobalamin the rate of that reaction falls, accompanied by an accumulation of 5-methyltetrahydrofolate. This leads to the inability to form other folic acid vitamers, such as 10-formyl- and 5,10-methylene-tetrahydrofolate, which are required for purine and pyrimidine synthesis. Lack of these vitamers leads to megaloblastic anemia. This hypothesis, known as the methyl folate trap (118), has been the subject of much debate and experimentation; and no clear-cut evidence is available to prove that trapping of 5-methyltetrahydrofolate actually occurs as a result of failure to convert homocysteine to methionine by the cobalamin-dependent methyl transferase.

Indirect evidence has been offered by Scott et al (141a) who have shown in monkeys exposed to nitrous oxide that subacute combined degeneration of the spinal cord develops, and that this lesion can be minimized by maintaining the methionine pool with supplemental dietary methionine. Scott & Weir (141b) have further discussed the hypothesis that kwashoirkor as well as subacute combined degeneration might be related to a deficiency of available methyl groups.

D. Dietary Sources

Cobalamin is made only by microorganisms in nature. Feces are a rich source since colonic microorganisms make large amounts of the vitamin. The usual dietary sources of cobalamin are meat and meat products and to a lesser extent milk and milk products. Fruits and vegetables have very little or no cobalamins, and hence vegans and other strict vegetarians probably derive their limited intake from foods or water contaminated with microorganisms contained in manure. Cobalamin biosynthesized in the colon is not absorbed there. All strict vegetarians slowly develop cobalamin deficiency.

Cobalamin present in foods is stable to high temperatures encountered during cooking but becomes labile if ascorbic acid is also present in the heated diet. The dominant forms present in foodstuffs are the enzymatically active forms—i.e. adenosyl and methyl cobalamins, generally linked to polypeptides.

E. Nutritional Requirements

The recommended dietary allowance (RDA) for cobalamin for an adult is 3 μg (56). However, the requirements in patients may vary due to changes in the gastrointestinal tract, since damage either by a gastrectomy or ileal resection would eliminate normal absorption. Scott & Weir (141), Sullivan & Herbert (155), and others (13, 93) have confirmed the earlier studies of the Vanderbilt group (17a, 40a) that 0.5–1 μg of the vitamin given parenterally daily provides maximum hematologic response. Using radioactive cobalamin, the daily turnover has been shown to be about 0.02% of the

body stores (1 μg lost from 5000 μg stores), and hence deficiency of cobalamin in an adult takes months to occur. However, an enterohepatic circulation of cobalamin is present and accounts for delivery of approximately 5–10 μg/day into the intestinal lumen. The cobalamin in bile is thought to be bound to R protein (see below). Therefore, when malabsorption is present, daily losses may exceed 5 μg/day, and deficiency occurs more rapidly. Normal serum and liver levels of cobalamin are over 200 pg/ml and over 0.28 mg/gm liver.

The joint FAO/WHO expert group (50) recommends daily intakes of 2 μg, 0.9 μg, and 3–4 μg for adults, children (1–3 years), and pregnant women, respectively. These compare with the RDAs of 3.0 μg, 2.0 μg, and 4.0 μg for these physiologic categories, respectively.

F. Deficiency Manifestations in Humans

The initial symptoms that follow prolonged deficiency of cobalamin are pallor, weakness, fatigue, dyspnea on exertion, paraesthesias, and a sore tongue. Deficiency also causes patchy or diffuse and progressive demyelinization, producing neuropathy. In most patients one or more of these symptoms predominate. Severely anemic patients may evidence cardiac failure. Loss of appetite, diarrhea, skin disorders, and loss of hair may occur in more depleted patients.

The occurrence of macrocytic anemia is typical in cobalamin deficiency; macrocytosis appears early, the erythrocytes being larger than normal, varying in size, but with normal hemoglobin saturation. The bone marrow is megaloblastic as opposed to the usual normoblast pattern. Because of decreased conversion of ribonucleotides to deoxyribonucleotides the cells have a higher RNA/DNA ratio.

Neuropathy usually leads to symmetrical paresthesias in feet and fingers and is due to a generalized demyelinization of nervous tissue. The process begins in peripheral nerves and progresses centrally to involve posterior and lateral columns of the spinal cord and the brain. Loss of memory, irritability, and abnormalities of smell and taste are observed. Even though cobalamin deficiency commonly causes structural damage in two major organ systems the two groups of symptoms are not always manifested together (164).

III. ABSORPTION OF COBALAMIN (cbl) IN HUMANS AND OTHER ANIMALS

A series of complex events takes place before the dietary cobalamins can be delivered for intracellular utilization. Three phases of this process are now well described: (*a*) the gastric phase involves release of dietary coba-

lamin and complexing of the released cobalamin with R protein, a glycoprotein secreted by stomach and salivary glands. (*b*) In the intestinal luminal phase, cobalamin is transferred from R protein to intrinsic factor. This transfer is mediated by pancreatic proteases. (*c*) In the mucosal phase, the intrinsic factor-cobalamin complex attaches to ileal mucosal receptors, a necessary step before the cobalamin can be absorbed.

A. Gastric Phase

1. GASTRIC RELEASE OF DIETARY COBALAMIN Cooper & Castle (34) demonstrated that the low pH of the stomach and peptic digestion release most of the protein-bound cobalamin in food. The acid peptic digestion of food protein–bound cobalamin appears to be a necessary step for the release of cobalamin in some circumstances, since patients with hypo or achlorhydria, or partially gastrectomized patients, fail to absorb egg [^{57}Co]-cyanocobalamin (44). However, most patients with cobalamin deficiency after gastric surgery also have insufficient intrinsic factor (135). The absence of gastric proteolysis alone probably rarely causes deficiency, because the cobalamin bound to proteins in food can also be released by the pancreatic proteases.

2. COBALAMIN BINDING TO R PROTEIN AND INTRINSIC FACTOR (IF) R proteins are a family of cobalamin binding proteins that move more rapidly than intrinsic factor on electrophoresis (63, 65). These R proteins are present in gastric juice (64) and in saliva (15), from which they have been purified (6, 19) and shown to bind cobalamin at both acidic and neutral pH (7). Allen et al (7) further showed that human salivary R protein–bound cobalamin with an affinity that was 50- and 3-fold higher than those of human intrinsic factor (IF) at pH 2 and pH 8.0, respectively. Even cobalamin bound to IF was transferred to an equal amount of R protein with $T_{1/2}$'s of 2 and 90 minutes at pH 2 and pH 8.0, respectively, whereas the reverse reaction did not occur. Digestion of R protein and R protein-cobalamin complex with pancreatic proteases led to a 150-fold decrease in the affinity of R protein for cobalamin and a complete transfer of cobalamin to intrinsic factor in 10 minutes. These observations are of considerable physiological importance and are discussed in Section III B.

In the absence of R proteins, binding of cobalamin (cbl) to IF is rapid (78) and occurs over a range of pH values from 3 to 9 (7, 131). The binding is stable; the complex can be broken only with 5 M guanidine hydrochloride and alkaline buffers (66). Binding of cbl induces conformational changes resulting in the formation of IF dimers and oligomers (5, 6). Affinity of binding for cbl is independent of the nature of the axial ligand X (Figure

1). Change in the structure of either corrin ring side chains or the nucleotide portion to produce analogs of cobalamin results in a decrease in affinity for IF, but does not alter the affinity for R proteins. Unlike R protein, pure hog IF or IF-cbl is stable to proteolysis.

3. PROPERTIES, SYNTHESIS AND SECRETION OF R PROTEINS Immunologically identical proteins that bind cbl have been identified in leukocytes and in plasma and other biological fluids. Various R-type proteins have different electrophoretic mobilities because of the different number of sialic acid residues.

The R proteins of gastric mucosa and salivary glands appear to be synthesized in mucosal cells of each organ and probably are not derived from granulocytes (87). In leukocytes the R protein is mainly associated with secretory granules (95, 148). In vitro release is inhibited by the sulfhydryl inhibitor N-ethylmaleimide (36) and by sodium fluoride (140) and activated by lithium (140) and the calcium ionophore A23187 (149). The R proteins of normal human plasma are of two types: transcobalamin I (TC I) and transcobalamin III (TC III). The former binds to anion exchange resins tightly and the latter weakly (20). TC I contains more sialic acid residues and fewer fucose residues than TC III (20). TC I is 70–100% saturated with cobalamin and accounts for 80–90% of the total endogenous vitamin content in normal plasma (72, 85). Both TC I and TC III are immunologically identical, exhibit heterogeneity, and have been thought to be released from granulocytes. It has not been conclusively proven that TC III and granulocyte R protein are identical, though they appear to have similar elution profiles on DEAE cellulose, carbohydrate composition, and other properties (26). Moreover, using isoelectric focusing Hall (73) has shown that the R protein released from granulocytes was not TCI but TC III.

R proteins have been purified from saliva, milk, normal granulocytes, and human hepatomas. Based on their amino acid compositions all these proteins have a molecular weight of around 60–66 $\times 10^3$. A review comparing the various R proteins has recently been published (92).

4. PROPERTIES OF INTRINSIC FACTOR Using the technique of affinity chromatography (3) Allen & Mehlman have obtained a very pure intrinsic factor from human gastric juice (5). It has a molecular weight of 42–45 $\times 10^3$ and 15% carbohydrate per milligram of protein. It has a single cobalamin binding site with an association constant of 1.5×10^{10} M^{-1}. Intrinsic factor found in the gastric mucosa of several other animal species (46) appears to have similar physical properties. Hog intrinsic factor purified by Allen & Mehlman (6) has a molecular weight of 52–57 $\times 10^3$ with 17% carbohydrate. IF from both human and hog can bind about 30 μg of cbl per mg protein.

Human IF has serine as its N terminal amino acid. The N terminal 27 amino acids have been identified (121, 159). Cyanogen bromide cleavage of human IF (116) resulted in four fragments only one of which contained a tyrosine residue. This paucity of tyrosine residues could be the reason for the difficulties observed in the radioiodination of IF.

5. SYNTHESIS AND SECRETION OF INTRINSIC FACTOR Using the technique of immunofluorescence (90, 91) and autoradiography with [^{57}Co]cbl (83) IF has been localized in the parietal cells of the cardiac and fundic regions. Donaldson and his coworkers (146) have shown that radiolabelled IF can be isolated from cultures of rabbit gastric mucosal biopsies incubated with [^{3}H]leucine or [^{35}S]methionine. Using electron microscopic immunocytochemistry Levine et al (106) suggested that in human and guinea pig, IF was associated with the rough endoplasmic reticulum of parietal cells. IF was not located in the contents of secretory granules. IF is presumably transported to the tubulo-vesicular system before secretion into the lumen.

The presence of food in the stomach stimulates the secretion of IF (40, 55). This secretory response is attributed in part to vagal and in part to hormonal stimulation. Atropine (163) blocks the basal IF secretion, and insulin stimulation of vagal activity increases IF secretion (11). Vagotomy decreases both basal and stimulated secretion of IF (111, 162). Atropine abolishes the increase in acetylcholine-induced output of IF in organ cultures of rabbit gastric mucosa (96). Histamine and pentagastrin administered either intramuscularly or intravenously stimulate IF secretion (89, 104), but this increase may be due either to an actual increase of newly synthesized IF or to a "washout" effect of IF already present in parietal cells. Peak IF secretion usually precedes peak acid secretion in tissue (23, 94). Histamine H2-receptor antagonists reduce both basal and stimulated levels of IF (16, 53). However, it is unlikely that such treatment would lead to cobalamin deficiency, since the reduction of IF is only partial. An interesting possibility is that IF may be synthesized as a high molecular weight precursor that is then converted to mature IF in the cell by protease treatment. Such a preprotein has been described for many other secretory proteins.

B. Intestinal Luminal Phase

Cobalamin bound to R proteins in the stomach must be made available to IF before it can be absorbed because R protein does not mediate cobalamin absorption. Okuda (120), Vonderlippe (165), and Toskes (161) suggested that pancreatic proteases might inactivate an endogenous inhibitor allowing cbl to be bound to IF. Allen et al (7) extended this concept and showed that pancreatic proteases such as trypsin and chymotrypsin can partially de-

grade both free R protein and R protein-cbl complexes. This process releases cbl that will eventually be bound to IF. Both [^{125}I]-R protein and R protein-[^{58}Co]cbl complex with an apparent molecular weight of 150,000 are converted to proteins of lower molecular weight (7). This partial degradation releases cbl. Based on these in vitro observations it was suggested that cbl malabsorption in pancreatic insufficiency (7) was due to a failure to partially degrade R protein to which cbl binds with much greater affinity, especially at acid pH. Allen et al (8) further extended their in vitro studies on patients with pancreatic insufficiency. They showed that cobinamide, an analog of cobalamin that binds to R protein with an affinity much greater than for IF, could correct cbl malabsorption in these patients without the addition of proteases. In such a situation most of the R proteins would preferentially bind cobinamide, and cbl would be free to bind to IF. The Schilling tests carried out on these patients using [^{57}Co]cbl either with the cbl analog or trypsin gave normal results. It was concluded that the transfer of cbl from R protein to IF was a normal physiological event. Thus both in vitro and in vivo experiments demonstrate the role of pancreatic proteases in the release of cbl bound to R proteins.

Based on these observations Burgge et al (22) have developed an absorption test for diagnosing pancreatic insufficiency. In this test equal amounts of either [^{57}Co]- or [^{58}Co]cbl are bound to IF and to R proteins. Normal subjects absorb and excrete in the urine equal amounts of both isotopes. In pancreatic insufficiency the isotope bound to R protein is unavailable since it is not transferred to IF. Thus the ratio of excreted isotopes will vary. Although this test is no more complex to perform than a regular Schilling test, it is not yet commercially available.

Marcoullis et al (110) have extended the observations of Allen et al (7, 8) and showed that ingested ^{57}[Co]cbl assayed in jejunal aspirates is bound to IF in normal healthy volunteers and largely to R protein in patients with pancreatic insufficiency. Nicolas et al (117) proposed the "inhibited cobalamin absorption theory" to explain the observations of Allen et al (7, 8). They further demonstrated that IF-cbl complex traverses the human intestinal tract without any structural alterations. Anderson & Von der Lippe have provided further evidence for the protease sensitivity of R proteins (9).

However, some preliminary reports (152, 153) have raised questions about the hypothesis of Allen et al, since incomplete correlations were observed between cobalamin malabsorption and levels of trypsin or chymotrypsin activity or gastric acidity. In addition, cobinamide was not always effective in correcting cbl malabsorption in all patients. In our opinion these reports do not invalidate the overall scheme outlined above for transfer of cbl from R proteins to IF. It is possible, however, that pancreatic proteases are not the only intraluminal factor that affects the binding of cobalamin

to intrinsic factor. Some evidence exists that bile acids may be one of these factors.

A role for bile in the absorption of cbl was suggested by Teo et al (157) when they observed that some patients with obstructive jaundice and T-tube bile duct drainage malabsorbed cbl. The malabsorption was corrected by replacing bile in the T-tube. However, in vitro studies (158) carried out by the same group showed that there was a time- and concentration-dependent inhibition of cbl binding to IF rather than the enhancement predicted by the in vivo studies. Inhibition was a function of dihydroxy bile salts and not trihydroxy bile salts. In order to explain these conflicting results Teo et al proposed the hypothesis that bile salts may bind to the excess free IF present in the intestinal lumen. They argue that such binding might prevent the excess free IF from competing with the IF-cbl for attachment to ileal receptors.

This hypothesis appears to be incorrect since free IF does not bind to the receptor even when present in 100-fold excess (86). Moreover, for their in vitro experiments Teo et al used crude gastric juice as a source of IF. Coronato & Glass (37) have shown an effect of bile salts on the receptor molecule present in the ileal mucosa but not on IF itself. The physiological role, if any, of bile acids in the sequence of events leading to absorption of cbl is still unclear.

C. Mucosal Phase

1. IN VITRO STUDIES Cobalamin is absorbed in the ileum (17, 30), and Herbert (77) suggested that the absorption of cbl was by means of specialized receptors for IF-cbl complex in the ileum. The presence of ileal binding and presumed receptors has been confirmed from various animal species (38, 39, 70, 108). In humans, binding is present over the entire ileum or second half of the small intestine. In fact the terminal ileum does not contain the most active binding (70). Partially purified receptor fractions have been obtained from various animal species (38, 39, 108).

Kouvonen & Grasbeck (102, 103) have obtained relatively pure receptor for IF-cbl from guinea-pig homogenates and have shown that it has a molecular weight of 200×10^3. However, the purity of receptor was not ascertained by protein stains on electrophoresis. Seetharam et al (142) obtained highly purified homogeneous receptor from canine ileal mucosa in large enough quantities to define the protein. They showed that the receptor has a molecular weight of 220×10^3 and is comprised of two subunits of molecular weight 62×10^3 and 48×10^3. They further showed that free cbl, free IF, and abnormal IF-cbl R-cbl does not bind to the receptor. The canine receptor had little or no carbohydrate and the receptor did not

cross-react with anti-IF antibody. In both of these properties it appears that the canine receptor is different from the partially purified porcine and human receptors (103). However, it is possible that these latter preparations contain other proteins, including possibly IF. Using an artificial bilayer (143) the receptor has been shown to be oriented in such a way that greater than 70% of the protein mass and all of the IF-cbl binding sites were on the outside surface. The receptor thus is probably anchored to the membrane by a relatively small hydrophobic anchor piece. Using papain, Seetharam et al (144) have purified the water-soluble IF-cbl binding unit of the receptor from canine mucosa and have shown that this fraction of the receptor has a molecular weight of 180–190 $\times 10^3$. The remaining fragment of molecular weight about 36×10^3 probably represents the anchoring structure of the receptor. The topology of the canine receptors as proposed by Seetharam et al (144) is at variance with that proposed by Kouvonen & Grasbeck (103) for porcine and human receptor. The main differences between the studies is the molecular weight of the functional unit (IF-cbl binding) and anchoring units of the receptor. The canine receptor has a functional unit of molecular weight around 180,000 whereas the molecular weight of the proposed functional unit in porcine and human receptor is 70,000. However, this proposed functional unit of 70,000 molecular weight has not yet been directly shown to bind IF-cbl.

2. IN VIVO STUDIES Even though the nature and structure of IF and its receptor are now well documented, the sequence of events that follow the binding of IF-cbl to receptor is largely unknown. After the attachment of IF-cbl to the receptor there is a delay of up to 3–4 hours before cbl enters the circulation (29, 34, 80). One of the early studies by Rosenthal et al (133) showed that in guinea pigs peak radioactivity was associated with microsomes and mitochondria 1.5 hr after an oral dose of [^{57}Co]CNcbl. Cytosolic radioactivity peaked only after 4 hr. The role of mitochondria in transintestinal transport was confirmed by the work of Peters et al (123) and Peters & Hoffbrand (122). These workers concluded that cobalamin was not absorbed by pinocytosis since no significant amount of radioactivity was detected in the lysosomal fraction. Using everted sacs Hines et al (80) showed that after the attachment of IF-cbl to the receptor only cbl enters the enterocyte, leaving free IF on the cell surface that could accept additional cobalamin. Since free IF does not bind to the receptor, these data suggest the interesting possibility that after discharging its cbl from the IF-cbl complex, the remaining IF is in some way modified to remain bound to the receptor. Using the technique of immune electron microscopy Levine et al (105) concluded that IF is not internalized during absorption. Supporting this concept is the observation of Cooper & White (35), who failed to demonstrate IF in the portal circulation. On the other hand, Kapadia et al

(97) have shown that labelled rabbit IF does penetrate the enterocytes and is present in the cytosol. Using ileal loops Kapadia et al (98) have demonstrated that IF-cbl is absorbed by the enterocytes. After 4.5 hr cbl bound to IF accounted for as much as 40% of cytosolic cbl, while 40% of the radioactivity was found as free cbl. However, Marcoullis & Rothenberg (110a) failed to detect any free cobalamin in canine ileal mucosa 3–5 hr after instilling ^{57}[Co]cbl into the stomach. The question regarding the fate of IF is still not fully answered, and much more biochemical and morphological work is needed before all the details of the transepithelial movement of cobalamin are known. However, we interpret the available data as suggesting that IF is not absorbed into the enterocytes along with cobalamin. Figure 2 demonstrates an overall scheme for the absorption of cobalamin, including all three phases—gastric, luminal, and mucosal.

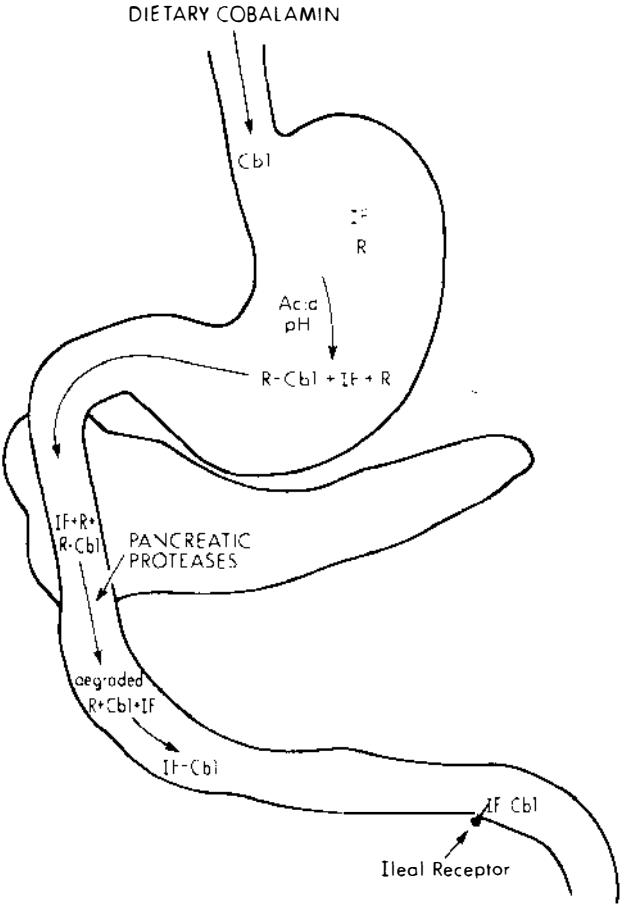

Figure 2 Proposed scheme for the utilization of dietary cobalamin. (Each of the reactions is further discussed in the text.)

IV. TRANSPORT OF COBALAMIN IN THE BODY

After the attachment of IF-cbl to ileal mucosal receptors, cobalamin enters the enterocyte by a mechanism yet to be elucidated. Most of the absorbed cobalamin is found in the mitochondria, and studies using [^{57}Co]CNcbl show that part of this absorbed CNcbl is converted to ado-cbl (122, 123). Cobalamin that exits from the enterocyte is usually not found in free state. In human plasma there are at least three cbl binders—transcobalamins I, II, and III. Most of the circulating levels of cobalamin (200–300 pg/ml plasma) are bound to the R protein, transcobalamin I. Transcobalamin II accounts for most of the unsaturated cbl binding activity found in human plasma (138).

A. Synthesis and Properties of Transcobalamin II (TC II)

TC II is the transport protein that delivers cobalamin to the tissues. Consequently, much work has been carried out on this protein in recent years. Tan & Hansen (156) showed that TC II accumulated in the perfusate of an isolated liver system. Moreover, in an intact animal removal of kidney, stomach, or spleen did not result in any decreased synthesis. De novo synthesis of TC II has been shown in liver perfusates and in cultured rat liver parenchymal cells (33, 49, 136). Extrahepatic synthesis of TC II has been found in dogs (151). TC II production has been demonstrated in macrophages (128) and in fibroblasts (67).

A highly purified TC II has been obtained by Allen and by Puutula & Grasbeck (4, 127). TC II has a molecular weight of 38,000, is not a glycoprotein, and is immunologically different from all other cbl binding proteins. The association constant (Ka) for cbl binding to TC II is $10^{11}M^{-1}$, indicating high affinity (81). The binding is tight, but cbl can be removed by 7.5 M guanidine HCl. TC II has 347 amino acids (2) and a Stokes radius of 2.66 nm (109). One molecule of TC II binds one molecule of cbl and, unlike IF, will bind a wide range of cobamides and other corrinoids at equally high affinity. Molecular microheterogeneity for TC II has been reported by some workers (41, 57) suggesting possible autosomally inherited polymorphism. This observation was based primarily on the different bands obtained on gel electrophoresis. On the other hand, other workers (115, 127) have obtained single bands. The differences between the homogeneity and the heterogeneity of TC II obtained by different workers could also be due to alterations that occur during purification procedures.

B. Function and Metabolism of TC II

The main function of TC II is to transport cobalamin from the intestine after it is absorbed (75) and to deliver the vitamin to other tissues (129). It

has been shown by various workers that the TC II mediated uptake of vitamin takes place in a variety of tissues— e. g. Hela cells and human kidney cells and human liver (54), reticulocytes (130), human placental membrane (58), human fibroblasts (132), Erhlich ascites cells (124), and canine spleen and heart (76).

Seligman & Allen (145) have obtained a receptor for TC II in pure form from human placenta, and suggested that TC II and its receptor might have evolved from a common gene. They suggest that TC II might have evolved as a nonglycosylated protein and the latter as a glycoprotein receptor suitable for membrane insertion. The purified receptor had an association constant of $5.6 \times 10^{10} M^{-1}$ for holo TC II, which was twice that for apo TC II ($2.3 \times 10^{10} M^{-1}$). The higher affinity for the TC II-cbl complex confirms the role of the receptor in mediating transmembrane movement of cbl. This high affinity of the plasma membrane receptor for TC II-cbl is probably the explanation for the reduction in TC II levels in plasma after an injection of cbl either intravenously (43) or intramuscularly (14). The TC II level returns to preinjection levels after several hours. However, the in vivo experiments of Schneider et al (138) suggested that there was a 30% faster rate of plasma clearance of [^{125}I]TC II compared to that of [^{125}I]TC II-[^{57}Co]CNcbl. This observation is hard to reconcile with other studies (14, 43, 145) indicating preferential affinity for the receptor for holo TC II compared to apo TC II.

The uptake of TC II-cbl by endocytosis and the involvement of lysosomes in the degradation of TC II appear to be well recognized. Involvement of lysosomes in accumulating administered [^{58}Co]cbl was shown in earlier studies using rat kidney (114). Using partially purified lysosomes from rat liver and kidney, Pletch & Coffey (125, 126) showed that one hour following an intracardiac injection of [^{57}Co]cbl the radioactivity was transiently associated with a protein fraction with a size similar to that of TC II.

The internalization of TC II-cbl and subsequent degradation of TC II by lysosomes have been demonstrated more conclusively using cultured fibroblasts (169, 170). Decreased sensitivity to trypsin of [^{125}I]TC II bound to cell surfaces with an increase in temperature provides evidence for internalization. Decreased release of acid soluble radioactivity into the medium after the addition of 50 μM chloroquine strongly suggests the involvement of lysosomes in the degradation of TC II (126).

The exact mechanism of internalization of TC II-cbl is not known, and more morphological work is needed to ascertain whether cell surface redistribution occurs after binding and results in the formation of specialized structures such as coated pits. Such a mechanism has been demonstrated morphologically for the internalization of low-density lipoproteins (10).

C. Function of R Proteins

The R proteins from saliva or gastric secretion do not appear to have any role in the intestinal absorption of cobalamin, nor do the plasma R proteins TC I and TC III facilitate cellular transport of cobalamin. First, the R-protein–[^{57}Co]cbl complex does not bind to the ileal receptors. Second, two brothers who had deficiencies of all R proteins were hematologically normal (74). Some evidence suggests that binding of cbl by R proteins protects cobalamin from bacterial utilization. Lactoferrin, an iron binding protein with a well-described antibacterial action, has a tissue distribution very similar to that of R proteins. Furthermore, high concentrations of R proteins are present in granulocytes. Gilbert (60) and Gullberg (68, 69) have suggested that the R proteins may play a role in host defense against bacteria. Allen (2) has suggested a role in clearing the cbl analogs found in the intestinal lumen and in the tissues (101).

D. Binding and Uptake of TC I-cbl and TC III-cbl by Hepatic Asialoglycoprotein Receptor

Ashwell & Morell (12) have shown that liver plasma membranes bind a wide variety of plasma glycoproteins as the initial step in their removal from plasma. These glycoproteins bind to this plasma receptor only if the terminal galactose is intact and no sialic acid is present on the glycoprotein. The asialoglycoproteins are internalized and degraded by the lysosomes within 90 minutes. Some R proteins seem to be cleared by the receptor-mediated system. Using the rabbit, Burger et al (21) have shown that [^{125}I]human granulocyte R-[^{57}Co]cbl and [^{125}I]human TC III[^{57}Co]cbl are cleared rapidly ($T_{½}$=5 min) from plasma, whereas [^{125}I] human TC I-[^{57}Co]cbl and [^{131}I] bovine serum albumin have prolonged plasma survival. The faster rate of clearance of R-cbl and TC III-cbl can be prolonged by saturating the liver plasma membrane receptor by a prior injection of desialated fetuin. Moreover, the TC I-cbl complex can be cleared more rapidly by desialating it before injection. The physiological significance of asialoglycoprotein receptor in clearing R-cbl from plasma is not fully known. Allen (2) indicates that this mechanism may help in the clearance of cbl analogs that might be formed by bacteria, especially since R proteins are known to bind tightly a wide variety of cbl analogs (8, 62). Analogs will then be secreted into bile after removal by the liver. Reabsorption of the analogs will be poor since IF binds analogs of cobalamin with a lower affinity than cobalamin itself (18). It is interesting to note that in studies involving two brothers (25) who

had congenital R protein deficiency, one had neurological illness. However, no harmful effects of noncobalamin analogs of vitamin B_{12} have yet been documented.

Patients deficient in TC II develop pernicious anemia (71, 82) but do not have elevated levels of methylmalonic acid and homocysteine (139) in their urine. It is possible that in these deficient patients cbl is delivered to the liver (but not other tissues) not by TC II internalization but via TC III and the asialoglycoprotein receptor. In one day the amount of TC III produced is so large that it can bind 100–150 μg of cbl.

E. Role of Lysosomes and Mitochondria in Cobalamin Metabolism

The role of lysosomes in the receptor-mediated absorptive endocytosis and the role of mitochondria in further metabolic reactions are not well documented in the intestinal mucosa. Furthermore, it is unclear whether these organelles in other tissues and cells play an important role in the metabolism of cbl bound to TC II. The work of Ryel et al (134) using mouse leukemic lymphoblasts and Pierce et al (124) using Ehrlich ascites carcinoma cells showed that TC III-cbl is not taken up by the classical endocytosis mechanism and TC II was not broken down by lysosomal enzymes. Using rat liver mitochondria Gams et al (59) showed that TC II-cbl was taken up by mitochondria at a rate of 10–30-fold higher than free cbl, and that the uptake was Ca^{2+}-dependent and not affected by 2,4-dinitrophenol. These studies using cyanocobalamin are in sharp contrast to the studies of Fenton et al (51) using hydroxycobalamin. These authors have shown in vitro that swollen mitochondria takes up only free cobalamin by an energy-independent process. In the mitochondria the vitamin was found to be associated with a protein of mol wt 120×10^3.

These studies suggesting noninvolvement of lysosomes in the degradation of TC II-cbl are in conflict with the studies using rat kidney (114), rat liver (126), and human fibroblasts (169, 170). The dynamics of cbl movement out of the lysosomes is poorly understood. Lysosomal membranes are fully permeable to molecules of mol wt 400 or less, and hence it is hard to understand fully a mechanism whereby TC II-cbl would enter lysosomes to be degraded with release of free cbl without involving endocytosis. It is also possible that TC II may not penetrate lysosomes but may be degraded on lysosomal membranes or by cytoplasmic proteases. These conflicting results may also be due in part to varied integrity and purity of the isolated organelles.

V. CAUSES OF COBALAMIN DEFICIENCY IN HUMANS

Our current knowledge of normal cobalamin absorption has come from patients with malabsorption of the vitamin and to some extent from in vivo studies on animal models. Deficiency of cobalamin arises with dietary lack, gastric, intestinal, or pancreatic disease, or due to various genetic abnormalities. In many instances the symptoms are insidious and develop over two to three years. Table 1 summarizes causes leading to the deficiency of the vitamin and relates them to the corresponding physiological defect.

VI. DIAGNOSIS OF COBALAMIN DEFICIENCY

A. Tests Determining Intake and Absorption

No reliable method assesses intake of cobalamin, but the Schilling test accurately reflects absorption. This test utilizes the fact that free cobalamin does not occur in the plasma or elsewhere until all binding proteins are saturated. Then the free cobalamin is filtered through the glomerulus. A parenteral injection of 1000 μg of nonlabelled cyanocobalamin is given to saturate tissue and serum binding proteins. Any serum to be drawn for assessment of body stores (see below) must be obtained beforehand. [^{57}Co]- and [^{58}Co]cbl linked to intrinsic factor (IF) are then given by mouth. Excretion of the labeled cobalamin in urine for 24 hr should exceed 10% of the administered dose if absorption is normal. In cases of possible bacterial overgrowth, absorption can be tested after administration of tetracycline, 1 g per day. The use of simultaneous isotope administration allows the test to be performed in one 24 hr period. The problems with the test involve the collection of urine and the intertest variability. When urine

Table 1 Physiological causes of malabsorption of dietary cobalamin

Physiological step	Disorder
1) Impaired food digestion	Gastrectomy, achlorhydria (45)
2) Decreased IF secretion	Pernicious anemia (28) Gastrectomy (79)
3) Impaired transfer to IF	Pancreatic insufficiency (7, 160) Zollinger-Ellison syndrome (137)
4) Abnormal IF	Decreased ileal binding (99)
5) Competition for uptake	Bacterial overgrowth (42) Fish tapeworm infestation (119)
6) Impaired attachment to ileal receptor	Ileal disease or resection (42)
7) Impaired passage through ileal cell	Familial cbl malabsorption (107)
8) Impaired uptake into blood	Transcobalamin II deficiency (24)

collection is incomplete or renal disease is present, a low cobalamin excretion is an unreliable index of absorption. Intertest variability can be as much as 30–50%, though the use of ratios with two isotopes diminishes this somewhat. Thus excretion of less than 7% is clearly low, and over 10% is clearly normal. When excretion is 7–10% interpretation must be cautious. It is a mistake to attach too much importance to precisely 10% excretion as an arbitrary normal limit. Stimulation of urinary excretion by two-fold or more upon addition of intrinsic factor is suggestive of intrinsic factor deficiency, even if the excretion without IF is in the 7–10% range.

Absorption of cobalamin in food requires breakdown by gastric proteases to liberate free cobalamin. Therefore, the Schilling test may not correlate always with physiological alterations in cobalamin absorption, especially when gastric physiology is altered. However, the tests utilizing food sources of labelled cobalamin are not practical. It is unlikely that cobalamin deficiency develops without at least a partial defect in intrinsic factor production.

When absorption of cobalamin with intrinsic factor is low and intestinal disease is not suspected, the following factors should be considered: The preparation of intrinsic factor may not be active and the patient have pernicious anemia; urine collection may be inadequate; or the mild intestinal dysfunction of cobalamin deficiency may have produced the low excretion.

B. Tests Determining Body Stores

Serum cobalamin usually correlates with body stores. Cobalamin concentration in blood cells is not higher than in serum. Thus hemolysis is not a major factor in producing false results. TC II, the serum carrier protein that delivers cobalamin to tissues, is responsible for only 10–30% of total serum cobalamin. Most of the rest is bound to an R protein, transcobalamin I. Therefore, with TC II deficiency serum cobalamin can be normal, but the vitamin is not delivered to tissues and body stores are low. Furthermore, the normal range for serum B_{12} assumes that 2.5% of patients with normal stores will have low levels. Low levels have been found in normal persons, especially the elderly, with no harmful effects. Therefore serum cobalamin does not always correlate with cobalamin body stores.

The assay for vitamin B_{12} used to be microbiological, using *Euglena gracilis* or *Lactobacillus leichmanii.* Although these assays detected a metabolically active vitamin, they required sterile technique, were time-consuming to perform, and were not sensitive below 100 pg/ml. Radioisotopic dilution assays are now generally available and are based on the principle that endogenous serum cobalamin competes with radioactive cobalamin for binding to a limited amount of cobalamin binding protein (113). However, cobalamin analogs bind to cobalamin protein binders and

can interfere with the serum assay. This is especially true since the commercially prepared binding proteins contain some R protein rather than 100% intrinsic factor. Cobalamin analogs bind more avidly to R proteins than to intrinsic factor. Therefore cobalamin deficiency can be masked by the presence of analogs in the serum resulting in a falsely normal serum value (100). As many as 20% of cobalamin deficient patients might be mistaken in this way. If suspicion still exists that deficiency is present, the microbiological assay should be used to confirm this diagnosis.

Further problems with the serum vitamin B_{12} assay have been noted (48). These levels can be low when the patient has normal body stores. Thirty percent of patients with folate deficiency have low serum vitamin B_{12} levels, though the reason is not clear. Protein deficiency will lower the amount of total serum vitamin B_{12} without having much effect on delivery of cobalamin to tissues, because most of the vitamin B_{12} binding activity in serum is due to transcobalamin I. Up to 75% of strict vegetarians have low serum cobalamin levels without evidence of deficiency (28). It is likely that these patients would develop some signs of anemia with continued inadequate intake. Finally, in pregnancy serum vitamin B_{12} level is low due to dilution and redistribution of the binding proteins. Less than 120 pg/ml of vitamin B_{12} is associated with low body stores if the above factors are not present.

High levels of vitamin B_{12} ($>$ 1000 pg/ml) are seen with acute liver disease due to release from the hepatocytes, and with increased white cells, since they produce an R protein that increases the total serum vitamin B_{12} binding capacity.

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