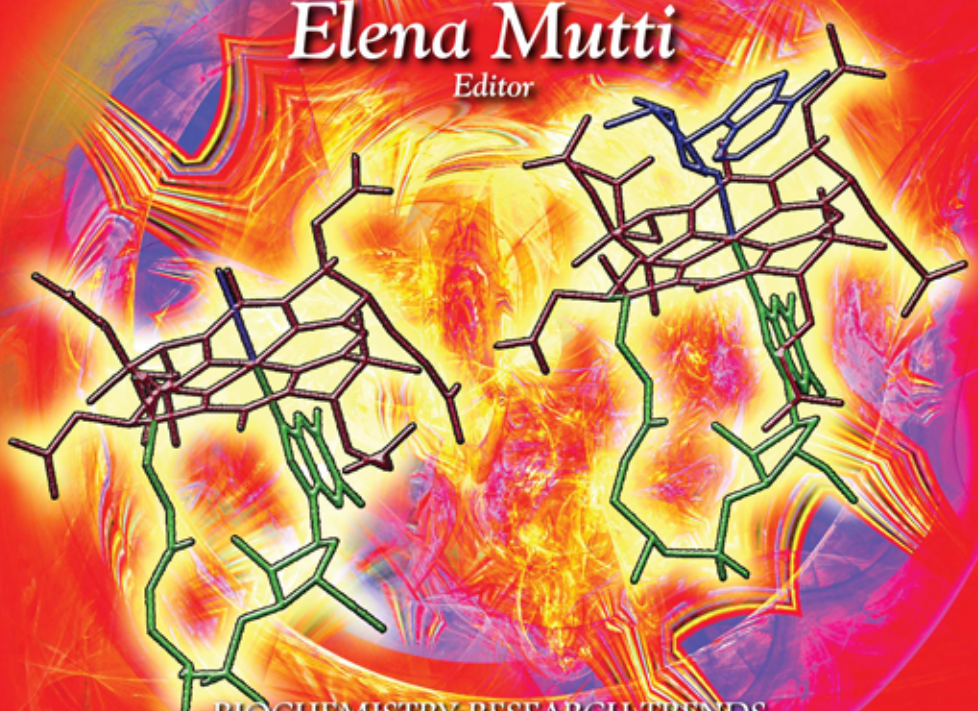
Vitamin B_{12}

Chemical Aspects, Transport, Cause and Symptoms of Deficiency, Dietary Sources, and Health Benefits

Elena Mutti
Editor



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VITAMIN B_{12}

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VITAMIN B_{12}

CHEMICAL ASPECTS, TRANSPORT, CAUSE AND SYMPTOMS OF DEFICIENCY, DIETARY SOURCES, AND HEALTH BENEFITS

ELENA MUTTI
EDITOR

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PREFACE

Although Thomas Addison had described a lethal form of anaemia associated to severe neurological signs in the 1850s, it has been necessary to wait one century till Dorothy Hodgkin was able to identify the structure of the molecule the lack of which is responsible for these disorders, the so called vitamin B_{12} (cobalamin (Cbl)).

Since then, another half of a century has passed and the corrinoid structure of Cbls is now known. New analogues (e.g. antivitamins) have been found and/or realized and their interesting applications in biomedicine described. Significant progress has also been achieved, allowing the understanding of complicated steps of Cbl transport mechanisms that include almost ten actors (e.g. amnionless, megalin, soluble CD320, etc.).

Besides the function of the co-enzyme, a new role for Cbl was identified almost twenty years ago. In fact, Cbl has an epigenetic involvement in the regulation of cytokines, growth factors and Cbl-linked transporters/enzymes. These epigenetic mechanisms could also be involved in the oncogenetic process.

In addition, the involvement of Cbl in new areas of medicine has been identified. For example, in Cbl-deficient status an alteration of immune function or severe failure to thrive have been identified. In nutrition, the precise identification of Cbl-rich food has been done.

Despite the process in understanding the importance of Cbl, the research must go on. In this book, readers will understand that there is still need of analysing several aspects like the mechanisms of epigenetic regulation as well as the real effect and the pharmacological application of Cbl in some human organs (i.e., the role of Cbl in cardiovascular disorders).

This book is written from three groups of “cobalaminlogist” and one pharmacologist with the intention of not only providing technical information

for clinical and biological purposes, but also laying the foundation for a new and alternative approach to Cbl, including nutrigenomics and a holistic study of the Cbl-deficiency effects.

ACKNOWLEDGMENT

The Editor (E.M.) would like to thank Prof. Giuseppe Scalabrino (University of Milan), who has introduced her in vitamin B_{12} field and guided in various phases of her research and Prof. Ebba Nexø (University of Aarhus, Denmark) for her invaluable leadership and discussions during her biochemical studies at the University of Aarhus.

ABBREVIATIONS

ACA	ATP:cob(I)alamin adenosyltransferase (named here: adenosylcobalamin-β-ligase)
Ado	5'-deoxy-5'-adenosyl
AdoCbl	5'-deoxy-5'-adenosyl-cobalamin (often named adenosyl-cobalamin, coenzyme B_{12})
ALAS	aminolevulinic acid synthase
ATP	adenosine-5'-triphosphate
B_{12r}	cob(II)alamin
B_{12s}	cob(I)alamin
BDE	(homolytic) bond dissociation energy
Cbl	cob(III)alamin (DMB-cob(III)amide); cobalamin
Cbl(I)	cob(I)alamin
Cbl(II)	cob(II)alamin
CblC	methylmalonic aciduria type C and homocystinuria (named here: cobalamin-β-deligase)
Cbl-D	Cbl-deficient
CNCbl	vitamin B_{12} (cyanocob(III)alamin)
CNS	central nervous system
DMB	5,6-dimethylbenzimidazol
EGF	epidermal growth factor
EGF-R	EGF receptor
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
Gth	glutathionyl
H_2OCbl^+	aquocobalamin (cation)
HBA	hydrogenobyrinic acid
HC	haptocorrin

hMS	(human) methionine synthase
HOCbl	hydroxocobalamin
holoTC	holotranscobalamin
IF	intrinsic factor
IFD	IF deficiency
IGS	Imerslund–Gräsbeck syndrome
IL	interleukin
MCM	methylmalonyl-CoA mutase (old: MMCM)
MeCbl	methylcobalamin
MMA	methylmalonic acid
MRP1	multiple drug resistance protein 1
N_2O	nitrous oxide
NADPH	dihydronicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NMR	nuclear magnetic resonance
PBG	porphobilinogen
PBGS	porphobilinogen synthase
PBGD	PBG deaminase
PrP^C	cellular prion protein
SAM	S-adenosylmethionine
SC	spinal cord
SN_2	bimolecular nucleophilic substitution
TC	transcobalamin
tHcy	total homocysteine
TNF-α	tumor necrosis factor-α
uro'gen III	uroporphyrinogen III
UROS	urophorphyrinogen synthase
δ-ALA	δ-aminolevulinic acid
ΨCNCbl	pseudovitamin B_{12}

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Chapter 1

NATURAL COBALAMINS – THEIR STRUCTURE, CHEMICAL REACTIVITY AND CO-ENZYMATIC ROLE

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ABSTRACT

The "antipernicious anemia factor" vitamin B_{12} (cyanocobalamin, CNCbl) is a unique "complete" corrinoid, classified as a cobalamin (Cbl). In humans, instead of the "vitamin" CNCbl, the organometallic B_{12}-derivatives methylcobalamin (MeCbl) and coenzyme B_{12} (adenosylcobalamin) serve as cofactors of methionine synthase and of methylmalonyl-CoA mutase, respectively. Cytoplasmatic methionine synthase catalyzes the synthesis of methionine from homocysteine. The mitochondrial methylmalonyl-CoA mutase isomerizes methylmalonyl-CoA to succinyl-CoA. The Cbl-processing enzyme CblC prepares Cbls for their biosynthetic conversion into B_{12}-cofactors. In mitochondria, an adenosyl-transferase installs the organometallic group of coenzyme B_{12}. In all of these enzymatic processes, the bound B_{12}-derivatives engage (or are formed) in exceptional organometallic reactions. This chapter recapitulates chemistry of vitamin B_{12} relevant in B_{12}-dependent enzymes and in the biosynthesis of the B_{12}-cofactor forms.

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Keywords: cobalamin, coenzyme B_{12}, methionine synthase, methylcobalamin, methylgroup transfer, methylmalonyl-CoA mutase, radical reaction, vitamin B_{12}.

INTRODUCTION

The availability of an (extrinsic) "antipernicious anemia factor" in raw liver was discovered in the 1920s, when the pathologist G.H. Whipple treated dogs that suffered from pernicious anemia. Minot and Murphy subsequently also cured their anemia patients in this way. About a quarter of a century later, vitamin B_{12} (cyanocobalamin, CNCbl) was isolated by the groups of Folkers (USA) and Smith & Parker (England) as a red cyanide-containing cobalt-complex (Figure 1). [1]

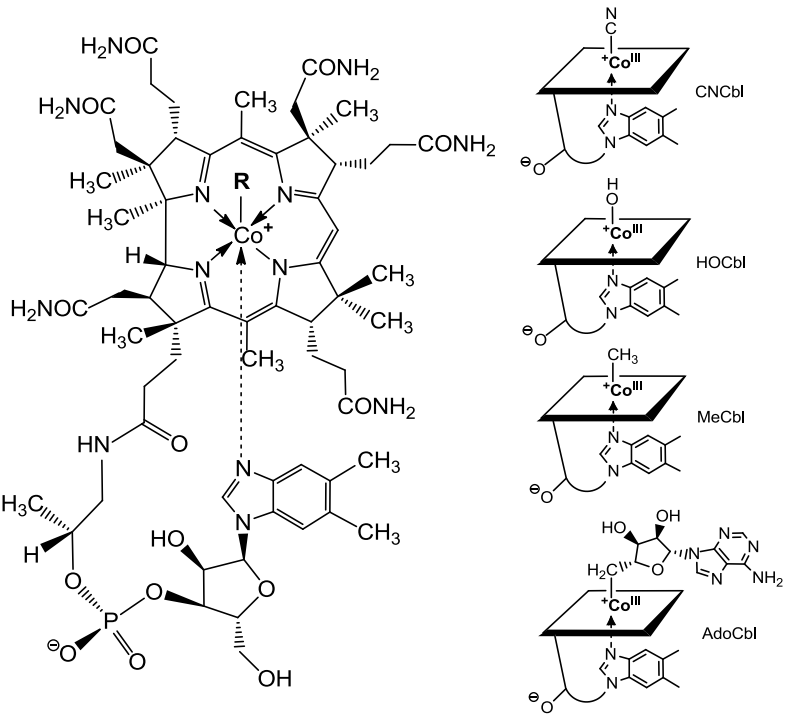

Figure 1. Left: Structural formula of cobalamins (Cbl). Right: Symbols of selected cobalamins: cyanocobalamin (CNCbl, vitamin B_{12}), hydroxycobalamin (HOCbl), methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl, coenzyme B_{12}).

The structure of B_{12}-derivatives was deduced by the pioneering X-ray crystal studies of Hodgkin et al. (Figure 2, left). Crystal studies also deciphered the amazing organometallic nature of coenzyme B_{12} (Figure 2, right). [2] Accurate structures of a range of B_{12}-derivatives have been analyzed, as reviewed recently. [3, 4]

Important earlier contributions to the biologically relevant chemistry of B_{12} were reviewed. [5, 6] During the last decades [4, 7-9], new insights were obtained concerning the role of vitamin B_{12} in humans and animals, driven by the first structures of B_{12}-dependent enzymes and B_{12}-transport proteins, [4, 10-14] by mechanistic studies of B_{12}-dependent enzymes, [8] as well as by investigations on B_{12}-deficiencies. [15-18] Cobalamins (Cbls) are essential to humans and animals, and Cbl-deficiency results in deadly diseases, as outlined elsewhere in this book.

NATURAL COBALAMINS

Cobalamins (5',6'-dimethylbenzimidazolylcobamides) are widely occurring as ‘complete’ corrinoids, which are covalent conjugates of cobyric acid (an ‘incomplete’ corrinoid) and a nucleotide function. In cobalamins, the latter carries a 5,6-dimethylbenzimidazole (DMB) base. CNCbl is the most important commercial form of the naturally occurring Cbls. It is a relatively inert Co(III)-corrin and crystallizes readily. Interestingly, a direct physiological function of CNCbl is not known. [15, 19] The physiologically relevant B_{12}-derivatives in humans are the light-sensitive organometallic cofactors coenzyme B_{12} (5'-deoxy-5'-adenosylcobalamin, AdoCbl) and methylcobalamin (MeCbl). [7, 9, 20]

Structure of Cobalamins

Natural cobalamins (Cbls) carry different cobalt-bound “upper” or β-ligands, e.g., cyanide in vitamin B_{12} (CNCbl, Figure1). Their central cobalt(III)-ion is coordinated six-fold, in a pseudo-octahedral arrangement. The four nitrogen atoms of the corrin ligand occupy the ‘equatorial’ positions at the cobalt center. The typical helical (non-planar) geometry of the corrin moiety derives from a saturated and direct *trans*-junction between the ‘Western’ rings A and D. [23] The DMB-base is coordinated at the “lower” (α-position) in the typical (and thermodynamically more stable) ‘base-on’

form of the Cbls. The Co_{β}-ligand influences the 'base-on/base-off' equilibria, [4] and its increasing σ-donor property [23, 24] correlates with a sizable stretch of the axial Co_{α}-N bond to DMB. At low pH, protonation of the nucleotide base generates the 'base-off' constitution of cob(III)alamins. The ease of this process reflects the stabilization of the 'base-on' form by the intramolecular DMB-coordination (Figure 3).

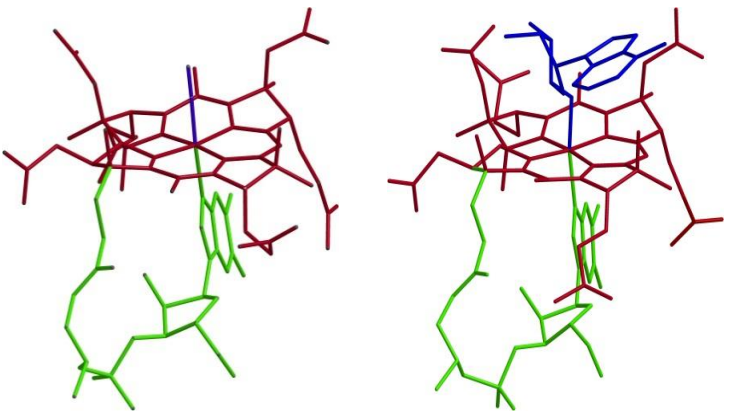

Figure 2. Crystal structures of vitamin B_{12} (CNCbl, left) [21] and of coenzyme B_{12} (AdoCbl, right) [22] - color code: red = cobalt-corrin moiety; green = nucleotide moiety; blue = axial β-ligand.

Nuclear Magnetic Resonance (NMR) spectroscopy has become another important method for studying the structure of B_{12} derivatives in (aqueous) solution. Using heteronuclear NMR experiments, the structures of cobalamins were analyzed in aqueous solution, giving insights into the dynamic behavior of B_{12}-derivatives, such as AdoCbl. [25, 26]

CHEMICAL REACTIVITY

Redox Chemistry

Under physiological conditions, B_{12}-derivatives exist in three oxidation states (as Co(III)-, Co(II)- and Co(I)-corrins), all differing strongly in their reactivity and coordination properties. The number of axial ligands of cobalamins decreases in parallel to their oxidation state (see Figure 3). [27, 4]

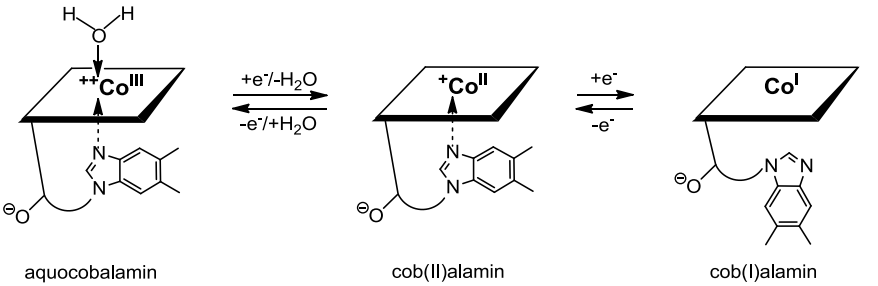

Figure 3. Redox-transitions between six-coordinated aquo-cob(III)alamin, five-coordinated cob(II)alamin and four-coordinated cob(I)alamin.

Cob(III)alamins, such as aquocobalamin (H_2OCbl^+), are red, when 'base-on'. One-electron reduction results in cob(II)alamin, loss of the β-ligand and a colour change to brown. The paramagnetic (low-spin) cob(II)alamin is a radicaloid, occurring in the 'base-on' form in neutral aqueous solution. Further one-electron reduction gives the green, diamagnetic cob(I)alamin with a four-coordinate and very nucleophilic Co(I)-center. Cob(I)alamin reacts rapidly with alkyl halides to givevarious organocob(III)alamins. [4, 27-29]

Two general thermodynamic trends for B_{12}-redox systems have been established:

i. coordination of axial ligands (DMB-base and/or strongly coordinating or nucleophilic ligands) stabilizes the corrin-bound cobalt center against one-electron reduction (Co(III)-/Co(II)-redox couples shift to more negative potentials). As a consequence, redox-potentials of 'base-off' cobalamins (as obtained by DMB-base protonation) are less negative than those of the corresponding 'base-on' forms.
ii. one-electron reduction of organometallic Co(III)-corrins requires more negative potentials than most Co(II)-/ Co(I)-redox couples.

Organometallic Chemistry

Organometallic chemistry is the basis of the cofactor activity of the B_{12}-coenzymes AdoCbl and MeCbl, which relies on the controlled formation and cleavage of their organometallic Co-C bonds. [4, 7, 20] When the Co-C bond of organocob(III)alamins is cleaved by homolysis to a radical and cob(II)alamin, formally, a one electron reduction of the Co-ion has occurred.

Organocob(III)alamins are rapidly formed, in reverse, from combination of cob(II)alamin and organic radicals, the 'homolytic' mode of formation of the Co-C bond. AdoCbl-dependent enzymes operate by this mode: AdoCbl functions as a reversible radical source [30], and cob(II)alamin is a very efficient trap of the Ado-radical (see Figure 4). [4]

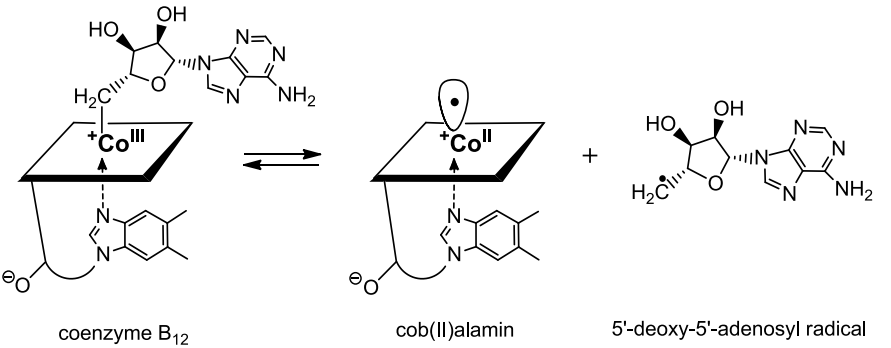

Figure 4. Reversible homolytic cleavage of the Co-C bond of coenzyme B_{12} (AdoCbl) generates a 5´-deoxy-5´-adenosylradical in AdoCbl-dependent enzymatic reactions.

The homolytic Co-C bond dissociation energy (BDE) of AdoCbl has been determined as about 30 kcal/mol. [3, 29, 30] AdoCbl, when dissolved in water, has a half-life of 10^{10} sec at room temperature (in the dark). However, at higher temperatures, AdoCbl undergoes rapid homolysis of the organometallic bond, which furnishes cob(II)alamin. The latter has a structure remarkably similar to that of the Co(III)corrin part of AdoCbl. [31] This explains why the radicaloid cob(II)alamin is a highly efficient "radical trap" and its radical recombination reactions have such remarkably high rates in solution.

The nucleophile-induced demethylation of methylcobalamin (MeCbl) furnishes cob(I)alamin, which may, in turn, react with methylating agents to give MeCbl. This heterolytic mode of cleavage or formation of the Co-C bond is, formally, a two electron reduction or oxidation process. Alkylations at the 'supernucleophilic' Co(I) center normally proceed via a bimolecular nucleophilic substitution (SN_2) in which cob(I)alamin preferentially reacts at its β-side (Figure 5). The immediate product of the β-alkylation is likely to be a 'base-off' Co_β-alkyl-Co(III)-corrin. In aqueous solution, the 'base-on' form of MeCbl is more stable by about 4 kcal/mol than 'base-off' Co_α-aquo-Co_β-methyl-cobalamin. [24] Thus, methylation by the SN_2-mode takes place in a two-step mechanism (Figure 5). [4]

Alkylation of Co(I)-corrins is a general method to prepare organometallic B_{12}-derivatives. Co(I)-corrins are generated via chemical [4] or electrochemical reduction. [20] The electrochemical method is specifically useful for the synthesis of complex organometallic B_{12}-derivaties, such as, e.g., recently prepared DNA-B_{12}-conjugates. [28] In certain cases, alkylation of Co(I)-corrins occurs via an alternative two-step one-electron transfer path where Co(I)-corrins act as strong reducing agents and the reaction proceeds via Co(II)-corrin intermediates. [4, 9] Alternatively, MeCbl has also been prepared from cob(II)alamin with methyl iodide in a radical alkylation process. [32]

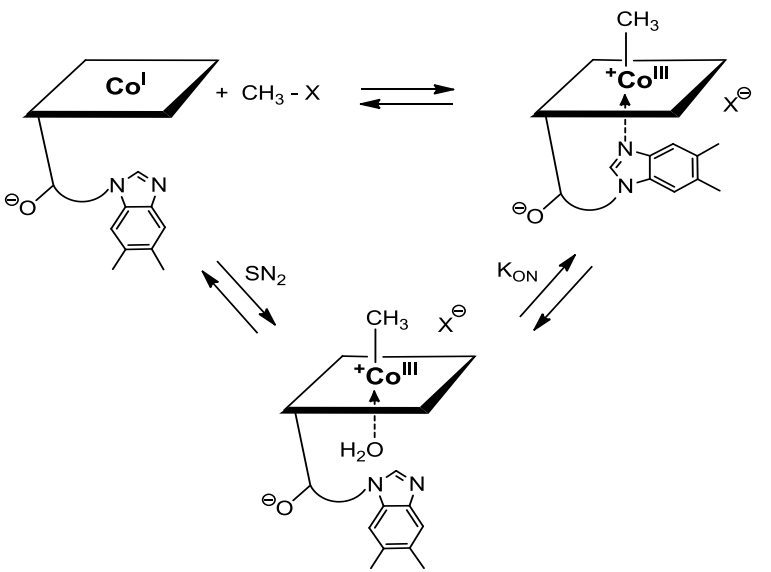

Figure 5. Nucleophile-induced formation and heterolytic cleavage of the Co-C bond of the cofactor methylcobalamin (MeCbl).

HUMAN B_{12}-DEPENDENT ENZYMES

Three major classes of B_{12}-dependent enzymes are known: AdoCbl-dependent enzymes, [33] methyltransferases, [34] and corrinoid dehalogenases. [35] In human metabolism, only two B_{12}-dependent enzymes play a role, namely methionine-synthase (hMS) and methylmalonyl-CoA mutase (MCM) (Figure 6).

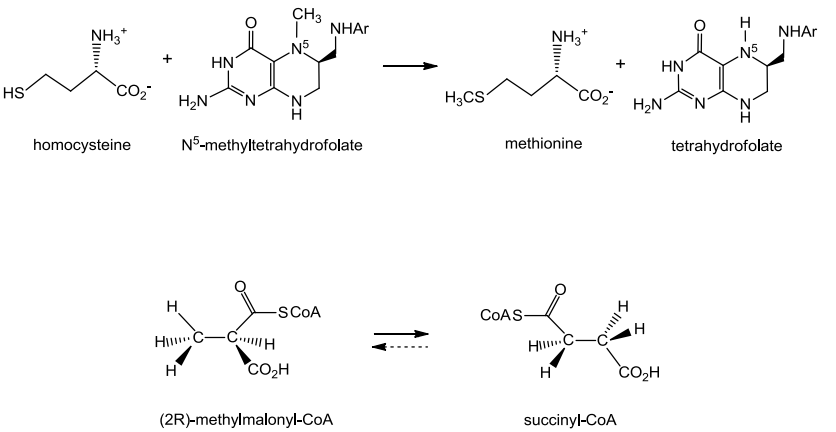

Figure 6. Transformations catalyzed by methionine synthase (top) and by methyl-malonyl-CoA mutase (bottom).

Methionine-Synthase

B_{12}-dependent methionine-synthase is the only methyl transferase in humans and animals. [36] This enzyme uses MeCbl as cofactor and catalyzes the methylation of homocysteine to methionine. Human methionine-synthase (hMS) (E.C. 2.1.1.13) is essential for the amino acid metabolism and is encoded by the cblG locus. [37] hMS is a monomeric protein of ~140 kDa located in the cytoplasm. It shares 55% identity in deduced amino acid sequence with MetH from *E. coli* and appears to have a similar modular structure as its bacterial counterpart. [34, 38] A closely related catalytic mechanism is considered for the two methyl transferases, which involves two SN_2-type half-reactions (Figure 7). [36] First, the methyl group of MeCbl is abstracted by homocysteine to give methionine and cob(I)alamin. Second, the strongly nucleophilic cob(I)alamin removes the methyl group from N^5-methyltetrahydrofolate to give tetrahydrofolate and to re-generate MeCbl. In MetH, the two methyl-transfer reactions occur in a rapid sequence with k_{cat} of 27 sec^{-1}. [34] Oxidation of bound cob(I)alamin to inactive cob(II)alamin occurs occasionally. In humans, this is repaired by the hMS-reductase module via reductive methylation. [4, 38]

Surprisingly, as discovered by X-ray crystallography, MeCbl is bound in MetH in a ‘base-off/His-on’-constitution in which the DMB-base is replaced by a protein-derived histidine. [39] Observation of this type of Cbl-structure was puzzling. Structure-based rationalization of the observed displacement of

the DMB-base by histidine has been a pertinent topic. However, methylcorrinoids lacking a nucleotide base are demethylated by thiolates approximately 1000 times faster than MeCbl, which demonstrates the stabilizing effect of the coordinated nucleotide base. [4, 24] Such axial base effects are expected to be relevant for methyl-transfer reactions catalyzed by Cbl-dependent methyl transferases. [4, 11]

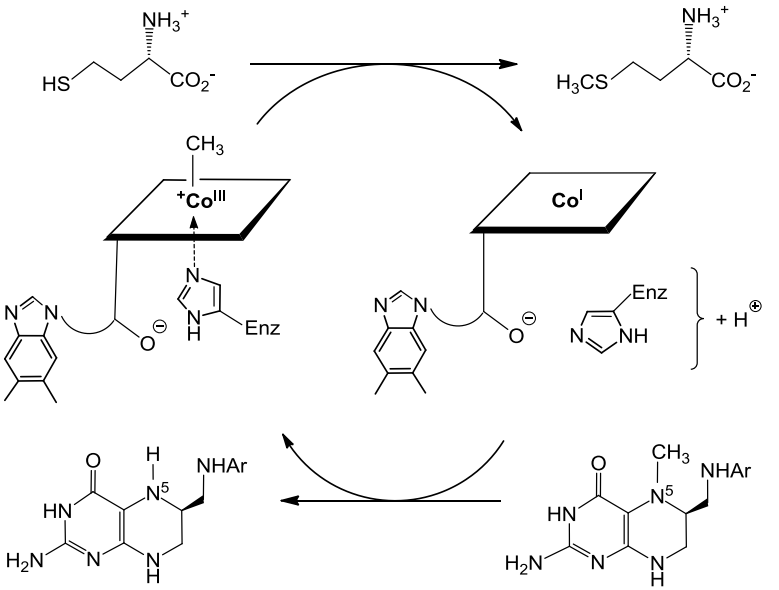

Figure 7. Methionine-synthase (MetH) catalyzes the B_{12}-dependent formation of methionine (top, right) from homocysteine (top, left) and demethylation of N^5-methyltetrahydrofolate (bottom, right) to tetrahydrofolate (bottom, left). The protein-bound Cbl mediates methyl group transfer by shuttling between 'base-off/His-on' MeCbl and cob(I)alamin.

In the well-studied MetH four (nearly independently functioning) modules bind (beginning at the N-terminus) homocysteine, N^5-methyltetrahydrofolate, the B_{12}-cofactor MeCbl and S-adenosylmethinoine (SAM). The B_{12}-cofactor is bound to the B_{12}-binding domain, which provides both an anchoring site for the nucleotide tail, as well as the crucial cobalt-ligating histidine residue of the "*His*-Asp-Ser-triad", as part of the conserved B_{12}-binding sequence (Gly-X-X-His-X-Asp). [11, 39, 40] The catalytic turnover is accompanied by significant structural changes of the B_{12}-cofactor as well by controlled domain shuttling of the protein. Indeed, the B_{12}-binding domain needs to interact sequentially

with each of the three other domains to achieve the overall ‘job’ of the methyl transfer reaction.

Methylmalonyl-CoA-Mutase

Enzymes using coenzyme B_{12} (AdoCbl) as cofactor catalyze metabolically important radical reactions by carrying out ‘difficult chemistry’. [41] The coenzyme B_{12}-dependent enzymes are subdivided into three families: carbon skeleton mutases (e.g. methylmalonyl-CoA mutase (MCM)), B_{12}-dependent isomerases (e.g. diol dehydratases and amino mutases) and B_{12}-dependent ribonucleotide reductase. [7, 9, 33, 41]

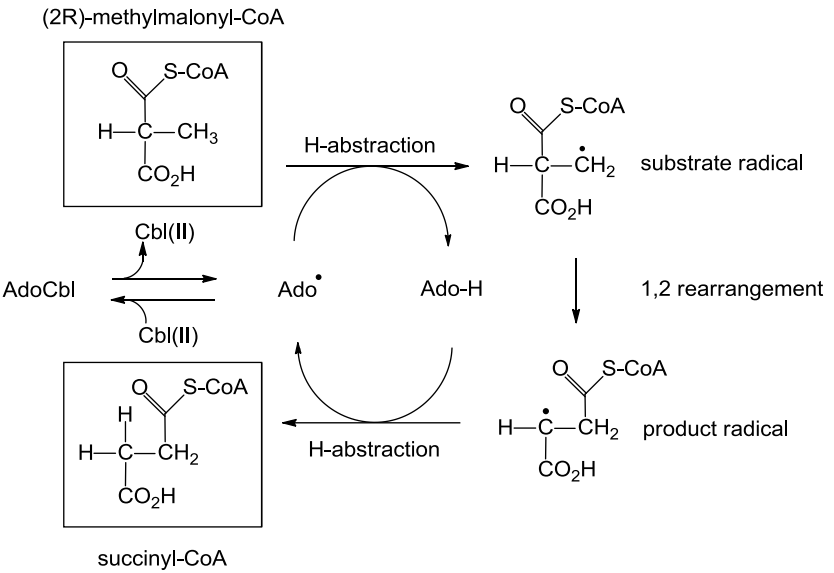

Figure 8. Coenzyme B_{12}-dependent methylmalonyl-CoA-mutase. Mechanism of the 1,2-isomerisation of (2R)-methylmalonyl-CoA to succinyl-CoA. This carbon skeleton rearrangement involves H-atom abstraction, radical rearrangement and back transfer of a H-atom. The protein-bound cofactor AdoCbl serves as a reversible source of the 5'-deoxy-5'-adenosyl radical and of cob(II)alamin (see Figure 4).

In human metabolism, only the carbon skeleton mutase methylmalonyl-CoA mutase (hMCM) is indispensable. [33] hMCM (EC 5.4.99.2) is a ~ 200 kDa protein located in the mitochondria. [42] As a part of the catabolic

pathway of uneven-numbered and branched chain fatty acids,[15, 43, 44] hMCM catalyzes the isomerisation of (2R)-methylmalonyl-CoA to succinyl-CoA (Figures 6 and 8). 'Base-off/His-on' binding of AdoCbl involving the "triad" His-Aps-Lys was discovered by X-ray analysis of MCM from *Propionibacterium shermanii* (pMCM). [13] pMCM, as well as hMCM, bind AdoCbl at the interface of the B_{12}- and substrate-binding domains. [42, 45]

Rapid formation of the radicaloid cob(II)alamin by homolytic Co-C bond cleavage of bound AdoCbl only occurs when substrate binds the pMCM-holoenyzme. [46] The resulting enzyme-bound Ado-radical abstracts a H-atom from the methyl group of (R)-methylmalonyl-CoA to give the 2-methylmalon-2'-yl-CoA radical, which undergoes (an intramolecular) rearrangement to a succin-3'-yl-CoA radical (Figure 8). The latter radical then abstracts an H-atom from 5'-deoxyadenosine to give succinyl-CoA and an Ado-radical, which recombines with cob(II)alamin to regenerate AdoCbl. The selective H-atom abstractions and the radical rearrangement are highly stereoselective and are tightly controlled by the protein environment in the active site. [46, 47] The crystal structure [42] revealed the major protein contributors that interact via H-bonds with the substrate and may help displace the Ado-ligand of the bound AdoCbl-cofactor.

COBALAMIN PROCESSING ENZYMES

Cobalamin-β-Deligase

After arriving in the cytosol, cob(III)alamins carrying various upper axial (β)-ligands are β-deligated to cob(II)alamin, the 'obligate' intermediate for the synthesis of MeCbl and AdoCbl. Malfunction of the crucial Cbl-β-deligase has been shown to be a cause of MMACHC (methylmalonic aciduria type C and homocystinuria). Cbl-β-deligase has been identified as the product of the *cblC* locus, and is, thus, commonly named CblC. [48] Mutations in Cbl-β-deligase (CblC) impair both human B_{12}-dependent enzymes and result in general 'functional B_{12}-deficiency'. [16]

Cobalamin-β-deligase (CblC) cleaves Co-C bonds of alkylcobalamins (such as MeCbl) as well as of CNCbl. (Figure 9) When MeCbl is bound, CblC catalyzes a nucleophilic displacement reaction in the presence of glutathione (Figure 9). Mechanistically, the dealkylation by (human) CblC resembles the first half-reaction catalyzed by hMS. [49] The resulting cob(I)alamin is subsequently oxidized to cob(II)alamin. CblC also catalyzes the reductive

decyanation of CNCbl in the presence of FMN or FAD and NADPH to give cob(II)alamin, as well. (Figure 9) [50]

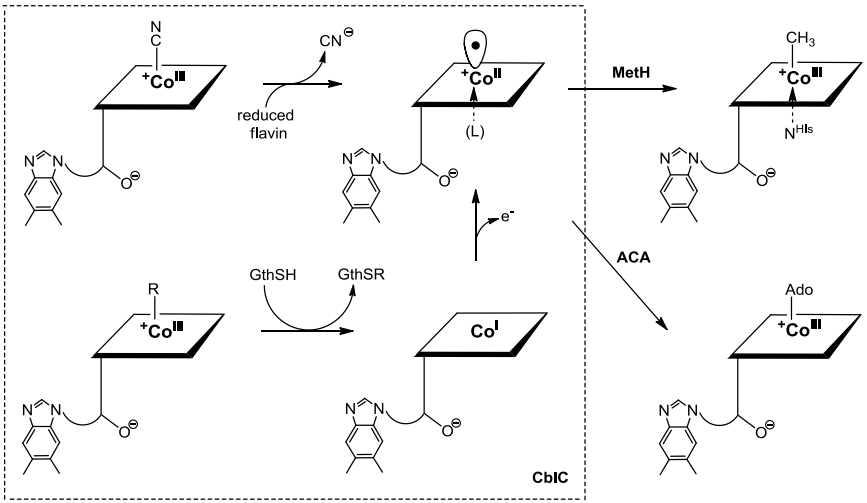

Figure 9. Mechanism of cobalamin-β-deligase (CblC). Cob(II)alamin is formed either by reductive decyanation of CNCbl or by nucleophile substitution of alkylcobalamins, followed by oxidation of the resulting cob(I)alamin. Cob(II)alamin is subsequently handed over to MetH, to be converted into MeCbl, or to adenosylcobalamin-synthase (ACA) giving AdoCbl (GthSH: glutathione; L: tentative ligand).

According to X-ray analysis, Cbls are bound to CblC in a large cavity at the domain interface in a five-coordinate, activated 'base-off' form. The Cbl-nucleotide moiety of MeCbl bound to CblC is bound in a pocket dominated by hydrophobic interactions [51] resulting in 'base-off' MeCbl.

Adenosylcobalamin-β-Ligase

Coenzyme B_{12} (AdoCbl) is enzymatically produced in human mitochondria by the ATP-dependent adenosylcobalamin-β-ligase (ATP: cob(I)alamin-adenosyltransferase, ACA, EC 2.5.1.6, formerly EC 2.4.2.13) encoded by the *cblB* locus. [52] Human ACA (hACA) is a bifunctional enzyme that not only forms AdoCbl via nucleophilic substitution (SN_2) but also transfers 'base-off' AdoCbl subsequently to the Ado-depedent enzyme hMCM (Figure 10). [53]

Cob(II)alamin is bound to hACA in a four-coordinate ‘base-off’ state [52, 53] facilitating reduction to cob(I)alamin by decreasing the redox potential significantly. [27] A crystal structure analysis of LrPduO (PduO-type adenosyltransferase from *Lactobacillus reuteri*) revealed an ATP molecule located above the cob(I)alamin β-side. The substrate orientation favors nucleophilic attack of the protein-bound cob(I)alamin at the 5´-carbon of ATP generating five-coordinate ‘base-off’ AdoCbl. [54] A chaperone-type delivery of AdoCbl by ACA to MCM has been proposed. [53]

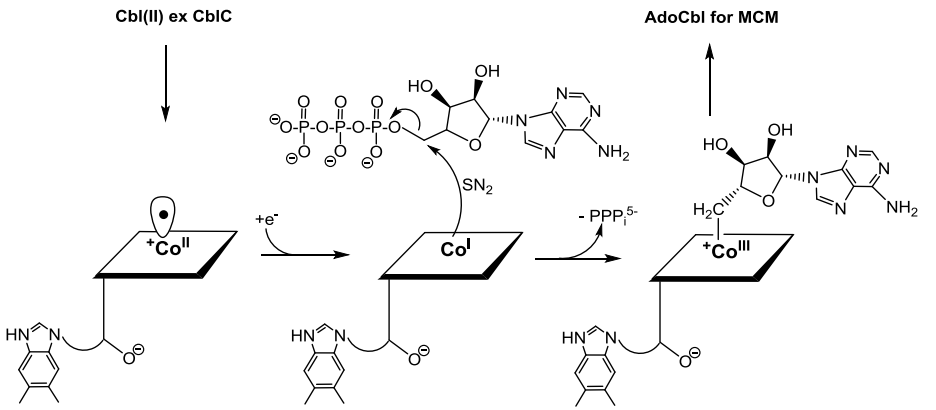

Figure 10. Mechanistic scheme of adenosylcobalamin-β-ligase (ACA). Cob(II)alamin is bound four coordinate ‘base-off’, thereby facilitating reduction to the highly nucleophilic cob(I)alamin, which attacks the 5´-carbon of ATP forming AdoCbl.

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Chapter 2

VITAMIN B_{12} ABSORPTION AND TRANSPORT IN MAMMALS

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ABSTRACT

Mammals are incapable of synthesizing vitamin B_{12} (cobalamin (Cbl)). Instead, they have a complex multi-step pathway for specific and efficient transport of this essential vitamin from its food source to the target body cells. Dysfunction in any of the transport steps may lead to Cbl deficiency.

Keywords: cobalamin, vitamin $B_{12,}$ absorption, transport, transcobalamin, CD320, intrinsic factor, stomach, intestine; blood

INTRODUCTION

The transport of Cbl from its food source to reach the body cells is mediated by a sophisticated set of carrier, receptor and transporter proteins [1]. The selective multi-step pathway of Cbl transport includes the liberation of

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Cbl from its matrix in the ingested food, gastrointestinal transport, absorption to reach the blood circulation, transport through the circulation, and cellular uptake from the circulation. These sequential steps of the transport process can be followed by tracking the Cbl content of the ingested food through the locations it passes by, starting from mouth to stomach, small intestine, ileum enterocyte, blood circulation until it reaches the target cells in different organs.

The Digestive Tract

Different food types have varied Cbl content. Cbl is absent from plant-source food but present in different amounts in meat based food (see chapter "Food source of vitamin B_{12}"). Cbl is mainly protein bound in its food source. The encapsulating matrix of Cbl affects its bioavailability as the vitamin has to be liberated from its matrix to become accessible to the transport mechanism [2, 3]. The efficient active Cbl absorption process guarantees uptake of minute amounts, but still passive diffusion of Cbl happens along the entire gastrointestinal tract (~1% of the ingested dose). This passive absorption is relevant upon using high Cbl oral-doses for deficiency treatment [4].

1) Mouth

The absorption of Cbl is negligible or does not exist under physiological conditions in the mouth, but its obvious role in chewing make food more accessible to the stomach acidity and enzymes' proteolytic activity, which both play a major role in liberating the Cbl from its food matrix. The mouth salivary glands secrete haptocorrin (HC) to saliva (about 50 nM) [5]. HC, a glycoprotein that binds Cbl and its non-coenzyme analogues, so it's not specific for Cbl. The Cbl-HC binding is unlikely to happen in the mouth, since the Cbl is not yet released from the food components. HC of salivary origin proceeds to the stomach, where some amount of gastric HC is also produced [6].

2) Stomach

Stomach acidity with the help of proteolytic activity of pepsin liberate encapsulated food Cbl. The released Cbl subsequently binds HC [7]. This binding protects the vitamin from chemical modification or acid hydrolysis in the stomach [8, 9]. Gastric dysfunction and diminished acid secretion (gastric atrophy, gastric surgery or treatment with acid suppressing drugs) may lead to Cbl malabsorption [10].

Parietal cells of the gastric mucosa secrete the intrinsic factor (IF), a Cbl-binding carrier. IF is a 60 kDa heavily glycosylated protein found mainly in the gastric juice and ileal fluid of mammals [11]. Despite high excess of gastric IF (about 50 nM) [7-9] almost no binding occur in the stomach to Cbl as the interaction is pH sensitive [7, 12]. In contrast to HC, IF is specific for Cbl and does not bind Cbl analogues therefore sorting out Cbl-analogues from the transport mechanism [7, 13]. IF function is essential for Cbl absorption as will be described below.

3) Lumen of the Small Intestine

Upon reaching the duodenum, the HC-Cbl complex is degraded by pancreatic proteases and the liberated Cbl bind to IF [7, 12]. HC is sensitive to proteolytic activity of trypsin, chymotrypsin and elastase, while IF is less sensitive to these intestinal enzymes, especially in its Cbl-bound form [7]. Thus, the different susceptibility of IF and HC to luminal proteolysis explain the transfer of Cbl from HC to IF. Reduced pancreatic enzyme secretion (of 80-90% as in some cases of chronic pancreatitis) leads to impaired degradation of HC-Cbl and the trapped Cbl become inaccessible to IF [14]. Deficiency of IF as in the case of Pernicious anemia (autoimmune attack on the parietal cells) or Hereditary IF Deficiency (IFD) (rare inborn errors of synthesis) leads to Cbl deficiency [15]. IF deficiency is corrected by exogenous-IF feeding [16].

4) Ileum Enterocytes

a) Entrance of Cbl

In the distal ileum, the Cbl-IF complex (IF-Cbl) complex is recognized by the receptor cubam on the apical plasma membrane of the enterocyte [17]. Once bound, the IF-Cbl complex is taken up by receptor mediated endocytosis [18]. Cubam recognizes IF-Cbl but not the Cbl-unsaturated IF nor the free Cbl [17, 19].

Cubam is a protein complex between the 400 kDa cubilin receptor and the 48 kDa amnionless protein [20]. Cubilin is a peripheral membrane protein that binds IF-Cbl while amnionless is a transmembrane, endocytic protein [19, 21]. Cubilin depends on amnionless for its endocytic internalization [22]. Without amnionless, cubilin is detached from the membranes, whereupon the cell loses the ability to absorb IF-Cbl [23].

The physiological importance of the cubam for Cbl absorption is obvious in patients suffering from Imerslund-Gräsbeck syndrome (IGS), a rare Cbl

absorption disorder caused by defects of cubam [24, 25]. IGS patients carry mutations in cubilin or/and amnionless [26, 27].

b) Transport within the Enterocyte

Upon cubam-mediated internalization to endosomes, IF-Cbl is liberated from cubam and transferred to lysosomes. Cubam, on the other hand, recycled to the cell surface. In the lysosome IF is degraded by the actions of lysosomal protease (most likely by cathepsin L) [28] and the Cbl is released. Inhibitors of lysosomal function block secretion of Cbl from cultured cells [29]. Released Cbl exit from lysosome to reach the cytoplasm, a process that probably involves the proteins LMBD1 and ABCD4. LMBD1 is a 61 kDa lipocalin receptor-like, lysosomal membrane protein while the ABCD4 is an ATP binding cassette (ABC) transporter [30]. Mutations in the genes encoding LMBD1 or ABCD4 are responsible for the rare inborn defects named *cblF* and *cblJ* respectively, both have similar phenotypes and clinical symptoms (For more details see chapter "Causes of vitamin B_{12} deficiency"). Defects in LMBD1 or ABCD4 resulting in trapping of free Cbl in enterocytes' lysosomes and therefore Cbl fail to reach the blood stream [30-32]. The mechanism of LMBD1 and ABCD4 contribution in the intracellular transport of Cbl is currently unclear. However, it was proposed that the translocation mediated by LMBD1 and regulated by ABCD4. Cbl transport after the lysosomal exit until its export from the cell is still unknown. Recent studies indicate that Cbl exit from the lysosome is bound by cobalamin-ß-deligase (CblC) protein in the cytosole, which is involved in decyanation of cyano-Cbl and dealkylation of alkyl-Cbl [33-35] then probably passed on to the cytosolic CblD protein [36] which plays a role in Cbl traffic to apomethylmalonyl-CoA mutase in the mitochondrion and apo-methionine synthase in the cytosol.

c) Exit from the Enterocyte

Cbl that exit in its free form (unbound to proteins) from the ileal enterocytes is exported by the basolateral multiple drug resistance protein 1 (MRP1) to reach the bloodstream [37]. MRP1 is a 190 kDa ATP-binding cassette transporter protein. MRP1-lacking mice have increased Cbl levels in the ileum, implying a physiological function of MRP1 in Cbl transport out of the enterocytes. However, the efflux of Cbl is only partially inhibited in these mice. This observation supports the existence of an alternative Cbl export mechanism that might exist with redundant activities to MRP1 [37].

Circulation

Upon the exit of Cbl from the enterocyte to the bloodstream, Cbl binds mostly to transcobalamin (TC) [38] though HC also exists in comparable concentration to TC. This is because of the high concentration of unsaturated TC in plasma compared to the concentration of unsaturated HC [39, 40]. TC is a non-glycosylated protein that has an essential role in transporting Cbl through blood circulation and from circulation to inside the body cells [41, 42]. Most tissues synthesize TC, which reaches the bloodstream by internal secretion [43, 44]. The TC importance is clear in children with an inborn defect of TC synthesis (absence or inactive TC) [45] (For more details see chapter "Causes of vitamin B_{12} deficiency"). The affected child develops severe Cbl deficiency within months of birth.

TC-Cbl (holoTC) is available for uptake by body cells. The main targets for the plasma holoTC are the liver [39] and the kidney [46]. Cbl bound to HC can be taken up by hepatocytes via the asialoglycoprotein receptor, but is unavailable to other cells. The physiological relevance of HC and its uptake are unknown [47, 48]. In humans HC is present in saliva, breast milk and plasma along with other body fluids [49].

From Circulation to Cells

TC-Cbl complexes enter the body cells by CD320 receptor-mediated endocytosis. CD320 is a 58 kDa heavily glycosylated endocytic LDL receptor, expressed virtually in all tissues [50]. Upon endocytosis, TC is degraded to liberate Cbl in the lysosome [51, 52] probably in the same way as described above for enterocytes. In support of its role in Cbl uptake, newborn with a deletion mutation in the CD320 gene affecting the LDL receptor domain causes a decrease in TC-Cbl binding and uptake by cells, though it is not clear that this disorder has any clinical phenotypes [53].

Cbl Recycling

The minute amount of Cbl absorbed are retained in the body, both through reabsorption in the kidney and through the enterohepatic circulation, therefore deficiency needs years of insufficient dietary intake to happen if the uptake

system is intact. The secreted Cbl in bile is reabsorbed via the IF-dependent manner.

TC-Cbl are prone to glomerular filtration because of the relatively small size, 46 kDa with 2.5 nm radius [44]. The filtered TC-Cbl complex is reabsorbed by the high affinity receptor megalin, thereby preventing urinary loss and mediates the vitamin return to the blood circulation. Megalin is a 600 kDa LDL receptor located in the apical membrane of proximal tubule cells [54, 55]. Upon megalin receptor mediated endocytosis, the liberated Cbl from TC reaches and accumulates in lysosomes of the kidney (indicating a storage function for this organelle) [56]. The exit mechanism of Cbl through the basolateral membrane of renal tubule to return to bloodstream is still unknown.

CONCLUSION

The discovery of genes and proteins involved in Cbl transport over the last 50 years make it possible to describe the pathway of Cbl transport from food to the body's cells. This knowledge helps in the diagnosis and treatment of Cbl deficiency caused by a defect in the transport mechanism. Contrasting the detailed gastrointestinal transport information mentioned in this book, much is still to be learned about Cbl transport in other organs and transport to and within the central nervous system (CNS), fetal-maternal Cbl transport in the placenta, as well as transport from the lactating mammary gland into milk. Also, the role of HC and the soluble form of CD320 receptor that present in many body fluids are not clarified, [57] and it’s not clear the role of TC-Cbl and HC-Cbl in Cbl absorption as these found to be taken up to some degree by ileal enterocytes [48, 58].

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Chapter 3

B_{12}-BIOSYNTHESIS, NATURAL COBALAMIN ANALOGUES, SYNTHETIC COBALAMINS AND NOMENCLATURE

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ABSTRACT

Microorganisms have developed two distinct biosynthetic pathways to synthesize vitamin B_{12}, nature's most 'beautiful' cofactor. Both routes require more than 30 enzymatic steps to complete vitamin B_{12} biosynthesis. Although artificial total synthesis of vitamin B_{12} has been found to be challenging, significant interest remains on the synthesis of vitamin B_{12} derivatives with medical applications.

Remarkably, structurally analogue 'complete' corrinoids distinguishable from cobalamins by their different lower ligands are found in archaea and bacteria. Although humans only require cobalamins as cofactors for methylmalonyl-CoA mutase (MCM) and methionine synthase (MS) other members of the 'corrinoid family' have an indirect

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influence on human health as well as ecosystem function as they are mandatory for several bacteria in microbial communities.

Here we discuss the biosynthesis of B_{12}, the chemical synthesis of B_{12} and the B_{12}-analogues, as well as the structural differences, the distribution in nature and the biological relevance of natural analogues of cobalamins, and finally nomenclature.

Keywords: 'complete' corrinoids, B_{12}-analogues, biosynthesis, chemical synthesis, B_{12}-nomenclature

INTRODUCTION

The majority of prokaryotes, animals and humans depend on corrinoids, a family of molecules that includes cobalamin (5,6-dimethylbenzimidazolyl cobamide, Cbl). Only a minority of archaea and bacteria can synthesize corrinoids *de novo*. The elucidation of the biosynthetic pathway to cobalamin took several years and is still in progress. All pigments of life (e.g. chlorophyll, heme and vitamin B_{12}) share the same pathway in their early stage of biosynthesis. The starting material is δ-aminolevulinic acid (δ-ALA), which is converted into the first cyclic intermediate uroporphyrinogen III (uro'gen III) by three enzymatic steps. Uro'gen III represents a branch point in the biosynthesis of all pigments of life. At the next biosynthetic intermediate precorrin 2, the pathway divides into an aerobic and an anaerobic branch. The branches converge in the final stages of the biosynthesis.

When the crystal structure of vitamin B_{12} was solved, chemists focused on its chemical synthesis. This challenging task was completed by Eschenmoser and Woodward in the 1970's. Despite the complex structure, the chemistry of vitamin B_{12} has been studied for many years, which led to the development of synthetic methods to introduce modifications in the vitamin B_{12} molecule.

Corrinoids are structurally complex essential cofactors (Figure 1) for fundamental processes such as the biosynthesis of amino acids and catabolism of branched carbon chains in humans and additionally DNA synthesis, fermentation of diverse carbon sources and anaerobic respiration in bacteria. Several structurally distinct corrinoids with variation in the base of the nucleotide moiety have been reported so far, although the significance of this diversity is still unclear.

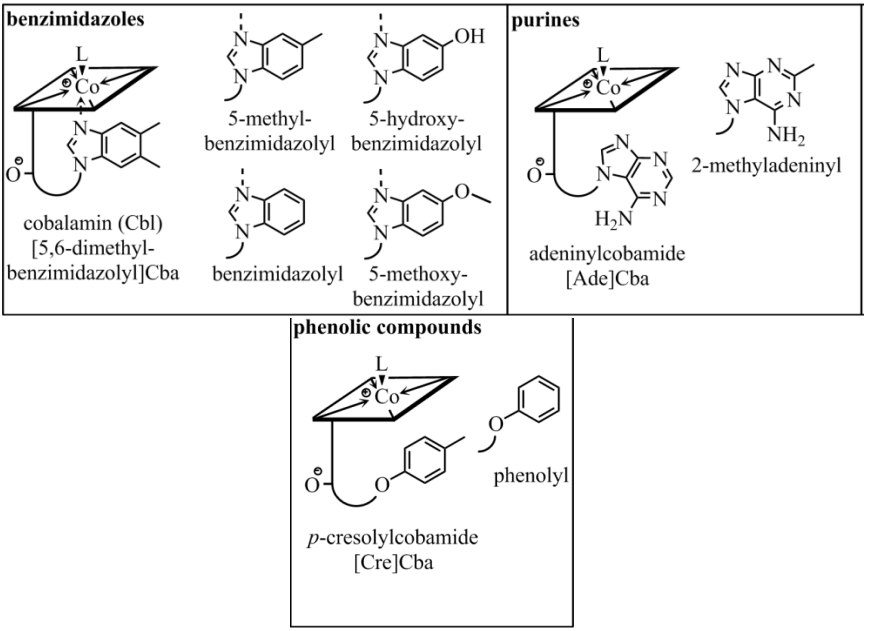

Figure 1. Schematic structures of the three classes of 'complete' corrinoids with the most abundant lower ligands, L: upper ligand (e.g. Ado, methyl, hydroxo, cyano).

BIOSYNTHESIS OF B_{12}

From δ-Aminolevulinic Acid to Uroporphyrinogen III

There are two different pathways for the biosynthesis of δ-ALA (Figure 2). In the Shemin pathway [1], found in non-photosynthetic eukaryotes and some bacteria, glycine and succinyl CoA are condensed to δ-ALA by aminolevulinic acid synthase (ALAS) [2]. In plants and most bacteria, δ-ALA is produced from glutamic acid in the so-called C5-pathway [3-4].

In the next step two δ-ALA molecules are condensed to the pyrrolic intermediate porphobilinogen (PBG) [5]. The enzyme responsible for the transformation is porphobilinogen synthase (PBGS). Subsequently, four PBG molecules are polymerized into the linear tetrapyrrolehydroxymethylbilane by the enzyme PBG deaminase (PBGD) [6]. The cyclization of hydroxymethylbilane to uro'gen III is carried out by uroporphyrinogen synthase

(UROS) [6]. UROS catalyzes not only the cyclization, but also the inversion of the fourth pyrrole ring to form uro'gen III [7].

The cobalamin biosynthesis is continued with two methylations at C2 and C7 to give precorrin 2. The reaction is catalyzed by Cob A, which uses S-adenosylmethionine (SAM) as a methylating agent [8-9]. The B_{12} biosynthetic pathway diverges at this point, depending on the presence or absence of oxygen. In aerobic microorganisms the cobalt insertion is carried out at a later stage, whereas in anaerobes cobalt is introduced at the beginning of the biosynthetic pathway (Figure 2).

The Aerobic Pathway

The first step in the oxygen-dependent biosynthesis of B_{12} is the addition of a methyl group at C20 in precorrin 2, catalyzed by Cob I and using SAM as cofactor (Figure 3) [10-11]. The methylation at C20 seems somewhat surprising, as this carbon is absent from the structure of vitamin B_{12}. Precorrin 3A is then hydroxylated at the new methyl group at C20 using molecular oxygen O_2, followed by lactonization of the a-side chain. This reaction is either catalyzed by Cob G or Cob Z, depending on the organism [12-13].

Precorrin 3B is now ready for the crucial step of ring contraction. The methylation and hydroxylation at C20 have generated a spring-load that enables the molecule to undergo ring contraction [12]. In the biosynthesis this reaction is carried out by Cob J. The product precorrin 4 not only has a contracted ring but also bears a methyl group at C17 (derived from SAM) [12, 14]. Precorrin 4 is further methylated at C11 catalyzed by Cob M to form precorrin 5, [12, 15] followed by replacement of the acetate group at C1 by a methyl group catalyzed by Cob F [12]. The resulting precorrin 6A is reduced to precorrin 6B by the NADPH-dependent enzyme Cob K at the double bond between C18 and C19 [16]. Next two methyl groups are introduced at C5 and C15, and the carboxylate side chain at C12 is reduced to a methyl group. These three reactions are all carried out by the multifunctional enzyme Cob L, to obtain precorrin 8 [17]. To complete the corrin ring synthesis, the methyl group at C11 must migrate to C12, forming hydrogenobyrinic acid (HBA) [18]. This step is catalyzed by Cob H, and the transfer of the methyl group also changes the conjugated π-system, resulting in a color change from yellow to pink. The acetate side chains a and c in HBA are then converted into acetamide groups by Cob B using glutamine as the nitrogen donor [19]. In the next step the cobalt(II) ion is inserted into HBA-a,c-diamide, giving

cob(II)yrinic acid-a,c-diamide. This challenging step of cobalt insertion into a ring-contracted tetrapyrrole is catalyzed by a cobalt chelatase, a large protein complex consisting of Cob N, Cob S and Cob T. This complex also requires Co(II) and Mg(II)-adenosine-5'-triphosphate(ATP). Cob N binds HBA-a,c-diamide and Co(II), whereas Cob S and Cob T form a chaperon-like complex and are predicted to bind Mg(II)-ATP [20].

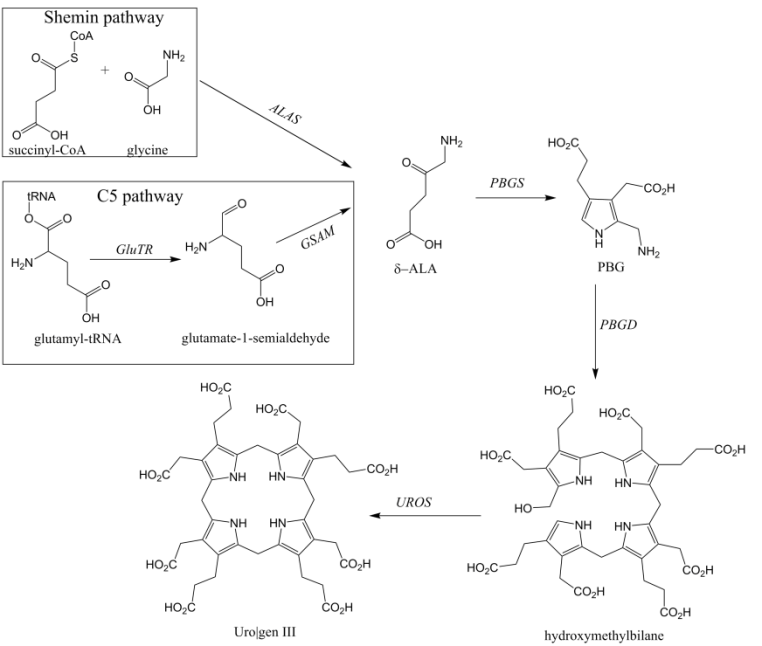

Figure 2. Biosynthesis of Uro'gen III.

The upper axial ligand is attached to the cobalt ion via a bimolecular nucleophilic substitution (SN_2) reaction, which requires cobalt(I), a highly nucleophilic species and also a strong reductant. This one electron reduction is performed by the NADPH-dependent cobalt reductase Cob R which uses flavine mononucleotide (FMN) as the electron donor.

The axial ligand 5'-deoxyadenosin is derived from ATP and attached to cobyrinic acid-a,c- diamide via nucleophilic attack by the Co(I) center on the 5' carbon of the ribose moiety in ATP, [19, 21] giving adenosyl cob(III)yrinic acid ac diamide. The reaction is catalyzed by Cob O. In the final step before attachment of the lower axial ligand, the remaining carboxy groups (except the f-side chain) are converted into amides by cobyric acid synthase (Cob Q) [22].

The Anaerobic Pathway

The main difference between the aerobic and the anaerobic pathways are the timing of the cobalt insertion and ring contraction. In the anaerobic pathway, cobalt insertion occurs at an early stage (Figure 4). Precorrin 2 is oxidized to factor II by an NAD^+-dependent reductase (CysG), which also catalyzes the addition of two methyl groups to uro'gen III [23-24]. Interestingly, CysG also function as a ferrochelatase in the formation of siroheme from precorrin 2 [25]. It has been shown that in B_{12} biosynthesis, the metal insertions are carried out by distinct cobalt chelatases. In *Bacillusmegaterium* CbiX is responsible for the cobalt insertion, [26] whereas in *Salmonellaenterica* the metalation is catalyzed by CbiK [27].

Cobalt-factor II is then methylated at C20 by CbiL in a SAM-dependent reaction, [28] analogous to the aerobic pathway. The resulting cobalt factor III is then further methylated at C17 and the macrocycle is contracted by CbiH. This enzyme forms a δ-lactone between the a-acetate side chain and C20 which subsequently results in contraction to the corrin ring [29]. Cobalt-precorrin 4 is methylated at C11 by the SAM-dependent enzyme CbiF to cobalt-precorrin 5A, which still contains the δ-lactone [30]. The next enzyme, CbiG opens the δ-lactone and releases acetaldehyde, thereby generating a unique double bond between C1 and C19 [31]. Precorrin 5B is then methylated by CbiD at C1 [32]. In this reaction, the methylation is facilitated by the cobalt(II)ion, which acts like a Lewis base and activates C1 [33]. The resulting double bond between C18 and C19 in cobalt-precorrin 6A is reduced by the NAD-dependent enzyme CbiJ. Subsequently, cobalt-precorrin 6B is methylated at C15 and the acetate group at C12 is replaced by a methyl group (CbiT), followed by addition of a methyl group to cobalt-precorrin 7 at C5 by CbiE. Both enzymes use SAM as the methyl donor. The final reaction to cobyrinic acid involves the 1,5 sigmatropic rearrangement of the methyl group at C11 to C12, catalyzed by CbiC [33]. Finally, amide groups are introduced by the ATP-dependent CbiA, which catalyzes the amidation of the a- and c-side chains using glutamine as the nitrogen donor [34]. The additional four amidations are catalyzed by CbiP in an ATP dependent manner to obtain cob(II)yric acid [35]. Although the cobyric acid has to be adenosylated at the cobalt ion for the final nucleotide attachment, nothing is known about the timing of the Cob A catalyzed reaction [36].

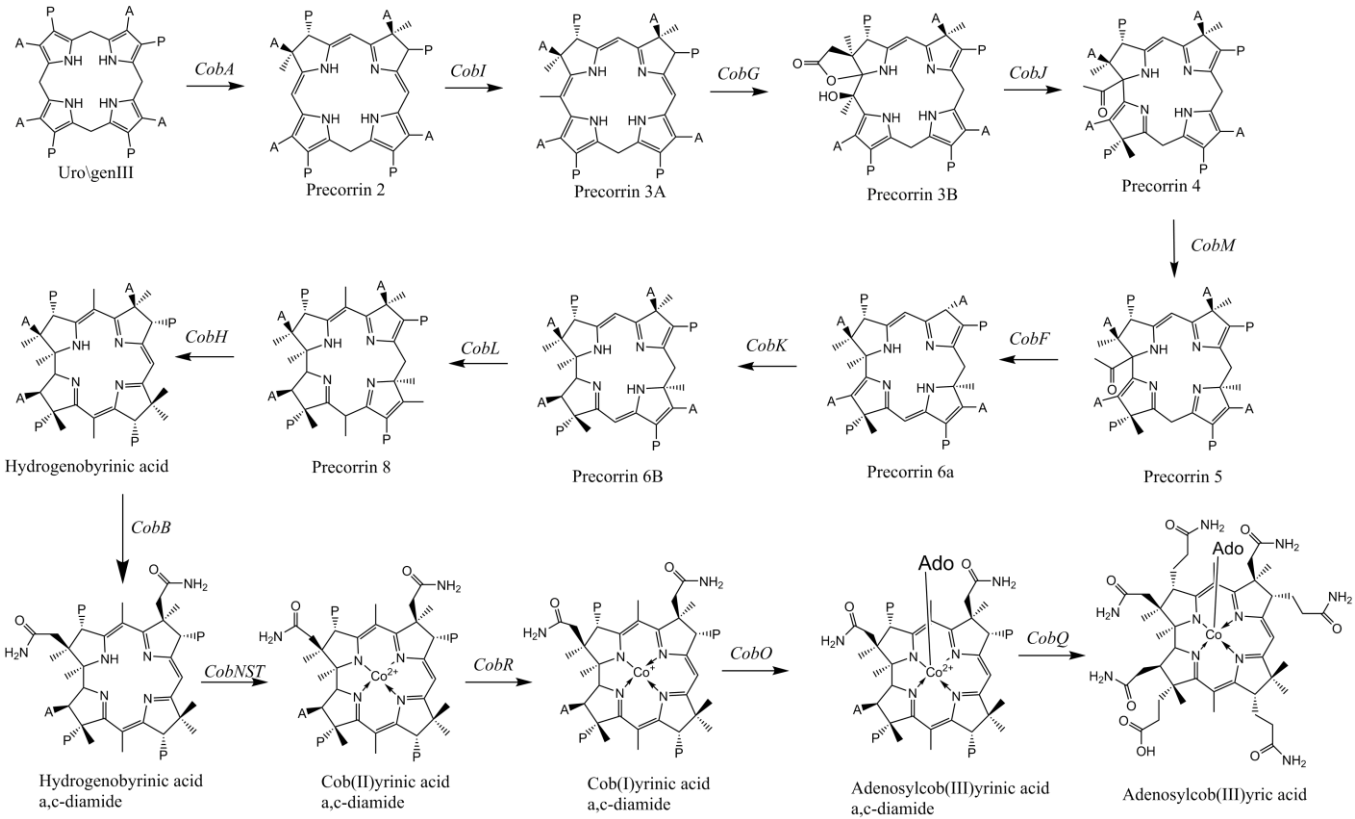

Figure 3. Aerobic biosynthetic pathway (A = acetate side chain, P = propionate side chain).

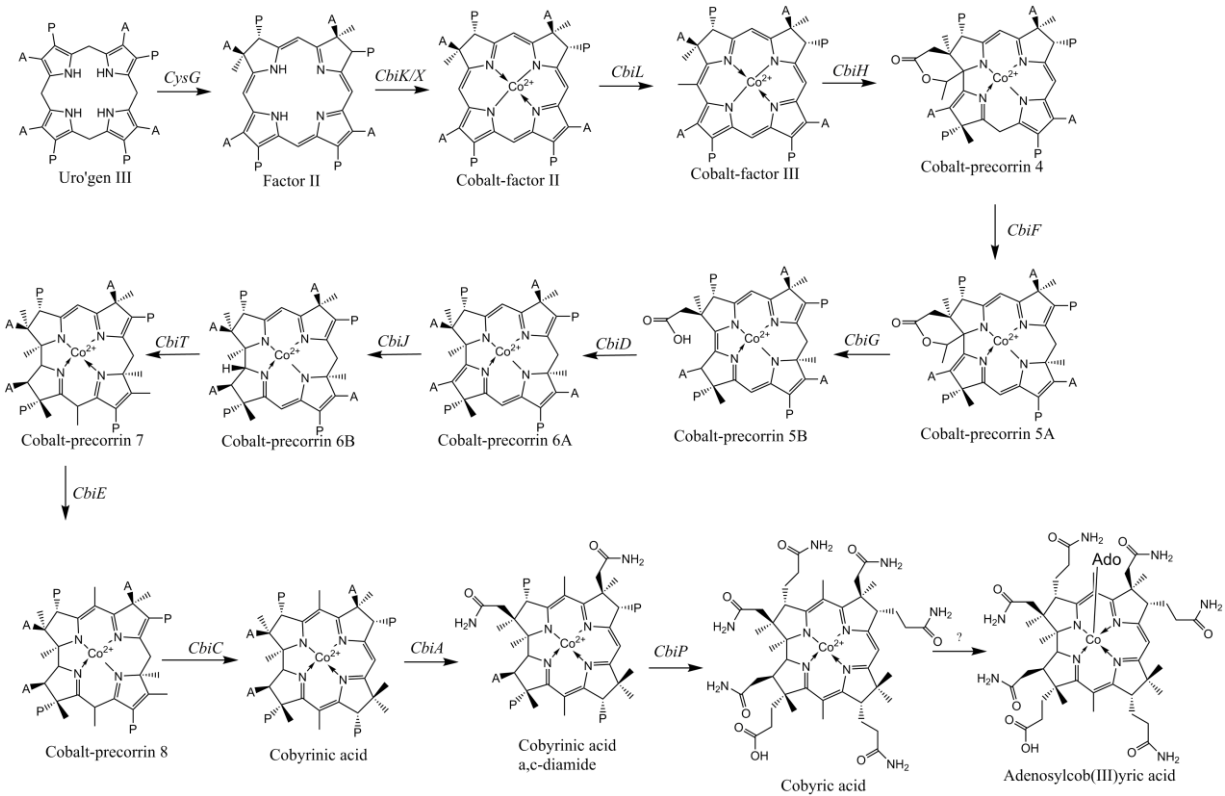

Figure 4. Anaerobic B_{12} biosynthetic pathway from uro'gen III to adenosylcobyric acid (A = acetate side chain, P = propionate acid side chain).

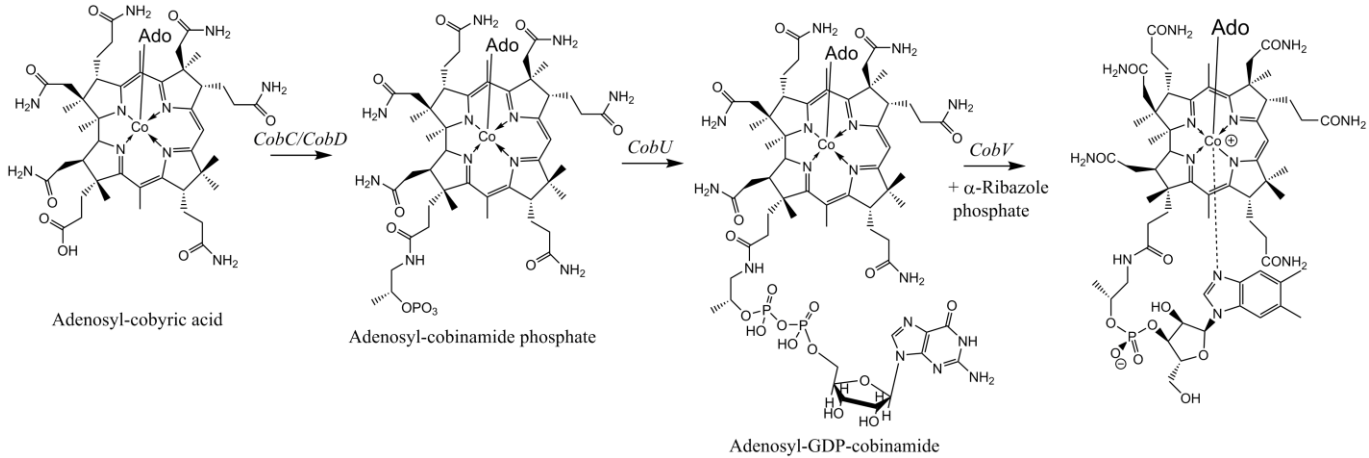

Figure 5. Final stages in the biosynthesis of coenzyme B_{12}.

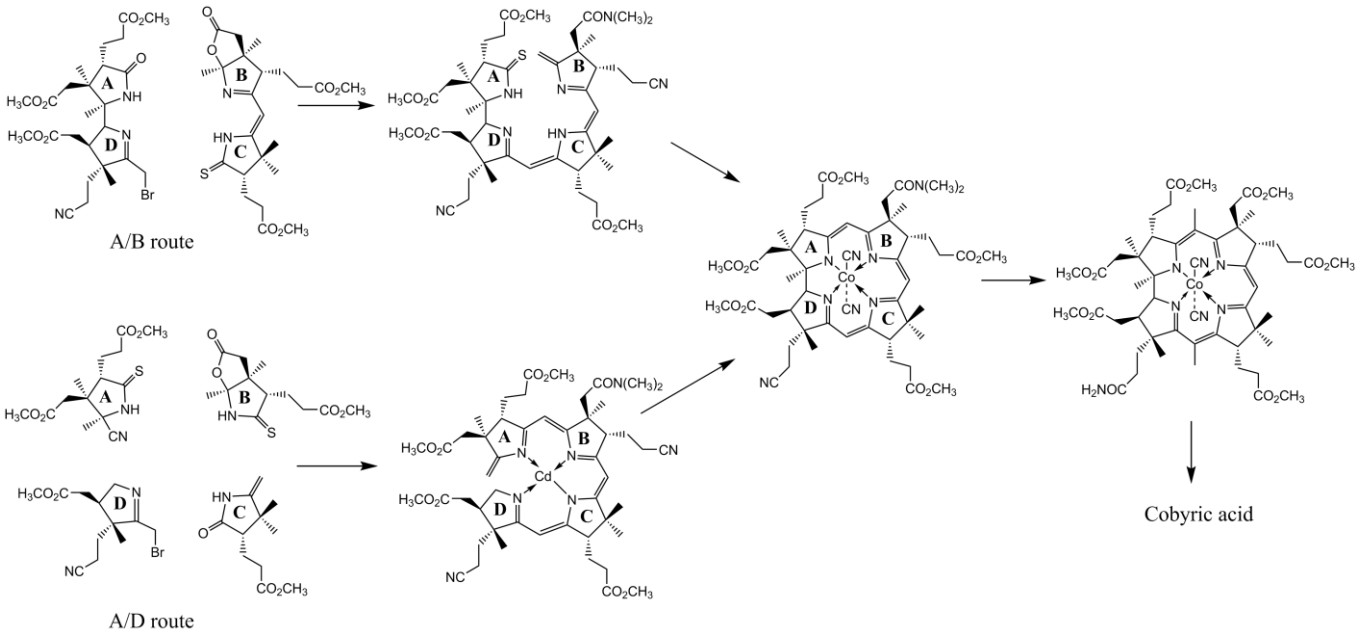

Figure 6. The two total synthetic routes leading to cobyric acid: top A/B route; bottom A/D route.

Nucleotide Loop Attachment

The formation of adenosylcobinamide phosphate requires the attachment of an L-threonine derived [37] aminopropanol-O-2-phosphate moiety to the propionic acid side-chain of ring D of adenosylcobyric acid (Figure 5) [38-39]. This attachment is catalyzed by a multienzyme complex (Cob C, Cob D and Protein α) and requires Mg^{2+} and ATP [38]. The assembly of the lower nucleotide loop which is the last step in the biosynthetic pathway can be considered to occur in three steps. These involve the activation of the cobinamide phosphate, the synthesis and activation of the α-nucleotide and finally their attachment. The activated cobamide form adenosyl-GDP-cobinamide is synthesized via attachment of guanosine monophosphate either by Cob P [21, 40] in aerobes or by Cob U [41-42] in anaerobes.

In the case of cobalamin the lower ligand attached to the activated cobinamide species is 5,6-dimethylbenzimidazol (DMB). Two pathways that produce DMB are known. The substrate of the oxygen-dependent pathway is FMN which is 'cannibalized' by BluB to synthesize DMB [43-45]. Isotopic labelling studies in the anaerobic bacterium *Eubacteriumlimosum* showed that DMB is constructed from erythrose 4-phosphate, formate, glutamine, glycine and methionine [46]. Recently, a gene cluster associated with the anaerobic biosynthesis of DMB was found and the functions of the biosynthetic genes were elucidated [47]. The origin of purine bases for corrinoid biosynthesis is still unknown. It is assumed that the basic nucleotides adenine, guanine and hypoxanthine are formed in the purine biosynthesis pathway. As cobamides contain α-glycosidic nucleotides the bases may result from salvaging of degraded tRNA [48]. In *Sporomusa ovata*, *p*-cresol is derived from tyrosine [49]. To date, nothing is known about the source of phenol in phenolylcobamide. Some speculate that it is formed from *p*-cresol [50]. The activated α-nucleotide is synthesized by the phosphoribosyltransferase Cob U [51] or Cob T [52], which transfers the phosphoribosyl moiety of nicotinate mononucleotide onto the base (e.g. benzimidazoles, purines and phenolic compounds). It has been shown that corrinoids structure depends on both lower ligand availability and phosphoribosyltransferase substrate specificity [53-54].

Finally the activated α-riboside phosphate is attached to adenosyl-GDP-cobinamide by a so-called cobalamin synthase Cob V [51] or Cob S [55]. Additionally it was shown for adenosyl-cobalamin (coenzyme B_{12}, AdoCbl) biosynthesis that a phosphatase Cob C is involved in either cleavage of the

phosphate from the α-riboside phosphate substrate before attachment to the activated cobinamide or from the adenosyl cobalamin-5'-phospate product to generate AdoCbl [21, 56].

NATURAL COBALAMIN ANALOGUES

Chemical and Structural Aspects

'Complete' corrinoids can be categorized into three classes: benzimidazoles, purines, and phenolic compounds (Figure 1). Benzimidazoles such as in vitamin B_{12} (cyanocobalamin) and purines such as in pseudovitamin B_{12} (ΨCNCbl) are linked α-N-glycosidically to the C1 atom of the ribose moiety. In the case of phenolic compounds such as *p*-cresolylcobamide the C1 of the ribose moiety is linked α-O-glycosidically. These lower nucleotide ligands determine the tendency of 'complete' corrinoids to exist in either of two constitutional isomers called 'base-on' or 'base-off' (see also Chapter "Natural cobalamins - their structure, chemical reactivity and co-enzymatic role"). Benzimidazoles and purines are capable of forming a 'base-on' state by a coordination of N3 (benzimidazoles) or N9 (purines) of the imidazole moiety of the lower ligand to the cobalt ion of the corrin ring. The 'α-pseudonucleotides' of phenolic compounds lack the ability to coordinate to the cobalt ion and are therefore always in a 'base-off' state [57-59]. Cobalamin, as an example for benzimidazoles, shows a significant thermodynamic preference for the base-on form in neutral aqueous solution at room temperature [58]. Their 5,6-dimethylbenzimidazole ligand creates a more stable coordination to the cobalt ion of the corrin ring than other lower ligands. In contrast to the biologically active form AdoCbl, its natural purine analogues pseudocoenzyme B_{12} and adenosyl-factor A (Coβ-adenosyl-2-methyladeninylcobamide) favour the 'base-off' state in aqueous solution [59]. This can be rationalized by the difference in nucleophilicity of the coordinating imidazole nitrogens of DMB and purines.

Distribution of 'Complete' Corrinoids in Nature

To date, 16 distinct 'complete' corrinoids with variation in the base of the nucleotide moiety have been described [48] of which the nine shown in Figure

1 have been the most commonly detected [57, 60-63]. Cobamides are utilized as cofactors by animals, some protists, and the majority of prokaryotes, but are synthesized only by a subset of prokaryotic species [64-66]. Strikingly, cobamides seem to play no role in the metabolism of plants, insects and fungi [66]. A majority of 76% of sequenced bacterial genomes encode corrinoid-dependent enzymes involved in radical rearrangement, ribonucleotide reduction, methyl-transfer and reductive dehalogenation, or many of these processes. However, less than half of these prokaryotes possess the full set of genes to synthesize corrinoids de novo [65, 67] following either of two related though genetically distinct routes requiring a total of at least 30 enzyme-mediated reactions [68]. In several microbial communities, significant levels of several members of the 'corrinoid family' have been detected [60, 69-70].

Biological Relevance of Natural Cobalamin Analogues

Although benzimidazolyl-, purinyl- and phenolylcobamides are formally capable of performing biologically relevant radical-mediated carbon skeleton rearrangements as well as methyl group transfer via the established mechanisms for AdoCbl and methylcobalamin (MeCbl) [71], organisms prefer specific corrinoids and therefore restrict uptake and use [72-75].

The restrictions may depend on the natural capacity of 'complete' corrinoids to switch between the 'base-on' and the 'base-off' forms [76]. This capacity is influenced by the lower ligand and changes the properties of the specific corrinoids in two ways.

First, coordination of the lower ligand to the cobalt atom modulates the strength of the cobalt bond to the upper ligand. This biologically unique cobalt-carbon is the reactive site of the cofactor. Thus, the organometallic reactivity and redox chemistry of the cobalt ion are adjusted via lower ligand coordination, for the chemical needs of the enzymatic catalysis [77].

Second, the 'complete' corrinoid structure, including the nucleotide moiety, is critical for selective and tight binding to transport proteins [78-82]. For example, human cobamide transport proteins intrinsic factor (IF), transcobalamin (TC) and haptocorrin (HC) have greater affinity for cobalamin and a many-fold reduced affinity for other corrinoids, indicating molecular specificity of transport processes for the binding of 'complete' corrinoids in the well-structured 'base-on' form [83-84]. Accordingly, cobalamins are the only physiologically active corrinoids in animals and humans.

In contrast to the strong selectivity toward cobalamin found in animals and humans natural 'complete' corrinoids in archaea and bacteria are diverse in their nucleotide moiety [85-87]. To date, three enzyme classes dependent on corrinoids can be distinguished namely methyl transferases, coenzyme B_{12}-utilizing enzymes and corrinoid dehalogenases [88]. Research on all three classes has provided evidence that cobalamin analogues are native cofactors for these enzymatic reactions [72, 89-93]. In addition corrinoid-dependent enzyme homologs (e.g. MCM and MetH) are present in bacteria that require [64] and respectively produce [57, 61, 63, 94-95] corrinoids other than cobalamin. Unfortunately very fewstudies have been performed comparing different 'complete' corrinoids as cofactors for corrinoid-dependent enzymes *in vitro* [79-81].

Further experiments in the future may help to elucidate the significance of the lower ligand diversity and help explain the restriction in uptake and use of corrinoids by B_{12}-dependent prokaryotes. This is highly relevant in microbial communities as corrinoids are mandatory for the majority of their members. New sequencing methods have made possible the analysis of highly complex microbial communities. Metagenomics led to an explosion of microbial genome sequence data that can be analyzed for information on the species composition and functional properties of diverse microorganisms. Based on these methods recent studies have led to a fundamental shift in our understanding of microbial communities in ecosystem function [96] and human health [97]. The only methods known to date to control the composition of a microbial community are antibiotic therapy, which targets broads taxonomic groups, or fecal transplantation of gut microbiota, in which the entire gut microbiome is replaced [98-99]. Recently, it was proposed that modulating corrinoid levels might impact community composition and thereby be a powerful tool to manipulate their structure [100].

TOTAL SYNTHESIS OF VITAMIN B_{12}

The total synthesis of vitamin B_{12} was one of the most challenging tasks in natural product synthesis, which led to the development of new synthetic strategies and the discovery of new mechanistic models (Woodward Hoffman rules [101]). The structure of vitamin B_{12} (see also chapter "Natural cobalamins - their structure, chemical reactivity and co-enzymatic role") consists of a highly substituted corrin ring with a central cobalt ion and possesses 13 chiral carbon centers, a nightmare for synthetic chemists. Starting

in 1960, it took more than 10 years for two research groups at Harvard University (R.B. Woodward) and ETH Zürich (A. Eschenmoser) to accomplish the synthesis of cobyric acid, a natural precursor of vitamin B_{12}, with more than 70 steps [102-103]. Two routes to the natural product were developed, which differ in their strategy for linking rings A and D (Figure 6). In the so-called A/B route, rings A and D were first connected together, followed by a stepwise connection with the dipyrromethan compound containing ring B and C via a sulfide contraction method. In the second synthetic route, ring D was connected to the B/C fragment, followed by addition of ring A. The bond between A and D was then formed by a photochemical cyclization (Figure 6).

SYNTHESIS OF ARTIFICIAL VITAMIN B_{12} DERIVATIVES

Mammals have developed a very selective transport system for vitamin B_{12} due to the low availability of cobalamins. This system can be used to transport imaging agents or drugs, linked to cobalamin ('Trojan horse strategy'). Due to their rapid growth, tumor cells have an increased demand for vitamin B_{12} and this has led to the development of cytotoxic vitamin B_{12}, derivatives.

Vitamin B_{12} possesses several sites that can be derivatized, but only modifications at the cobalt center, the 5R ribose OH and (to some extent) the e-side chain do not influence the binding to TC, the transport protein in blood, due to steric distortion [104].

Organocobalamins

The most common method to synthesize cobalt modified cobalamin derivatives involves reduction of the Co(III) center to the highly nucleophilic Co(I) species using either chemical reducing agents (e.g. metallic zinc, sodium borohydride ($NaBH_4$)) [88, 105] or electrochemical methods (Figure 7) [106]. Cob(I)alamin rapidly reacts with halogenalkanes via an SN_2 reaction. The resulting organocobalamin is highly light-sensitive. This ability may be used to release the attached molecule by irradiation with light.

A less frequently used mode for preparation of organocobalamins is the reaction of radicaloidcob(II)alamin with organohalides (Figure 8) [107-109]. Cob(II)alamin is prepared by using mild reducing agents (e.g. formic acid,

formate salt [107]) or by heterogeneous reduction with H_2/PtO_2 [110]. This method not only produces alkylcobalamins, but also aryl- [111] and alkynylcobalamins [112]. The latter molecules are light-stable organocobalamins whereas arylcobalamins are still sensitive to light. However, arylcobalamins are not processed by the enzyme cobalamin-β-deligase (CblC, MMACHC) which removes the axial ligand of cobalamins upon entering the cell, [113-115] and are thus considered 'antivitamin B_{12}' [111]. They can be employed to induce B_{12} deficiency in laboratory animals for investigation of the non-cofactor roles of vitamin B_{12} [116].

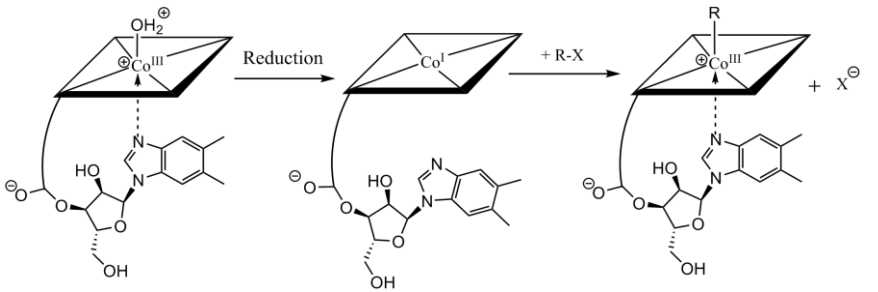

Figure 7. Preparation of organocobalamins via reduction to cob(I)alamin.

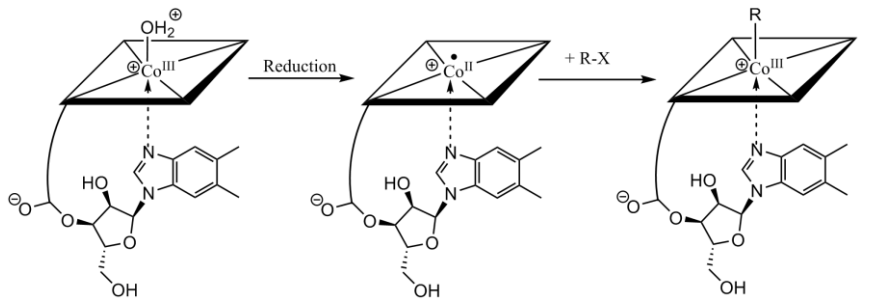

Figure 8. Radicaloid synthesis of organocobalamin via cob(II)alamin: R = alkyl, alkynyl, aryl.

Ribose Modified Cobalamins

The second position to functionalize without disturbing binding of cobalamins to the B_{12} transport proteins is the 5R-hydroxy group of the ribose moiety. The hydroxy group can be transformed into an amine-reactive

intermediate by the well-established reaction with 1,1'-carbonyl-di triazol (CDT) or 1,1 carbonyl-di-imidazole (CDI). The resulting carbamate group is stable to hydrolysis and thus resistant to proteases. [117]

Vitamin B_{12} and Medical Applications

Cobalamins linked to (bio)molecules are called B_{12}-bioconjugates. Several B_{12}-bioconjugates have already been investigated for possible applications in the delivery of therapeutic drugs and in molecular imaging using fluorescent molecules or radioisotopes via the B_{12}-uptake system. Fluorescent molecules such as cyanine-5, rhodamine or fluoresceine were tethered to the cobalt ion or to the 5R-ribose hydroxyl group [118]. In particular CBC-244, a rhodamine 6G labeled vitamin B_{12} derivative, was used to measure the binding kinetics of the B_{12} transport proteins [119]. In addition B_{12} derivatives containing metal chelator molecules were prepared to deliver Gd^{3+} (an MRI probe, but also cytotoxic), [120] vanadate (used in the treatment of diabetes) [121] or rhenium (fluorescent test compound to simulate ^{99m}Tc-uptake) [122] into the cell. Radiolabeled vitamin B_{12} derivatives containing ^{99m}Tc or ^{111}In were studied to develop radioimaging agents to detect cancer [123-124].

Anticancer drugs, such as colchicine or nido-carborane were conjugated to vitamin B_{12} to target tumor cells [125-126]. Cisplatin, one of the earliest chemotherapeutic drugs, was directly linked to vitamin B_{12} by coordination of the platinum to the nitrogen of the axial cyanide ligand [127].

This strategy is not limited to small molecules. One of the first conjugates was sepharose linked to the e-side chain of vitamin B_{12}, which allowed purification of B_{12} binding proteins by affinity chromatography [128]. A conjugate between vitamin B_{12} and human serum albumin was used to generate B_{12}-antibodies [129-130].

Vitamin B_{12} was also conjugated to granulocyte-colony-stimulating factor [131], erythropoietin [131] and luteinizing hormone-releasing hormone [132]. The drawback of B_{12}-bioconjugates is their accumulation in the liver and kidney, the natural B_{12} storage organs. This aspect, in addition to the complexity of vitamin B_{12} chemistry has hindered research related to B_{12}-conjugates in the past.

NOMENCLATURE OF VITAMIN B_{12}

The structure of vitamin B_{12} is based on a corrin ring with a central cobalt ion (see also chapter "Natural cobalamins - their structure, chemical reactivity and co-enzymatic role"). The four pyrrole rings are usually referred to as A through D. Per definition, the methyl group on C1 is bound on the 'α-face' or 'lower face'. The other substituents of the corrin ring periphery and the cobalt ligands are described as α or β when bound to the same or opposite side of the corrin ring plane, respectively. Side chains are referred to as "a" through "g" in a clockwise order, starting from ring A and going to ring D. The atom numbering starts at pyrrole ring A at the carbon connected to ring Dand proceeds in a clockwise direction. The side chain atoms are numbered by adding an additional number to the original corrin ring atom number (e.g. C82 = 2nd carbon atom of the d-side chain with its origin at C8) (Figure 9) [133].

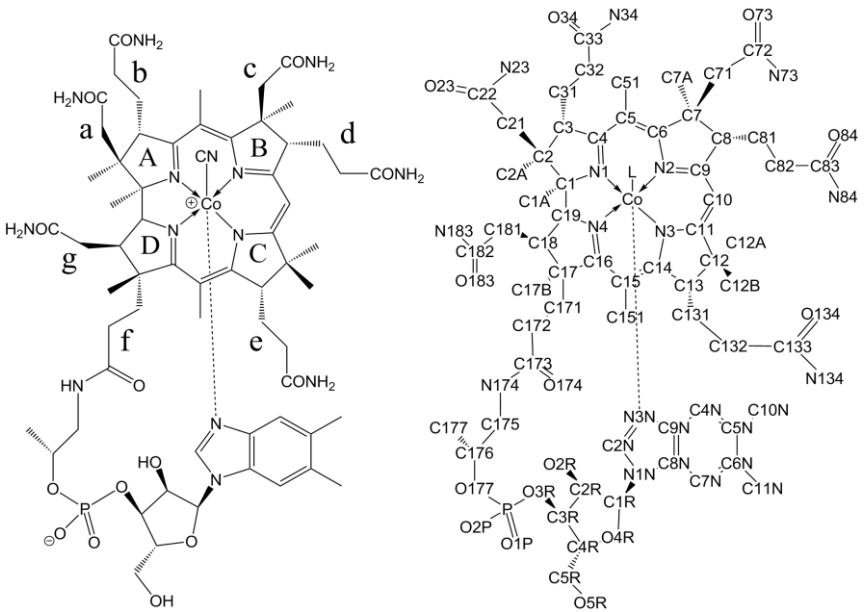

Figure 9. Structure of Co$_\beta$-cyano-cobalamin (vitamin B_{12}) the pyrrole rings labelled A through D and the side chains labelled a through g (left) and atom numbering (right).

In 'complete' corrinoids (e.g. cobalamins, Figure 1) the f-side chain is linked to a nucleotide moiety which consists of a propanol amine linker, a 3'-phosphoryl -D-ribose and a base (e.g. 5,6-dimethylbenzimidazole in

cobalamins). Commonly used terms for 'incomplete' corrinoids are 'cobamide' (base removed), 'cobinamide' (f-(2'R)-2'hydroxypropyl-amides), 'cobyric acid' (a,b,c,d,e,g-hexa-amide-f-acids) and 'cobyrinic acid' (a,b,c,d,e,f,g-hepta-acids).

Cobamides containing an additional base (benzimidazoles, purines, phenolic compounds, Figure 1) which are N-glycosyl or O-glycosyl derivatives respectively, at C1 of the ribofuranose unit are named by adding the name of the appropriate aglycon radical as a prefix to the name. The commonly used term cobalamin stands for a cobamide in which 5,6-dimethylbenzimidazole is the aglycon attached by a glycosyl link from its N1 to the C1 of the ribose (Examples: 5-methylbenzimidazolylcobamide, adeninylcobamide, *p*-cresolylcobamide). In most corrinoids the lower ligand is assumed to coordinate to the αside of the cobalt ion if not otherwise indicated and is therefore not additionally named. When another ligand occupies the cobalt-α position, it and its locant may be indicated. An additional upper ligand (β-side) is indicated the same way (Examples: Coα,β-dicyanocobalamin, Coα-(aqua)-Coβ-(adenosyl)-2-methyladeninylcobamide, Coα-(aqua)-Coβ-(methyl)-*p*-cresolylcobamide).

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Chapter 4

NON CO-ENZYMATIC ROLE OF VITAMIN B_{12}

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ABSTRACT

Since its discovery, vitamin B_{12}[#] (cobalamin (Cbl)) has been considered only as the co-enzyme in the two important reactions carried out by the enzymes methionine synthase (MS) and methylmalonyl-CoA mutase (MCM) (see "Natural cobalamins - their structure, chemical reactivity and co-enzymatic role").

In recent years, it has been demonstrated that the scenery of Cbl functions is more complex. In fact, Cbl is able to regulate the mRNA and/or protein levels of some cytokines and growth factors in the nervous system and elsewhere. Cbl is also implicated in the control of the level of some genes involved in Cbl transport and/or metabolism.

Keywords: cytokine, growth factor, nutrigenomic, transcobalamin (TC), vitamin B_{12} (cobalamin (Cbl))

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Vitamin B12 is referred here and in the other "non-chemical" chapters (chapt.s 2, 4-9) not in the precise chemical meaning of cyanocobalamin (see chapters 1 and 3) but in the general terms, commonly used, of human cobalamins (Cbl) (5',6'-dimethylbenzimidazolyl-cobamide).

INTRODUCTION

It is well known that some fat-soluble and water-soluble vitamins are able to affect gene expression.

In particular we mention: *i)* retinoic acid influences the expression of a multitude of cytokines that participate in traumatic reactions (i.e., interleukin(IL) 1 and 6, transforming growth factor- ß) (for a review see [1]); *ii)* 1,25-dihydroxyvitamin D_3 genes of osteoblast metabolism (i.e., osteocalcin) [2]; *iii)* vitamin E genes involved in lipid metabolism and cell cytoarchitecture [3]; and *iv*) vitamin B_2 inhibits IL-6 expression [4].

Recent studies on Cbl functions have been conducted in animal models of deficiency (totally gastrectomized or rat fed a Cbl-deficient (Cbl-D) diet) and it has been demonstrated that a status of Cbl deficiency causes a cytokine and growth factor imbalance especially in rat central nervous system (CNS) and peripheral nervous system, insofar as the levels and/or synthesis of some of these are up- or down-regulated [5-8].

In particular, Cbl deficiency up-regulates levels and/or synthesis of: a) tumor necrosis factor (TNF)-α and nerve growth factor (NGF) in rat cerebrospinal fluid, spinal cord, and peripheral nervous system [9-11]; and b) soluble form of both CD40 and its receptor CD40 ligand (which belong to the TNF-α, TNF-α-receptor super family) in rat cerebrospinal fluid, whilst not in serum [12]. On the contrary, Cbl deficiency down-regulates: a) epidermal growth factor (EGF) and EGF receptor (EGF-R) levels in rat CNS and/or cerebrospinal fluid [13-15]; and b) IL-6 levels in rat cerebrospinal fluid [16]. The specificity of these changes is supported by the fact that - in cerebrospinal fluid of Cbl-D rats - the leptin, somatostatin, vasoactive intestinal peptide does not change and/or these changes are corrected by Cbl-replacement treatment in Cbl-D rats [9-16]. An increased expression of proNGF (combined to an up-regulation of TNF-α converting enzyme and phosphatase 2A) has also been demonstrated in a cellular model of Cbl deficiency [17].

Additionally, Cbl influences the mRNA and protein synthesis of the cellular prion protein (PrP^C) which is claimed to have myelinotrophic properties, in CNS and duodenum of rats [18]. PrP^C protein levels were significantly higher in spinal cord (SC) of Cbl-D rats than in the controls whilst the PrP^C-mRNA levels were greatly reduced [19].

The severe neuropathological lesions caused by chronic Cbl deficiency in the rat CNS occur as a result of a deregulation in the physiological equilibrium of these growth factors and cytokines [6-8]. In fact, it has been demonstrated that repeated intracerebroventricular administration of the lacking molecules

(i.e., EGF or IL-6) or monoclonal antibodies inactivating the excess molecules (TNF-α, NGF, or PrP^C) restore the normal SC myelin in Cbl-D rats without modifying their Cbl-D status [9-11, 14, 16, 19]; indeed, the repeated intracerebroventricular administration of TNF-α, anti-EGF antibodies or PrP^C to normal rats reproduced the ultrastructural SC myelin lesions similar to those of Cbl-D neuropathy, without modifying their Cbl status [9, 11, 14, 19].

Data on animal models were confirmed in humans. In fact, in adult patients with clinically confirmed severe Cbl deficiency, an up-regulation of TNF-α and PrP^C levels in cerebrospinal fluid and serum has been observed and a down-regulation of EGF and IL-6 levels in serum and/or cerebrospinal fluid [20-23].

Moreover, a recent study in mice proves that a Cbl overdose can also modify the expression of some genes of the EGF family. The expression of one of the EGF receptors (HER3) and three of its ligands (heparin-binding EGF-like growth factor, transforming growth factor-α, and neuregulins 1α) was increased in SC of Cbl treated mice [24]. Vice versa, treatment with Cbl decreased expression of the EGF system in the kidneys in a dose-dependent manner [25].

Other studies have demonstrated that Cbl is also able to regulate the expression of its cell transporters. In fact, protein levels of CD320 (the receptor for the Cbl-transcobalamin (TC) complex) were elevated in duodenal mucosa, kidneys, liver, and SC of rats made Cbl-D and postoperative Cbl-replacement treatment normalized the protein levels of this receptor [26]. An increased need for Cbl, induced by partial hepatectomy or reproductive process, results in a marked increase of intrinsic factor content and mRNA level in rats [27].

Vice versa, TC and CD320 were down-regulated in salivary gland of normal mice chronically treated with high doses of Cbl [28].

Likewise a status of Cbl overdose or Cbl deficiency (induced by chronic treatment with a Cbl antagonist, cobinamide) in mice induces a significant decrease in the expression of the lysosomal membrane Cbl transporter in SC [24].

Finally, some authors have shown a regulatory role of Cbl on the level of some enzymes of Cbl pathway. In fact, under Cbl-D conditions MCM protein level is abnormally increased in rat liver [29], whilst MCM [29] and cystathionine beta-synthase mRNA levels are decreased [30].

Moreover, Cbl supplementation induces *in vitro* translational up-regulation of MS by shifting the mRNA from the ribonucleoprotein to the polysome pool [31] and a decreased transcript level of methylenetetra-

hydrofolate reductase in mouse kidneys [28]. A summary of the regulatory effect of Cbl is depicted in Figure 1.

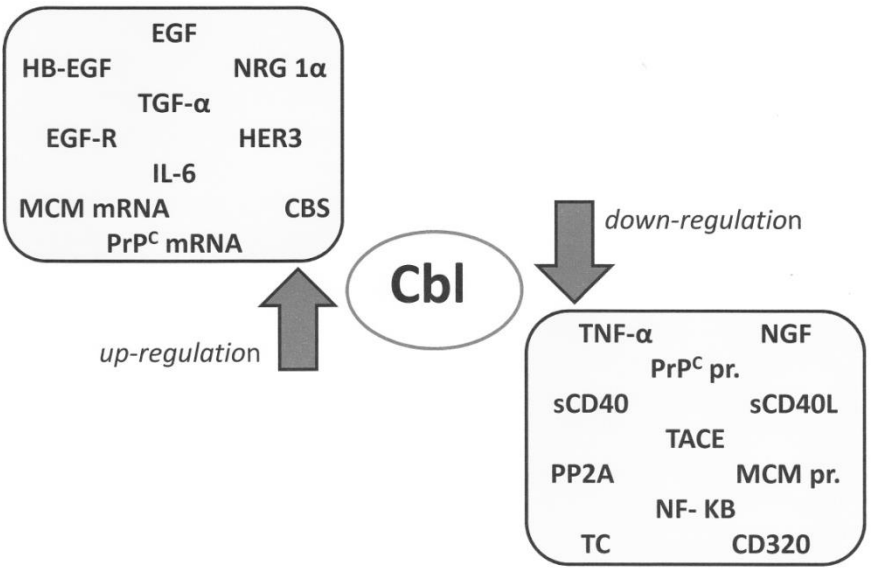

Figure 1. *Cbl regulatory role.* List of the principal molecules regulated by Cbl: Cystathionine beta-synthase (CBS); epidermal growth factor (EGF); heparin-binding EGF-like growth factor (HB-EGF); interleukin(IL)-6; methylmalonyl-CoA mutase (MCM); nerve growth factor (NGF); neuregulin(NRG)1α; nuclear factor–kappa B (NF-KB); phosphatase 2A (PP2A); protein (pr.); cellular prion protein (PrP^C); soluble(s); transcobalamin (TC); transforming growth factor(TGF)-α; tumor necrosis factor(TNF)-α; TNF-α converting enzyme (TACE).

A recent review by Guéant et al. provides an overview on the principal nutrigenomic effect of Cbl [32].

The molecular mechanism underlying the regulatory role of Cbl in mammals is not known. Scalabrino et al. have hypothesized that Cbl might need an inducible transcription factor, nuclear factor-kappa B as a step in the signal transduction pathway necessary to display its non-coenzymatic effects. In fact, it has been demonstrated that Cbl physiologically and indirectly down-regulates nuclear factor-kappa B levels in rat SC and liver [33].

Instead, studies from Oltean and Banerjee showed a remarkable regulatory role of Cbl which modulates internal ribosome entry site-dependent translation of MS [30, 34]. Other authors hypothesized that the down-regulation of mdr-1 gene expression in HepG2 cells by Cbl was linked to the increased MS

activity, while no modification of the mdr-1 promoter methylation status was detected [35].

It is of interest to recall other molecular mechanims of Cbl (for an overview see [36]), in particular, recent studies have showed that Cbl cofactors directly interact with mRNA in regulatory 'ribo-switches' in microorganism [37, 38] or act as a cofactor of regulatory proteins [38]. However, in mammals, such regulatory 'ribo-switches' are, until to date, unknown.

CONCLUSION

All of these findings show that Cbl plays a central role in the regulation of the physiological equilibrium of several genes and/or protein in the mammalian CNS and elsewhere, independently of or going beyond its classic coenzyme functions. All of these studies have shed new light on the new frontiers of nutrigenomics, a branch of epigenomics. Different dietary habits, leading in this case to a different intake of a nutrient (vitamin) can really alter gene expression. The alterations are referred to as non-heritable changes of gene expression induced by mechanisms such as methylation of deoxyribonucleotidic acid(s), histone acetylation/methylation, dysregulation of gene transactivation and synthesis of miRNA, etc. It is interesting to note that the studies on epigenetic role of Cbl could be applied also in the field of oncology (see subchapter "Role of Cbl in cancer").

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Chapter 5

CAUSES OF VITAMIN B_{12} DEFICIENCY

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ABSTRACT

Deficiency of Vitamin B_{12} (also called cobalamin (Cbl)) is the result of genetically determined or acquired defects. The first group includes defects in the transport and/or metabolism of Cbl and its co-enzymes. The second group is mainly composed by nutritional deficiencies and malabsorption problems.

A Cbl-deficient (Cbl-D) status in fetus, embryo and child in the first months of life is also worth a mention, and it is often traced to a Cbl-D status in the mother.

Keywords: acquired cause; amnionless; cubilin; haptocorrin (HC); inborn errors; intrinsic factor (IF); transcobalamin (TC)

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INBORN ERRORS OF CBL TRANSPORT AND/OR METABOLISM

Abnormalities in the transport and/or metabolism of Cbl and its co-enzymes are included in the genetically determined cause of Cbl deficiency.

Thirteen different inborn errors affecting the intestinal Cbl absorption, the transport of Cbl in blood, the uptake of Cbl by cells or the intracellular Cbl metabolism have been identified [1,2]. Each of these disorders is inherited as an autosomal recessive trait.

Cbl transport system is a complex and precise process (see for the details the Chapter "Vitamin B_{12} Absorption and Transport in Mammals"), and an alteration in any of the stages can produce a Cbl-D status.

A deficiency of haptocorrin(HC) was diagnosed only in a very limited number of children and is usually associated with a deficiency of other neutrophil granulocytes products, such as lactoferrin. At the best of our knowledge, mutations in HC gene (on chromosome 11 q11-12) have been identified only in two families [3]. In addition, patients with deficiency in serum HC levels could present a low serum Cbl levels but no symptoms of vitamin deficiency [4-6].

The Cbl malabsorption may be also the consequence of a defective intrinsic factor(IF)-Cbl complex. A defective complex formation is the result of inadequate proteolysis of food Cbl, reduced transfer of Cbl from R proteins to IF, or defective synthesis of IF, cubilin or amnionless [7,8]. An inadequate proteolysis and a reduced transfer to IF could be the consequence of an abnormal intragastric events and a reduced or inactive production of pancreatic protease, respectively. Genetically, mutations in the gastric IF gene (on chromosome 11q12.1) are causative of IF deficiency (IFD; OMIM: 261000). Pathogenic mutations in either cubilin or amnionless cause the so called Imerslund-Gräsbeck syndrome (IGS;OMIM: 261100) [8].

Almost 100 children with inherited IFD have been identified.They present the classical symptom of pernicious anemia (i.e, impaired intestinal absorption of dietary Cbl) but not atrophy of gastric intestinal cells and antibodies against IF. In some cases, an absence of IF in gastric secretions was observed, while other patients showed an abnormal IF with decreased affinity for cubilin or increased susceptibility to proteolysis (for a review see [1]). At least 60 mutations have been identified, the majority of which lead to truncate protein and loss of function [9].

IGS was first described in Finland and Norway, where the prevalence is about 1:200,000. Currently, approximately 300 cases have been published worldwide, with new cases predominantly appearing in eastern Mediterranean countries.

The molecular basis of IGS involves a mutation in one of two genes, cubilin (on chromosome 10p13) or amnionless (on chromosome 14q32.32), which both codify for components of the intestinal receptor of the Cbl-IF complex and the receptor mediating the tubular reabsorption of proteins from the primary urine [10]. In fact, IGS is also called selective Cbl malabsorption with proteinuria, since mild proteinuria (with no signs of kidney disease) is present in about half of the patients. Proteinuria could be the effect of the defective role of cubilin and/or amnionless in reabsorbing of filtrate proteins in the proximal tubule of the kidney.

It has been demonstrated that all IGS cases in Finland were due to cubilin mutations (three different mutations) and in Norway to amnionless mutations (two different mutations). In Turkey, Israel, and Saudi Arabia, two different amnionless mutations and three different cubilin mutations were present. Scandinavian cases appear to be typical examples of enrichment by founder effects, while in the Mediterranean region high degrees of consanguinity expose rare mutations in both genes [11].

Some inborn error of Cbl transport are due to the lack of transcobalamin (TC) synthesis or production of a not-functional TC, unable to bind Cbl and transport it from the blood into the cells.

TC deficiency (OMIM: 275350) is characterized by elevated total plasma homocysteine and methylmalonic acid concentrations but normal serum Cbl levels, since most Cbl in serum is carried on HC. Diagnosis of TC deficiency is confirmed studying the synthesis of TC in cultured fibroblasts, or by molecular analysis of the TC gene (on chromosome 22q12.2) [12].

Over 40 patients with TC deficiency have been described and mutations in TC gene have been identified [1]. Most of the reported mutations are deletions or insertions in the TC gene resulting in frame shifts that predict protein truncation. Nonsense mutations and point mutations that activate exonic cryptic splice sites have also been reported [1,13,14]. Polymorphic variants have also been described. In the white population, the most frequent polymorphism in TC gene is the substitution of guanine to cytosine in 775 position (775G> C), which causes the substitution of proline with arginine at codon 259 and a higher serum levels of methylmalonic acid if confronted with subjects with other genotype [15].

Defects in the TC receptor (CD320; OMIM: 606475) for cellular uptake of Cbl have also been identified. CD320 (on chromosome 19p13.2), expressed on the plasma membrane, binds TC saturated with Cbl (holo-TC) and mediates cellular uptake of Cbl.

Analysis of CD320 in five asymptomatic newborns with elevated methylmalonic acid and decreased uptake of holo-TC in fibroblasts, identified a homozygous single codon deletion, resulting in the loss of a glutamic acid residue in the low-density lipoprotein receptor type A-like domain [16]. Finally, it was also described a patient with elevated levels of both TC and the soluble form of CD320 that presents elevated levels of total plasma Cbl but neurological symptoms similar to Cbl deficiency [17].

Into the cells, Cbl is transported into lysosomes to be released from TC and exported in the cytosol. Abnormalities in lysosomal transport have also been identified, but they are still classified as metabolic defects by some authors.

Until now, two proteins have been identified to be responsible for Cbl lysosomal release into the cytoplasm, the so called lysosomal membrane transporter 1 (LMBRD1) and ABCD4 [2,18]. The so called *cblF* and *cblJ* are two inherited disorders due to mutation in LMBRD1 (on chromosome 6q13) and ABCD4 (on chromosome 14q24.3) genes, respectively [2,18]. In *cblF* (OMIM: 277380) and *cblJ* (OMIM: 614857) inborn error of Cbl metabolism, free vitamin accumulates in lysosomes, thus hindering its conversion to cofactors. Both patients present a diminished synthesis of both Cbl coenzymes, resulting in methylmalonic aciduria and homocystinuria.

The anomalies of the synthesis of Cbl coenzymes (see the chapter "Natural cobalamins – their structure, chemical reactivity and co-enzymatic role" for details on Cbl- enzymes and co-enzymes) cause the genetically determined diseases of intracellular Cbl metabolism. Specific diagnosis of these inborn errors has traditionally depended on "complementation analysis", which has resulted in the definition of six/eight types named: *cblA*, *cblB*, *cblC*, *cblD*, *cblE*, *cblG*, *cblF*, *cblJ*. Each of which is autosomal recessive and representing a mutation at a separate gene. In the "complementation analysis", patient cells are fused with fibroblasts from patients with known Cbl inborn defect and the function of Cbl-dependent enzymes (methionine synthase (MS) and methylmalonyl-CoA mutase (MCM)) is checked. If the mutations in the two cells affect different loci, the enzymatic function in the fused cells will be normal.

For *cblF* and *cblJ* see the paragraph on abnormalities in lysosomal transport.

The diseases named *cblA* (OMIM: 251100) and *cblB* (OMIM: 251110) are due to a decreased synthesis of 5'-deoxy-5'-adenosyl-cobalamin (AdoCbl) accompanied by a normal synthesis of methylcobalamin (MeCbl), with a consequent high serum levels of methylmalonic acid (methylmalonic aciduria). *cblA* and *cblB* patients present lesions in two genetically distinct loci. In particular, cells from *cblA* patients are unable to convert the hydroxoCbl in AdoCbl, and present mutations in the MMAA gene (on chromosome 4q31.21), which apparently play a role in transfer of AdoCbl to methylmalonylCoA and in the stabilization of the mutase bound AdoCbl [1,19]. One mutation, c.433C>T (R145X), represents 43% of pathogenic alleles in North America patients and a common haplotype was identified [18]. Fibroblasts from *cblB* patients, instead, present abnormalities at the level of the enzyme cob(I)alaminadenosyltrasferase (EC 2.5.1.17 (on chromosome 12q24.11)) [1,5]. Patients with a defect in the enzyme MCM (mut patients) present symptoms similar to *cblA* and *cblB* diseases.

Patients with *cblE* (OMIM: 236270) and *cblG* (OMIM: 250940) disorders present decreased synthesis of MeCbl with normal synthesis of AdoCbl, and decreased function of MS. Using a complementation analysis it is possible to distinguish the two complementation groups of patients with *cblE* or *cblG* disease. *cblE* patients present a mutation in the gene (on chromosome 5p15.31) coding for the enzyme methionine synthase reductase (EC 4.2.99.10), which is necessary for the reduction of Cbl to cob(I)alamin, and therefore allows the binding of Cbl itself to MS [20].The most frequent mutation in methionine synthase reductase, representing 25% of disease-causing alleles, is an intronic mutation (c.903 + 469T $\rightarrow$C) that creates a novel splice acceptor site in exon 6 resulting in the inclusion of 140 bases of intronic sequences in the mRNA [1]. *cblG* patients, instead, have abnormalities in the gene MTR (on chromosome 1q43.) that encodes the catalytic subunit of MS (EC2.1.1.13) [21].

cblC (OMIM: 277400) and *cblD* (OMIM: 277410) patients present a diminished synthesis of both Cbl coenzymes, resulting in methylmalonic aciduria and homocystinuria. *cblC* disorder is probably the most common inborn error of Cbl metabolism (more than 500 patients) and it is caused by mutations in the MMACHC gene (on chromosome 1p34.1). The gene MMACHC was identified in 2006 [22] and, although the exact function of the protein is currently unknown, appears to play an important role in the synthesis of Cbl intermediates. For instance, it has been shown that MMACHC can catalyze the decyanation of cyanoCbl and a dealkylation

reaction of AdoCbl or MeCbl [23] (see the chapter "Natural cobalamins – their structure, chemical reactivity and co-enzymatic role" for further details).

Finally, *cblD* patients present abnormalities in the MMADHC gene (on chromosome 2q23.2), which encodes a protein of unknow function [1].

See Table 1 (left part) for a summary of the principal inherited causes of Cbl deficiency.

Table 1. Principal causes of Cbl deficiency in Humans

Inherited	*Acquired*
Abnormalities in Cbl transport/absorption HC deficiency IF deficiency or defective IF Imerslund-Gräsbeck syndrome TC deficiency or defective TC Defects in CD320 Abnormalities in lisosoma transport cblF disease cblJ disease Abnormalities in Cbl metabolism AdoCbl deficiency (cblA and cblB diseases); Combined deficiencies of AdoCbl and MeCbl (cblC, cblD diseases); MeCbl deficiency (cblE, cblG diseases); Defects in MCM (mut patients)	Autoimmune chronic atrophic gastritis Chronic Helicobacter pylori gastritis Gastric resection Pancreatic insufficiency pancreatitis; pancreatectomy Intestinal disorders infestation from diphyllobothriumlatum; blind loop syndrome -tropical sprue; lymphomas or tuberculosis of the intestine; celiac disease; Crohn's disease. Long-term N_2O exposure Insufficient Cbl intake

Abbreviations: AdoCbl:5'-deoxy-5'-adenosyl-cobalamin; Cbl:cobalamin; HC: haptocorrin; IF:intrinsic factor; MeCbl:methylcobalamin; MCM:methylmalonyl-CoA mutase; MS:methionine synthase; TC: transcobalamin.

ACQUIRED CAUSES OF CBL DEFICIENCY IN ADULTS

Pernicious anemia is the most common cause of Cbl acquired deficiency. It is an autoimmune disease that affects parietal cells located in the body and

fundus of the stomach, although in the blood of patients it is also possible to identified auto-antibodies against cellular proton pump and/or against the IF itself. The consequence is a reduced production or inactivation of IF. The auto-immunization against parietal cells, mediated by $CD8^{+}$T lymphocytes, causes a chronic atrophic gastritis (type A) and reduces the production of hydrochloric acid (achlorhydria). Colonization of the stomach by Helicobacter Pylori can result in a chronic gastritis (gastritis of type B) that reduces the number of gastric parietal cells producing IF[24,25].

Total or partial gastrectomy could produce total or partial decrease of IF synthesis, and ultimately, Cbl deficiency[26]. Fortunately since the1980s, gastrectomy and terminal small intestine surgical resection decreased frequency, and so Cbl malabsorption from this cause has become rarer.

In 50-70% of patients with functional insufficiency of exocrine pancreas following chronic pancreatitis or pancreatectomy, a malabsorption of Cbl [27] has been shown. Poor Cbl absorption is probably due to a deficiency of pancreatic proteases, which in turn prevents the cleavage of R protein [27].

Some intestinal diseases, such as infestation from *diphyllobothrium latum*, blind loop syndrome (in which parasites and/or bacteria compete with the host organism for the use of the Cbl [28,29]), tropical sprue, lymphomas or tuberculosis of the intestine, celiac disease and Crohn's disease are other causes of Cbl malabsorption [30].

Prolonged use of nitrous oxide (N_2O), an anesthetic in dentistry, can cause a state of Cbl deficiency. In fact, N_2O irreversibly oxidizes the cobalt ion of Cbl from the (+) 1 to the (+) 3 valence state. Oxidation of the cobalt ion by nitrous oxide prevents MeCbl from acting as a coenzyme in the production on methionine [31].

Long-term ingestion of antiacids, such as H_2-receptor antagonists and proton-pump inhibitors, and metformin treatment could be also a cause of Cbl deficiency [30].

Some authors classified all of these disorders, except pernicious anemia, as food Cbl malabsorption syndrome. In fact, this syndrome is characterized by Cbl deficiency in the presence of sufficient food-Cbl intake and a normal Schilling test ruling out malabsorption or pernicious anemia (diagnosis of exclusion) [30].

Finally, Cbl deficiency may be also due to insufficient Cbl introduction with food (strict vegetarian diets, namely the veganism).

See Table 1 (right part) for a summary of the principal acquired causes of Cbl deficiency.

CBL DEFICIENCY IN FETUS, EMBRYO AND CHILD IN THE FIRST MONTHS OF LIFE

Recommended intake of Cbl for pregnant woman is increased to 2.6 µg versus 2.4 µg/day for adults to meet the need of the fetus [32]. During pregnancy, the fetus absorbs Cbl through the placenta. Case reports have long indicated that infants of Cbl-D mothers often manifest disorders connected to Cbl deficiency (i.e., megaloblastic anemia, irritability, failure to thrive, reduced cerebral growth) [32].

In newborns, Cbl deficiency is often due to a diet with low Cbl content milk (i.e., milk from Cbl-D mothers). Moreover, recent studies have demonstrated low concentrations of Cbl in foremilk and hindmilk 4 months after childbirth. Low concentrations of Cbl milk mirrored biochemical changes in infants, which suggests an impaired Cbl status and indicates that an exclusive nutrition with mother's milk may not be sufficient for the supply of Cbl from this age [33].

Finally, Cbl deficiency in children, if not due to genetically determined cause, is often associated to a diet low on animal source food [32].

CONCLUSION

Cbl deficiency results from several causes (inborn or acquired) inducing metabolic abnormalities and severe symptoms. Identifying the exact cause, we can remove this (i.e., changing eating habits during insufficient introduction) and/or go with the best therapy. In some cases the deep knowledge of cause is also necessary to prevent a status of deficiency (i.e., supplementary therapy immediately after gastric resection).

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Chapter 6

LABORATORY TESTING FOR VITAMIN B_{12} DEFICIENCY

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ABSTRACT

In this chapter, the principal tests for determination of vitamin B_{12} (cobalamin (Cbl)) deficiency will be analyzed. It will address a series of issues related to these assays: reference values, potential markers and technical disadvantages.

Even if partially outside the chapter topic, it is useful to recall also recent studies related to utilization of high Cbl levels as potential marker of some diseases.

Keywords: Cbl deficiency; holotranscobalamin (holoTC); hypercobalaminemia; methylmalonic acid (MMA); total homocysteine (tHcy)

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INTRODUCTION

A Cbl-deficient (Cbl-D) status could be classified in: *i*) uncommon and serious clinical Cbl deficiency characterized by megaloblastic anemia and/or neurocognitive dysfunction; or *ii*) more common subclinical (mild, asymptomatic) Cbl deficiency, defined by abnormal concentrations of Cbl-related biomarkers (also referred to as subclinical Cbl deficiency) [1]. The common biomarkers used for Cbl status are: serum Cbl, methylmalonic acid (MMA), and total homocysteine (tHcy) [1]. Holotranscobalamin (holoTC) is also considered a possible indicator of Cbl status [1].

Available biomarkers can be categorized as those that directly measure Cbl in blood and those that measure metabolites that accumulate with inadequate amounts of Cbl.

Serum Cbl and holoTC measure the amount of Cbl available for the body's cells. These biomarkers, therefore, reflect the broad Cbl status range from high risk of severe deficiency to adequacy. MMA and tHcy, which are functional (metabolic) measures of Cbl status, accumulate when concentrations of Cbl are inadequate and mirror any lack of Cbl within the cells. These functional measures are useful for identifying subclinical Cbl status and reflect early changes in Cbl status.

Since factors unrelated to Cbl status affect all 4 biomarkers (i.e., impaired renal function, genetic variation, disease conditions, drug use, pregnancy, technical problems) we could obtain false-positive or false-negative classifications of Cbl deficiency. For this reason, information from both categories of biomarkers can yield a more accurate assessment of Cbl status than information from only one Cbl status category [1]. Even, a study from Fedosov suggests to combine all the four parameters to obtain a more reliable indication of the Cbl status [2]. Furthermore, some authors suggest that the clinical picture is the most important factor in assessing the significance of test results assessing Cbl status. The interpretation of the result on Cbl status should be considered only in relation to the clinical circumstances [3].

Measurements of serum Cbls have been used worldwide since the 1950s. Despite limited specificity and controversy about their sensitivity, these measurements are still the standard investigation for Cbl deficiency. Patients with serum Cbl concentrations well below the reference interval are generally considered to have Cbl deficiency [4].

Opinions differ as to the optimal laboratory cutpoint for the serum Cbl test, due in part to the insidious onset and slow progression of the disorder and some limitations of current assays. Some studies have tended to dichotomize

low values at 148 pmol/L (200 pg/mL) [5], others suggest a value of 125 pmol/L (with a "gray area" at 125-250 pmol/L) [4].

An additional problem when comparing different assays and results from different laboratories concerns the units for reporting of serum Cbl levels. Some report in pg/mL (ng/L) and some in pmol/L (1 pmol/l = 1.355 pg/ml) [3].

Cbl assays require extraction of Cbl from its binding proteins in plasma and conversion of the native Cbl to cyanoCbl (CNCbl), the non physiologic form that serves as the assay standard.

Different methods exist for the measurement of the free CNCbl. Over the years, growth response of various microorganisms has been used in quantification of Cbls in biological materials and foods. The used microrganisms have been Lactobacillus delbrueckii, Escherichia coli, Euglena gracilis, and Ochromonas malhamensis [6]. Older assays were then replaced by radioisotopic competitive binding techniques using intrinsic factor (IF) as the Cbl-binding protein. In fact, the principle of this assay is based on the competition for the binding to IF between added isotope-labelled CNCbl versus CNCbl from a sample. Immunoenzymatic luminescence methods, that rely on competitive binding by IF, have now replaced isotopic assays [5].

Elevated concentrations of MMA are considered the most representative marker of metabolic Cbl insufficiency, but poor assay availability limits clinical utility. MMA is a sensitive marker of Cbl deficiency, and an elevated MMA concentration is often used as a gold standard for the classification of a patient's status as Cbl-D or Cbl non-deficient. Major drawbacks are the low specificity of marginally elevated MMA values, the complexity of the assay, the high cost, and often a slow turnaround time [4]. MMA assay is therefore not widely used. Gas chromatographic assay of MMA in urine has been available since the late 1950s but nowadays sensitive capillary gas chromatography/mass spectrometry assays made it possible to measure small concentrations in serum accurately [5].

Exceptionally high levels of plasma MMA (>750 nmol/l) almost invariably indicate Cbl deficiency [3]. Many laboratories defined cutoffs by 3 or 2 standard deviation from the mean (≈<370 or <270 nmol/L, respectively) but the most commonly applied MMA cutoff is ≈270 nmol/L [5].

tHcy has low specificity because it also increases in patients with folate and possibly thiamine and vitamin B_6 deficiency. It is considerably a good marker of Cbl deficiency only in a folate-fortified population [7,8]. In the clinical laboratory, plasma tHcy is measured by a variety of techniques, briefly classified into two groups: chromatographic methods and

enzyme/immunoassays [3, 7]. There is no consensus on the reference range, although most laboratories regard a concentration above 13-15 μmol/L as indicative of hyperhomocysteinemia [3, 8]. A major drawback to the clinical utility of tHcy is that sample collection and processing is critical as the serum sample must be kept cool and then centrifuged and removed from the red cells within 2 h of collection [3].

Since diagnostic accuracy of serum Cbl levels for the assessment of Cbl status is low, hence the need of a more sensitive marker. Emerging evidence indicates that holoTC seems more suitable than total Cbl for diagnosis of Cbl deficiency. Plasma holoTC transports the active Cbl (see the chapter "Vitamin B_{12} absorption and transport in mammals") and is decreased in a Cbl-D status.

In a study from Obeid and Herrmann [9], subjects with normal renal function (evaluated by normal serum creatinine) showed a negative correlation between serum holoTC and MMA, but in some cases (MMA > or =300 nmol/L and holoTC < or =35 pmol/L) concentrations of total Cbl were still well within the normal range. These data displayed a higher sensitivity and specificity for holoTC compared with Cbl for detecting concentrations of MMA > or =300 nmol/L [9]. There is also the added advantage of use in pregnancy as the holoTC fraction of Cbl does not seem to be subject to the physiological drop seen in total serum Cbl over the course of pregnancy [11].

It has been demonstrated that holoTC in physiological conditions has a reference interval of around 40–200 pmol/L [10, 12].

Recent studies questioned the values of the cut-off and the specificity of the results obtained from patients with values around the threshold. In patients with borderline values of holoTC the concordance between holoTC and MMA levels was poor [13,14]. In fact, Hermann and Obeid [15] suggest that all testing for Cbl deficiency should start with holoTC measurement but, in order to improve assessing, data with 23 pmol/L < holotTC < 75 pmol/L should be followed by MMA testing. Renal dysfunction and folate deficiency, that may affect the levels of holoTC, should be also considered [13, 14].

Further studies could be needed to evaluate all of these problematics and technical issue (test's cost and limited availability), but we associate to some authors that predict that holoTC will be an excellent marker for monitoring a population's Cbl status [10].

In some underdeveloped areas, the described assays on blood and/or serum are not feasible due to high costs and/or lacking infrastructure. Dried blood spot analysis could be an economical and field applicable substitute. The advantages of dried blood spot analysis include less invasive blood sampling, elimination of many blood preparation steps, no requirement for refrigerated

storage, and ability to easily mobilize large numbers of samples. There are some reports on the potential of dried blood spot for detecting total Cbl [16] or MMA [17]. These methods could be used also in large-scale population-based surveys without the requirement of an on-site laboratory infrastructure.

See Table 1 for a summary of the four biomarkers common used for Cbl status.

Table 1. Principal characteristics, advantage and disvantage of laboratory tests used to diagnose Cbl deficiency [4]

Test	Type of test	Rationale	Proposed cut-off	Advantage	Disvantage
Total Cbl	Direct	↑ in Cbl deficiency	<148 pmol/L or <125 pmol/L (with "gray area" at 125-250 pmol/L)	Accessible Cheap	Variation in reference interval due to different methods; Sensitivity and specificity debatable; Large % of indeterminate results (grey-zone)
MMA	Functional	↓ in Cbl deficiency	>370 nmol/L or >270 nmol/L	High sensitivity	Not easily accessible; Expensive; Requires mass spectrometry/gas chromatography; Low Specificity; False positive with reduced renal function and increases with age
Hcys	Functional	↑ in Cbl deficiency	>13-15 µmol/L		Low Specificity;
HoloTC	Direct	↓ in Cbl deficiency	<35 pmol/L (with a "gray area" at 23-75 pmol/L)	Expected to have high sensitivity	Low Specificity; Affected by renal function; Improved specificity over other tests; Changes occur early in Cbl depletion

Once Cbl deficiency is diagnosed, using one or more of the above markers, next challenge is to find the cause of Cbl deficiency. If a thorough history reveals dietary insufficiency, or gastric or ileal resection, usually, further laboratory tests are not recommended, alternatively patients could be subjected to tests aimed mainly at evaluating the function of the gastric

mucosa and/or the Cbl absorption [4]. Antibodies to IF and gastrin or pentagastrin I levels are often used to diagnose pernicious anemia.

For years, Shilling's test, in which labeled Cbl (with or without IF) is administered orally, has been used to investigate whether lack of the vitamin is caused by lack of IF. This test has been considered the gold standard for investigating Cbl absorption. Shilling's test is, however, no longer readily available due to increasing difficulties in obtaining labeled Cbl and IF. Nexo et al. proposed alternative approaches to measuring Cbl absorption, named CobaSorb [18] and C-CobaSorb [19]. In CobaSorb an oral dose of non-radioactive Cbl is administered, and active Cbl absorption is reflected by an increase in holoTC. In C-Cobasorb the increase of CNCbl bound to TC is measured instead of holoTC.

A recent study suggests the importance of CobaSorb and C-CobaSorb to identify patients not requiring Cbl injection therapy and to choose the best route of administration (injection respect to oral treatment is inconvenient for the patient and constitutes a cost for society) [20].

Interest in monitoring Cbl status increased with folic acid fortification of cereal grains in the United States in 1996, since this excessive intake of folic acid might obscure or mask Cbl deficiency and potentially delay its diagnosis until neurologic consequences become irreversible [21]. A study from Selhub et al [21] has shown that having low Cbl status, regardless of serum folate, was associated with a significantly increased prevalence of both anemia and cognitive impairment, but the worst combination was low Cbl status and high serum folate. Specifically, anemia and cognitive impairment were observed '5 times as often in the group with low Cbl status and high serum folate as in the group with normal Cbl status and normal serum folate. These data represent important epidemiologic evidence of an adverse interaction between high folate status and low Cbl. Consequently, they seem to support the idea that the neuropsychiatric consequences of Cbl deficiency are exacerbated by high folate status.

So far, researches have been focused on Cbl-D status and specific and sensible methods to detect it,neglecting hypercobalaminemia (high serum Cbl levels) that is a frequent and underestimated anomaly. Recent evidence has described that various pathological conditions (i.e., solid neoplasms, hematological malignancies and liver and kidney diseases) are linked to elevated serum Cbl [22,23]. The aetiological profile of high serum Cbl predominantly encompasses severe disease entities for which early diagnosis is critical for prognosis, hereof the potential importance of the Cbl assay as an early diagnostic marker [22,23].

However, so far, the use of high levels of Cbl as a marker of some diseases has not been thoroughly evaluated. Further studies are needed to better understand the clinical data related to high serum Cbl and the approach to adopt upon discovery of elevated Cbl levels.

CONCLUSION

A fast and correct diagnosis of Cbl deficiency is necessary for a speedy therapy. According to above-mentioned contents, we believe that it is important to improve all the methods but it will probably continue to be necessary a double or quadruple analysis using multiple methods. We also believe that the scientific community must finally take account of the possibility to detect hypercobalaminemia and to adopt this as a marker of some diseases.

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Chapter 7

PATHOLOGICAL EFFECTS OF VITAMIN B_{12} DEFICIENCY

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ABSTRACT

Vitamin B_{12} (cobalamin (Cbl)) acts on many organs and systems of the human body. Because of Cbl systemic action, a Cbl-deficient (Cbl-D) status recurs as numerous symptoms, more or less serious, at the level of the whole body.

Conventionally, symptoms are different between inherited disorders in which Cbl deficiency occurs at birth or juvenile age and acquired disorders that affect adults.

Deserve a special mention also the effects of a Cbl-D status when it develops during fetal development of the individual (i.e., in Cbl-D pregnant mothers).

For this reason, this chapter has been divided in subchapters describing the effects of deficiency during development, in children and

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adults, dividing the latter in the principal affected targets (nervous system, blood, heart, gastric system and immune system).

The role of Cbl in cancer is another important topic of this chapter. As a dietary methyl group donor, Cbl can partecipate in human cancerogenesis modulating epigenetic mechanisms: DNA methylation is, in fact, crucial for altering gene expression of oncogenes and oncosuppressors.

Keywords: cancer; cardiovascular disorder; Cbl deficiency; fetus development; lymphocyte; macrocyte; megaloblast; neuropathy; white matter

INTRODUCTION

Effects on Fetus Development

Several studies have focused attention on the maternal nutrition as the major determinant of the health of the offspring. In pregnant women with Cbl deficiency, the levels of Cbl transported to the fetus decline respect to the physiological one (intrauterine Cbl deficiency) [1].

An intrauterine Cbl deficiency induces several disorders in fetus. In fact, infants whose mothers were Cbl-D had a low birth weight compared to normal one [1,2]. In particular, studies demonstrated that Cbl status in the mother was related to neonatal Cbl status and low neonatal Cbl concentrations were adversely associated with low birth weights [3, 4].

Infants with intrauterine Cbl restriction are also at increased risk of type 2 diabetes later in life [1]. In a study on Indian population, low maternal Cbl status coupled with high folate increased the risk of insulin resistance in the child, of gestational diabetes and permanent diabetes [5].

Although authors report discordant data, according to some studies, Cbl insufficiency may also increase risk of neural tube defects (failure of the neural tube to close during gestation induces spina bifida, anencephaly, and encephalocele) [1, 6-8].

Just to quote one, decreased Cbl concentrations were found in amniotic fluid samples derived from neural tube defects pregnancies [9]. Interestingly, the fathers of babies with neural tube defects had significantly lower serum Cbl levels in comparison to father of normal babies [10].

Experimental studies look confirm the importance of Cbl during fetus development. In fetus from severe deficient mothers, disorders in hematopietic

system (i.e., decreased number of red blood cells, decreased level of hemoglobin [11] and immature hematopoietic elements [12]) and morphological alterations in almost all organs [12, 13] have been demonstrated.

A study in a sheep model demonstrated that clinically relevant reductions in the availability of Cbl, folate and methionine from maternal diet around the time of conception lead to widespread epigenetic modifications to the genome associated with increased adiposity, insulin resistance, altered immune function, and high blood pressure in adult offspring [14].

Finally, a status of Cbl deficiency looks also to be associated with reduced number of pregnancies, increasing male and female infertility and recurrent fetal loss [15-17].

Effects in Childhood

It is possible to observe a Cbl-D status in children raised on low diet on animal source foods or with inherited Cbl-related diseases (see the chapter "Causes of vitamin B_{12} deficiency").

Infants born to mothers with a normal Cbl status have stores of Cbl that are adequate to sustain them for the first several months postpartum (Cbl deficiency rarely occurs before about 4 months of age). Infants of Cbl-D breastfeeding mothers, or infants receiving low amounts of animal-source foods, may be vulnerable to Cbl deficiency between 6 and 12 months of age [18].

Some studies in Cbl-D children have highlighted a possible impact of Cbl status on cognitive functions [1].

In Cbl-D children the damage is usually limited to the brain and characterized by white matter loss with delayed myelination [19, 20], magnetic resonance imaging abnormalities included brain atrophy, callosal thinning, craniocaudally short pons, and increased T_2 FLAIR signal in periventricular and periatrial white matter [21-23].

Clinical seizures, developmental delay, microcephaly, hypotonia and nystagmus have been demonstrated in *cblC* patients and/or infants with nutritional Cbl deficiency [23-26]. Neurodevelopmental deficits were noted most prominently in motor skills, with relative preservation of socialization and communication skills [23]. Other studies indicated the presence of intellectual dysfunction, attention problems, concerns with behavioural aspects of executive function in *cblC* patients [27]. A negative correlation between the

plasma Cbl concentration and the mental development index score was also identified [28].

In addition to neurological symptoms, infants may experience other physical symptoms, including abnormal pigmentation, enlarged liver and spleen, sparse hair, food refusal, anorexia, failure to thrive and diarrhea [18, 24-26].

In infants, Cbl deficiency is also associated to megaloblastic/macrocytic anemia and neutropenia [29, 30]. The neutrophil could appear dysgranulopoietic and defective in function (low chemotaxis and elevated superoxide production) [31, 32].

Some case reports suggested an association of hemolytic-uremic syndrome (characterized by microangiopathic hemolytic anemia, thrombo-cytopenia, and renal failure) with *cblC* disorder [33-36].

Few studies have indicated *cblC* deficiency as the common denominator in cases of isolated pulmonary hypertension [37], renal thrombotic microangiopathy [38] and in the rare combination of pulmonary hypertension and renal thrombotic microangiopathy [39].

Finally, a study demonstrated the presence of structural heart defects (i.e., left ventricular non-compaction, secundum atrial septal defect, and mitral valve prolapse) in 50% of analysed patients with *cblC* [40].

Neurological Effects

In adults, the neurologic manifestations due to Cbl-D status are not necessarily secondary to the hematological symptoms. Only some of the patients with pernicious anemia develop a neuropathy and,viceversa, patients with neurological disturbances due to Cbl deficiency, but without anemia or macrocytosis, have been described [41, 42]. Moreover, in some patients the severity of the hematologic and neurologic manifestations may be inversely related.

In the central nervous system (CNS) the histopathological hallmarks of the neuropathy due to Cbl deficiency are: (*i*) a diffused but uneven vacuolation (so-called “spongiform vacuolation”) of the white matter (especially the spinal cord (SC)) that affects the posterior or lateral SC columns throughout their length; (*ii*) intramyelinic and interstitial edema of th ewhite matter of the CNS (especially the SC) [43-46]; and (*iii*) reactive astrogliosis [43, 45-46]. The most consistent magnetic resonance imaging finding is a symmetrical abnormally increased T_2 signal intensity.This abnormality is related to

demyelination or intramyelin edema and confined to posterior or posterior and lateral columns in the cervical and thoracic SC [19, 47]. In acute and severe cases, the SC might also present as swollen and involvement of anterior columns has also occasionally been reported [19].

Histopathological lesions, similar to those in the SC, have been seen more rarely in brain white matter (i.e. leukoencephalopathy) [48-50]. In some patients, fluid attenuated inversion recovery and T_2-weighted images demonstrate extensive areas of a high-intensity signal in the periventricular white matter [19].

Recent studies have also investigated a low Cbl status as a modifiable cause of brain atrophy [51].

Peripheral neuropathy (also called polyneuropathy) is another of the main neuropathological consequences of Cbl deficiency [52, 53]. The histopathological and ultrastructural hallmarks are intramyelinic and interstitial edema and gliosis [54, 55]. Cbl deficiency also leads to electrophysiological abnormalities in the peripheral nervous system [52, 53, 56].

In the CNS and peripheral nervous system, myelin sheaths seem to be more severely involved than the axons, and no axons devoid of myelin or any ultrastructural evidence of new myelin deposition occurring simultaneously with myelin lysis have been observed [49]. Only optic nerve fibers are often spared in patients with neuropathy due to Cbl deficiency [57]. Neurons do not seem to be structurally affected by Cbl deficiency. With reference to the above mentioned subject, some authors classified the neuropathy due to Cbl deficiency as a pure myelinolytic disease with no apparent loss of myelin [49, 58].

Moreover, no histopathological signs of inflammation or apoptosis have been observed in CNS of patients who have died with Cbl-D neuropathy [59].

The typical neurologic manifestations of the neuropathy due to Cbl deficiency are: (i) myelopathy with or without an associated neuropathy; (ii) optic neuropathy; and (iii) paresthesias without abnormal signs [60]. Optic neuropathy occurs only occasionally in adult patients. Optic nerve disease is characterized by symmetric, painless and progressive visual loss. Central and centrocecal scotomas are the main ophthalmologic findings [19].

The neurologic features typically include a spastic paraparesis or tetraparesis, extensor plantar response and impaired perception of position and vibration. The involvement of the posterior and lateral columns of the cervical and upper dorsal parts of the SC is responsible for the impairment of position sense, paraparesis and tetraparesis [19]. Almost all patients have loss of

vibratory sensation, often associated with diminished proprioception and cutaneous sensation and Romberg sign [19]. Accompanying peripheral nerve or rarely optic nerve involvement may be present [60].

The neuropsychiatric manifestations of Cbl deficiency include personality change, psychosis, emotional lability, and rarely delirium or coma [41, 57, 61]. Reported symptoms of psychosis include suspiciousness, persecutory or religious delusions, auditory and visual hallucinations and disorganized thought-processes [19].

Cbl deficiency has also been negatively correlated with cognitive functioning in healthy elderly subjects. Symptoms include slow mentation, memory impairment, and attention deficits [19].

Other neurologic manifestations possibly related to Cbl deficiency include cerebellar ataxia, orthostatic tremors, myoclonus, ophthalmoplegia, catatonia, vocal cord paralysis, a syringomyelia-like distribution of motor and sensory deficits, and autonomic dysfunction [60]. Fatigue, irritability, and lethargy are also reported in some patients.

The clinical features of the Cbl deficiency polyneuropathy are similar to those of a cryptogenic sensorimotor polyneuropathy [60]. Cryptogenic sensorimotor polyneuropathy is characterized by decreased or lost proprioception or sense of vibration and loss of ankle jerks [62]. The onset of symptoms is in the hands with concomitant involvement of upper and lower limbs.

Separate discussion is necessary for neurological diseases not etiologically linked to Cbl deficiency, but possibly with an abnormality in Cbl levels. In fact, some studies have reported an association between Cbl deficiency and diverse neurodegenerative processes (for a review see [63]). We will focus our attention on the more investigated: multiple sclerosis and Alzheimer's disease.

The debate concerning the possible role of Cbl in multiple sclerosis is long-lasting, and the results are still conflicting. In fact, although the majority of patients with multiple sclerosis do not have confirmed Cbl deficiency, a subgroup of patients share the association of both disorders (for a review see [64]). Moreover, a recent meta-analysis revealed that individuals with multiple sclerosis tend to have significantly lower serum Cbl levels respect to the controls [65]. Patients with multiple sclerosis and lower Cbl level present prolonged visual and posterior tibial somatosensory and evocate potential latencies respect to patient with multiple sclerosis and normal Cbl level [66].

Finally, combination therapy with interferon-ß plus Cbl greatly improved both the clinical and histopathological pictures of an experimental model of multiple sclerosis [67].

Viceversa, Scalabrino et al. demonstrated that Cbl levels are increased in cerebrospinal fluid of multiple sclerosis patients with relapsing–remitting and secondary-progressive clinical courses, but unchanged in those with the primary-progressive clinical course [68].

The pathological significance of the abnormal Cbl levels in multiple sclerosis remains a matter of speculation [68, 69] as well as the interpretation of conflicting results.

Some studies reported low serum Cbl levels in patients with Alzheimer's disease [70-73] but the clinical relevance of the association between low Cbl levels and Alzheimer's disease is untilnow not completely known.

It has been hypothesized that the associations between Cbl and some neurodegenerative disorders which are strongly represented by increased homocysteine levels, such as Alzheimer's disease, reflect the effects of chronic cerebral oxidative stress, effectively resulting in B vitamin depletion [63]. Other authors reported a greater basal production of interleukin(IL)-6 in Alzheimer's disease patients who had low Cbl level compared to normal Cbl counterpart. It suggests that IL-6 abnormality could be implicated in the pathogenesis of Alzheimer's disease [74]

Hematological Effects

Clinical hematological presentation of Cbl deficiency ranges from incidental increased mean corpuscular volume and neutrophils hyper-segmentation in otherwise asymptomatic patients to symptoms due to severe anemia, such as angor, dyspnea on exertion, fatigue or symptoms related to congestive heart failure, such as ankle edema, orthopnea and nocturia [19].

The bone marrow of Cbl-D patients shows characteristic megaloblastic erythroblasts and giant metamyelocytes (early granulocyte precursors) [75]. Megaloblastosis represents a unique morphological and functional aberration of erythropoiesis [76].

The biochemical characteristic of the abnormal erythroblasts (called megaloblasts) is a defective DNA synthesis. Defective DNA synthesis leads to disparity in nuclear-cytoplasmic asynchrony and Cbl-D cells slowly divide until mature daughter cells die in the marrow or are arrested at various stages of the cell cycle [76].

In fact, in physiological conditions the eukaryotic cell cycle consists of four distinct phases: G1 (Gap1) phase, S phase (synthesis), G2 (Gap2) phase (collectively known as interphase) and M phase (mitosis). M (mitosis) phase is

itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides in half forming distinct cells. In normal cell cycle most of the cells are in G1 phase and present a quantity of DNA equal to 2N (where N is the amount of DNA in the haploid genome) while only a few number of cells (in S and G2 phase, before mitotic division) have a DNA amount equal to 4N. Instead, in patients with megaloblastic anemia, erythroblast stops duplication in S phase and so presents a double quantity of DNA (4N).

It is interesting to note that although megaloblastic changes are most striking in the bone marrow and peripheral blood, many other proliferating cells (epithelial cells lining the gastrointestinal tract, epithelial cells pertaining to the female genital tract) may exhibit megaloblastic features.

Although the opinion of the scientific community is not unanimous, two principal mechanisms of impairment of DNA synthesis have been proposed: the "methylfolate trap hypothesis" and the "formate starvation hypothesis" (for detail see [77]).

The megaloblast nuclei are wider than those of normoblasts and chromatin is abnormally dispersed due to a delayed condensation. Chromosomal abnormalities such as excessive stretching, random breakage, elongation and despiralization (unwinding) [76] may be present in megaloblasts. In addition, the nucleus may be located eccentrically and may exhibit indentations and/or karyorrhexis.

The blood film shows oval macrocytes (macrocytosis is a rise in the mean cell volume of red cells above the normal range (in adults 80-100 fl)) and hypersegmented neutrophil nuclei (with six lobes) [75, 78, 79] (see Figure 1).

Also an abnormal degree of variation in the shape of the erythrocytes in blood (anisocytosis and poikilocytosis) has been reported [19], in particular teardrop poikilocytes are quite common [80, 81] (see Figure 1). Schistocytes, howell-Jolly bodies and Cabot rings have also been identified in blood [81-83]. Howell-Jolly bodies are small fragments of DNA whilst Cabot rings are unusual oval or figure-eight shaped inclusions of unclear origin.

Most of the megaloblastic progenitor erythroid cells die in the bone marrow and macrophages effectively scavenge them, leading to ineffective intramedullary erythropoiesis [19, 84, 85]. Moreover, advanced megaloblastic anaemia has also a poorly understood component of intravascular hemolysis responsible for the short survival of red cells transfused into Cbl-D patients [86-88].

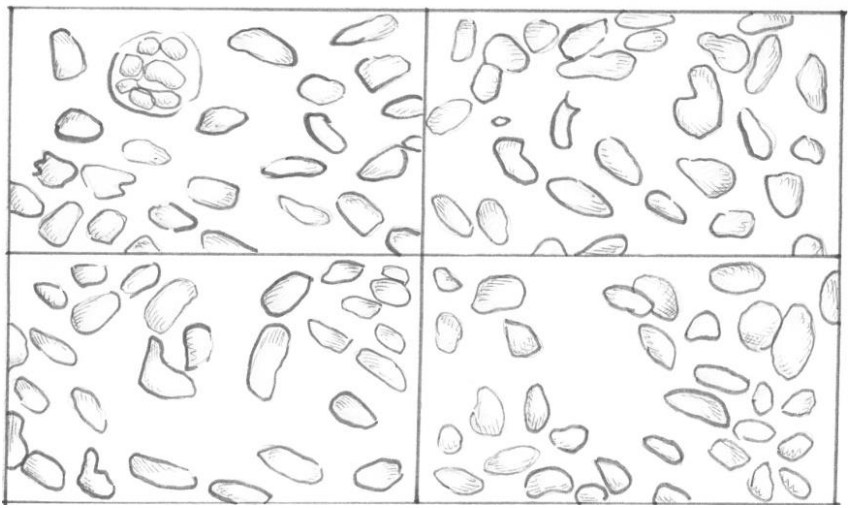

Figure 1. Representative picture of smear of peripheral blood from Cbl-D patients. The image deliberately emphasizes the presence and the salient features of the typical cells from a Cbl-D status (i.e., oval macrocytes, hypersegmented neutrophil nuclei, teardrop poikilocytes, and schistocyte). Picture is kindly drawn by Mr. Andrea Zadra.

Megakaryopoiesis may be involved, with hypersegmented nucleus and liberation of fragments of cytoplasm, generating "giant platelets" in the peripheral blood. However, platelet production and release are impaired, and as a result, various degrees of thrombocytopenia could occur [19]. Impaired platelet aggregation to adenosine diphosphate, collagen, epinephrine and ristocetin was also found in some patients with nutritional Cbl deficiency [89].

Effects on Immune System

Almost all nutrients in diet play a crucial role in maintaining an "optimal" immune response, so that deficient and excessive intakes can have negative consequences on immune status and susceptibility to a variety of pathogens.

Although just few studies have examined the relationship between Cbl and immune response, Cbl-defiency looks to have an immunomodulator effect for cellular immunity.

In severe cases, the white cell count and platelet count fall (pancytopenia) [75]. In particular, decreases in the number of lymphocytes and $CD8^+$ cells and in the proportion of $CD4^+$ cells, an abnormally high CD4/CD8 ratio, and

suppressed NK cell activity were noted in Cbl-D patients compared with control subjects [90]. All of these parameters were normalized after treatment with methylCbl (MeCbl) [90]. These results were confirmed in a study on patients with pernicious anemia before and after cyanoCbl treatment reporting that after treatment, absolute numbers and percentage of lymphocyte subgroups were elevated, as well as the levels of C3, C4, and immunoglobulins [91].

The elevation of the CD4/CD8 ratio by Cbl deficiency was also confirmed in rats and mice [92, 93]. In the same experimental group of Cbl deficiency it was also observed a decrease in the serum C3, IgM and IgG concentrations [92, 93]. Instead, in a recent *in vivo* study, it has been observed a significant decrease in the NK cytotoxicity in the spleen of Cbl-D group respective to the control group. Furthermore, in the same group of rats, it has been observed a significant decrease in the B lymphocyte (CD45RA) subsets but not for lymphocyte subsets helper T cells, cytotoxic-supressor T cells, matureT cells and natural killer cells. Consequently, it seems that Cbl deficiency decreases both B-cell diversity and NK activity [94].

Finally, it has been demonstrated in *in vitro* studies that MeCbl blocks the production by T lymphocytes of several cytokines (IL-6, interferon-γ, and IL-1ß) [95] and augments the proliferation of T cells in response to Concavalin A and autologous B cells [96].

More studies are necessary to clarify the exact role of Cbl in immune system.

Effect on Cardiovascular System

To the best of our knowledge, very few studies were carried out in order to test the direct effect of Cbl deficiency on cardiac structure.

Moreover, a recent systematic review of cohort studies has highlighted much heterogeneity in the few results of the association between blood Cbl levels and risk of cardiovascular diseases in adulthood [97].

In patients with Cbl deficiency it has been demonstrated an association with impairment of global and segmental myocardial deformation [98], low left ventricular ejection fraction [99] and, only in a patient, orthostatic hypotension [100]. Autoimmune pernicious anemia was identified as a cause of collapse, and heart failure in a young patient [101]. In Cbl-D sheep, a gelatinous edema was observed in the auricular-ventricular region of the heart and the right and left auricles were partially atrophied with presence of

hemorrhagic suffusion by cellular infiltrates of neutrophilic and eosinophilic granulocytes in connective tissue [102]. In Cbl-D rats, the hearts were characterized by large areas of white fibrotic streaks, marked reduction in muscle fiber mass and enlarged mitochondria [103]. Otherwise, another study has shown that fractional shortening, left ventricular dimension at end-diastole and end-systole, posterior wall thickness, and perivascular collagen level, were comparable in Cbl-D rats and controls but interstitial collagen (an indicator of cardiac remodelling and fibrosis) and brain natriuretic peptide-45 plasma concentration exhibited a trend to be lower in Cbl-D animals [104].

Some studies have assessed the prevalence of Cbl deficiency in patients with cardiovascular disorders. In patients with coronary heart disease it has been reported a percentage of Cbl deficiency between 22 [105]-45% [106] and in Indian subjects even 86.7% [107]. Cbl deficiency was also associated to venous thrombosis [108].

In a study on patients hospitalized for cardiovascular disease, low Cbl concentrations were found in 33% of patients, with a different incidence for different reasons for hospitalization [109]. The highest frequency was reported for patients with angina pectoris and myocardial infarction.

Even if partially outside the scope of this chapter, a short digression on the use of Cbl in prevention of cardiovascular disorders is also included, citing only a limited number of the most recent studies. In the so-called "homocysteine-lowering B vitamin therapy" Cbl is administered (or introduced by fortified food) in combination with folic acid and/or vitamin B_6 in order to decrease the homocysteine level, a suspected etiological factor for atherosclerosis. Obviously, in a combined therapy is hard to define the exact functions of each treatment (i.e., the precise role of Cbl) and, moreover, the scientific community is not unanimous on the effectiveness of the "homocysteine-lowering B vitamin therapy".

Some studies suggested that vitamin B supplementation is associated with lower levels of blood pressure [110], increased maximal coronary blood flow [111], improvement in coronary flow reserve [112] and reduced progression of early-stage subclinical atherosclerosis [113]. Otherwise some clinical trials have failed to show a benefit of B vitamin therapy in reducing composite outcomes of cardiovascular death, myocardial infarction, and stroke [114-116]. Some recent reviews suggested that some confounding factors may trouble the interpretation of the negative results, i.e., statin/aspirin therapy [117], folic acid fortification [117], age [118], and renal function [119].

According to above-mentioned contents, we believe that further investigations are needed to determine the effective role of Cbl in the

physiology of cardiovascular system, also in order to clarify some ambivalent results of the vitamin substitution therapy.

Effects on Digestive System

Some confusion exists whether some abnormalities in the digestive system are a cause or an effect of Cbl deficiency. It is well known that stomach and intestine play a major role both in the Cbl absorption than in the pathogenesis of Cbl deficiency (i.e., pernicious anemia, Imerslund-Grasbeck syndrome) (see chapters "Vitamin B_{12} absorption and transport in mammals" and "Causes of vitamin B_{12} deficiency") but changes in stomach and intestine due to Cbl deficiency have received less emphasis in the literature.

Studies have demonstrated that a normal endoscopic appearance was not correlated with Cbl status but atrophic gastritis was more common in the Cbl-D patients [120-121]. In some patients it is possible to observe with endoscopic examination a loss in the folds of gastric mucosa and its thinning.

Gastric emptying times were prolonged in patients with Cbl deficiency and this prolongation was partial corrected after Cbl replacement therapy [122]. Mucosal morphology and enzyme activities (dipeptidas and disaccharides) were studied in the intestine of Cbl-D patients. The villi were generally shorter than in control with demonstrated malabsorption of some nutrients [123-124]. In some of patients the disaccharide (maltase, isomaltase, sucrose, trehalase, and lactase) and L-alanyl-L -proline dipeptidase activity were depressed [124].

Biochemical and ultrastructural changes were also observed in liver of Cbl-D men and animals [125]. Cbl deficiency results in increased activity of the enzymes of fatty acid synthesis, such as acid synthetase and acetyl-CoA carboxylase. It has also been demonstrated that in the livers from Cbl-D animals there are an increased activity of the enzyme citrate synthase and increased mitochondrial cristae membranes [125].

Human data on abnormal hepatic metabolism of lipid look to be confirmed in ovine, where a Cbl deficiency is associated to a hepatic dysfunction called "ovine white liver disease" and characterized by pale, swollen, friable fatty livers, and showed marked accumulation of triglyceride and free fatty acid [126, 127].

Several experimental studies have demonstrated that Cbl has an hepatoprotective effect on different types of liver injured, such as dimethylnitrosamine-induced liver injury, arsenic intoxication or liver injury

by carbon tetrachloride [128-130]. Cbl administration to rat subject to partial hepatectomy normalized in four days the total lipid content and induced a more rapid true regeneration of liver (restoration of the dry fat free liver weight) [131]. Conversely, it is interesting to note that also hypercobalaminemia (high serum Cbl levels) is associated with etiologically different liver diseases [132, 133]. In fact, high serum Cbl levels were observed in some patients affected by acute and chronic liver diseases, although the exact pathophysiological mechanisms are not yet known [132, 133]. It has been considered that hypercobalaminemia may be an indicator of a functional deficit with clinical consequences paradoxically similar to those of Cbl deficiency [133].

A wide range of oral signs and symptoms may appear in Cbl-D patients as a result of basic changes in the metabolism of oral epithelial cells [134, 135]. The reported oral symptoms include a burning sensation, pruritus, lingual paresthesia, red tongue with erythematous macular lesions, recurrent ulcerations, reduced taste sensitivity and generalized sore mouth [134-136] (see Figure 2).

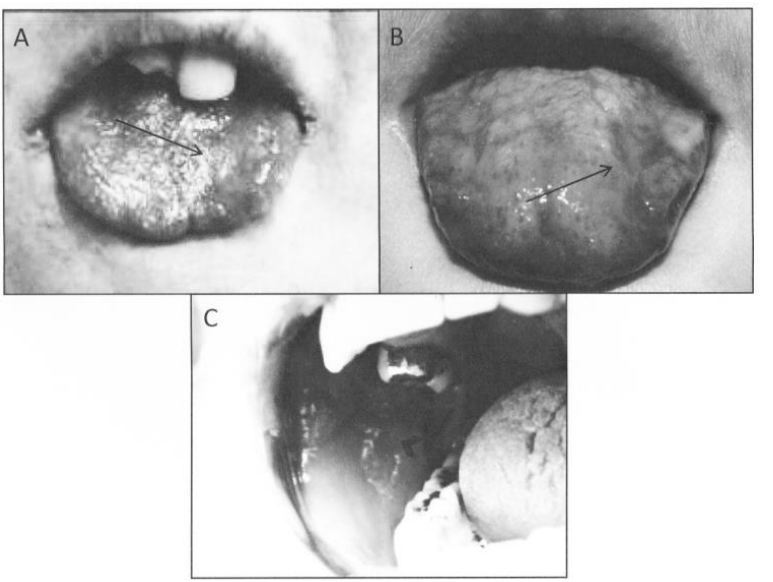

Figure 2. Clinical photographs of patients with oral lesions from Cbl deficiency. Lesions involve the anterior portion of the tongue in A and B (arrows) and the mucosa of the right cheek in C (head of arrow). Photos are kindly provided by Dr. Gianluigi Caccianiga (University of Milan-Bicocca).

Some authors suggested that oral lesions are the initial symptoms of a Cbl-D status, and so the dentist, who is often consulted first, should have a prime opportunity and responsibility to contribute to diagnosis [134, 136].

Patients with atrophic glossitis had a significantly higher frequency of Cbl deficiency than healthy control subjects [137]. Atrophic glossitis is manifested as partial or complete loss of filiform papillae on the dorsal surface of tongue and is characterized by ephitelial atrophy and varying degree of chronic inflammation in the subephitelial connective tissue.

Role of Cbl in Cancer

The classical theory of the multi-step process of cancerogenesis states that cancer results from the accumulation of mutations in oncogenes and tumor suppressor genes in neoplastic transforming cells. In the last decade, this theory has been largely revised following the observation that epigenetic modifications of several genes occurs in the genome of transforming cells, altering gene expression without affecting DNA sequence [138]. Therefore, the current opinion is that human cancer is the consequence of the accumulation of both mutations and epigenetic modifications of specific genes involved in the control of cell proliferation, adhesion and migration [139]. Methylation of DNA, modification of histones, and interfering microRNA (miRNA) collectively represent a group of epigenetic elements dysregulated in cancer.

Targeting the epigenome with compounds that modulate DNA methylation, histone marks, and miRNA profiles represents an evolving strategy for cancer chemoprevention, and these approaches are starting to show promise in human clinical trials [139].

Emerging evidence suggests that nutritional status plays an important role in etiology and pathogenesis of cancer by causing and/or enhancing tumor development, or by preventing and/or inhibiting tumorigenesis [140, 141]. This is supported by a plenty of epidemiological data showing positive or negative correlations between nutrients intake and human cancer incidence [142]. A lot of literature has been devoted to uncover the mechanisms underlying dietary components effects and many data refer to epigenetic modification of genes strictly connected with tumor development. Indeed, old evidence [143] demonstrated that a chronic deficiency of the major dietary methyl group donors – methionine, choline, folic acid and Cbl – can induce the development of cancer in different tissues in rodents. Over the years, a

number of comprehensive studies have demonstrated that diets lacking methyl donors may act as co-carcinogens [144, 145] and, more importantly, as complete carcinogens that can induce tumor formation in the absence of any exogenous carcinogens. This is especially true for human liver [144], prostate [146], colorectal cancer [147] and to a lesser degree, breast carcinoma [148].

DNA methylation represents one of the best studied epigenetic processes in cancer [139]: it consists in the addition of a methyl group to carbon 5 of the cytosine within the dinucleotide CpG. DNA methyltransferases are the key enzymes for DNA methylation while S-adenosylmethionine represents the methyl group donor. The synthesis of S-adenosylmethionine requires the one-carbon units provided by folate metabolism, better known as one-carbon metabolism.

DNA methylation is necessary for controlling gene expression of tissue-specific, housekeeping or imprinted genes and also for maintaining genomic stability. In cancer, hypomethylation usually occurs at repeated DNA sequences, enhancing DNA instability and gene expression [149]; on the contrary, specific methylation of CpG islands in the promoter region of a gene is commonly associated with gene silencing by inducing chromatin conformational modifications inhibiting the access of the transcriptional machinery [149].

Dietary methyl donors have their impact on carcinogenesis through their role in providing one-carbon moieties for the synthesis of nucleotides and of S-adenosylmethionine, the universal donor for nearly all methylation reactions, including that of DNA [150]. Related to their role as one-carbon donors, deficiencies of folate, Cbl and possibly other related nutrients are reported to cause epigenetic instability by promoting genomic hypomethylation and the seemingly paradoxical hypermethylation of specific gene promoters [150].

As previously mentioned, both genomic and gene-specific DNA methylation patterns in various tissues have been shown to be sensitive to one-carbon nutrient availability. During mammalian embryogenesis, DNA methylation patterns are highly labile and experience a wave of genome-wide demethylation, followed by a period of controlled and precise remethylation [151]. Therefore, the integrity of the developing epigenome may be especially sensitive to fluctuation in one-carbon nutrient and methyl group supply during this early stage of life. Beside the adult life, also childhood and infancy and especially the in utero life are, indeed, critical window of time during which cancer risk may be modified by diet. Recent experimental and epidemiological data suggest that maternal intake of dietary methyl donors during gestation

may have an impact on the risk of cancer in offspring later in life [see 149 for a review]. For example, maternal multivitamin use has been related to a reduced incidence in their children of acute lymphocytic leukemia [152], pediatric brain tumors [153] and neuroblastoma [154].

Current literature is nowadays lacking a deep meta-analysis of the real efficacy of a Cbl supplementation in maternal diet towards the risk of developing cancer in offspring; moreover, the association between maternal one-carbon intake and cancer in offspring have been limited to cancer that affect children.

No epidemiological evidence is now available to support or refute the idea that maternal one-carbon nutrient intake can impact the risk of developing adult cancer, as those of the colorectum or breast.

CONCLUSION

Alongside to traditional effects on the nervous and hematopoietic systems, new Cbl roles are emerging. The Cbl effect in the intrauterine period and first months of life is just one of the topic. Other aspects are the importance on the defence system, as well as the complicated interpretation on the gastric system and the role in cancer.

On light of the above, a deep study and knowledge of all possible effects of Cbl are necessary in order to recognize all Cbl-D situations and go with a correct therapeutic approach. Just to name one, the availability of dietary compounds modulating DNA methylation, as Cbl, might be an alternative method to control epigenome in cancer or might represent a useful tool for cancer prevention.

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Chapter 8

PHARMACOLOGICAL USE OF VITAMIN B_{12}: VITAMIN B_{12} IN THERAPY

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ABSTRACT

Vitamin B_{12} (cobalamin (Cbl)) deficiency is a common pathological condition in people with impaired Cbl assumption due to diet (vegans), pernicious anemia or gastric surgery. Specific therapeutic protocols are available according to severity of the disease and to clinical hematological and/or neurological consequences. In this Chapter, the available Cbl formulations (intramuscolar or oral), the range of doses used in different clinical conditions and some specific recommendations for drugs interaction and for specific clinical conditions will be treated.

Keywords: cyanocobalamin; injection; hydroxocobalamin; oral administration

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INTRODUCTION

As previously discussed (see Chapter "Pathological Effects of vitamin B_{12} deficiency"), anemia, cardiovascular disorders and permanent neurologic damage are the main adverse health effects of Cbl deficiency. As humans depend on exogenous sources of Cbl (see "Food source of vitamin B_{12}"), people consuming diets completely free of animal products (vegans) as well as those with pernicious anemia (i.e., malabsorption) are at a greater risk of developing Cbl deficiency [1].

For people with deficiency, the dose of Cbl required varies by severity. The daily requirement of Cbl has been set at 2.4 μg [2], but higher amounts — 4 to 7 μg per day — which are common in persons who eat meat or take a daily multivitamin, are associated with lower methylmalonic acid values (the levels of methylmalonic acid are markedly elevated in the vast majority (>98%) of patients with clinical Cbl deficiency) [3]. However, most patients with clinical Cbl deficiency have malabsorption and will require parenteral or high dose oral replacement. For these reasons, on average 1,000 μg/day is recommended.

Different nutritional guidelines for Cbl intake have been established and published for people with different causes and degrees of Cbl deficiency: general population must refer to the guidelines published by the Food and Nutrition Board [2]. Nutritional guidelines for vegetarians come from the American Dietetic Association [4], while there are no recommendations from the American Society of Hematology for the diagnosis and treatment of Cbl deficiency. The American Academy of Neurology recommends measurements of Cbl, methylmalonic acid and homocysteine in patients with symmetric polyneuropathy [5].

Cbl is available for injection or oral administration; combinations with other vitamins and minerals also can be given orally or parenterally. As it will be deeply discussed further in this Chapter, the choice of one or the other preparation always depends on the cause of the deficiency. Generally, oral preparations should be avoided in patients with a marked deficiency of Cbl leading to abnormal hematopoiesis or neurological deficits and in patients with lack of intrinsic factor (IF) or ileal disease; therefore, Cbl is preferentially administered by intramuscular or subcutaneous injection.

INJECTED VITAMIN B_{12}

There are many recommended schedules for injections of Cbl (cyanocobalamin (CNCbl) in the United States and hydroxocobalamin (HOCbl) in Europe) according to the age of the patients, to the severity of symptoms and to the cause of the deficiency.

The range of *CNCbl* administered doses is 1-1000 μg. Doses >100 μg are rapidly cleared from plasma into the urine, and administration of larger amounts of Cbl will not result in greater retention of the vitamin [6]. Anyway, about 10% of the injected dose (100 of 1000 μg) is retained.

CNCbl injection is safe when given by the intramuscular or deep subcutaneous route, but it should never be given intravenously. There have been rare reports of transitory exanthema and anaphylaxis after injection [7,8]. If a patient reports a previous sensitivity to injections of CNCbl, an intradermal skin test should be performed before the full dose is administered.

HOCbl is the drug of choice to treat Cbl deficiency in Europe. Current clinical practice within the UK is to treat Cbl deficiency with HOCbl in the intramuscular form [outlined in 9]. Standard initial therapy for patients without neurological involvement is 1000 μg intramuscularly three times a week for 2 weeks. Patients presenting with neurological symptoms should receive 1000 μg intramuscularly on alternate days until there is no further improvement, reviewing the need for continuation of alternate day therapy after 3 weeks of treatment [10]. Moreover, it has been reported that HOCbl, given in doses of 100 μg intramuscularly, have a more sustained effect than CNCbl, with a single dose maintaining plasma Cbl concentrations in the normal range for up to 3 months. However, some patients show reductions of the concentration of Cbl in plasma within 30 days, similar to that seen after CNCbl.

HOCbl is generally well tolerated, though side effects include itching, exanthema, chills, fever, hot flushes, nausea and dizziness; in the past, the administration of HOCbl was exceptionally associated with anaphylaptic reaction probably due to hypersensitivity to cobalt or any of the other components of the medication [7].

Due to cross-sensitivity of HOCbl and CNCbl, treatment of patients may be a challenge. Skin patch testing may help to choose an appropriate product [11]. If absolutely necessary, treatment may be considered under hydrocortisone cover in a hospital setting where severe hypersensitivity can be managed.

ORAL VITAMIN B_{12} PREPARATIONS

A wide discussion on the pros and cons of oral versus parental administration of Cbl in patients with different degree of Cbl deficiency is present in the recent literature: key point are not only the efficacy of the formulation and the improvements of clinical signs and symptoms, but also the cost of the therapy and the patient compliance. To this regard, a Cochrane review considering clinical trials about the use of oral Cbl suggests that oral therapy is as effective as intramuscular Cbl, with the benefit for patients of fewer visits to health centres and reduced discomfort of injections [12]. On the other hand, proponents of parenteral therapy state that compliance and monitoring are better in patients who receive this form of therapy because they have frequent contacts with health care providers [13]. In the following paragraphs, a review of the main concepts on this topic will be provided.

High Dose Oral Treatment

High dose oral CNCbl (1000–2000 μg) is licenced for use in several countries except the UK; as it is widely available also via the internet, this treatment is increasingly popular. Passive, IF-independent absorption of a small fraction (0.5 - 4%) of such large doses should suffice to meet daily requirements and might be useful for patients with pernicious anemia, as demonstrated in an old study [14]. Indeed, oral doses of 1000 μg are able to deliver 5 to 40 μg, even if taken with food.

Data from the literature concerning the efficacy of oral treatment are not conclusive. Some data suggest that, in patients with pernicious anemia, atrophic gastritis, or a history of ileal resection, high daily oral doses (2000 μg daily) of CNCbl produce similar reductions in the mean corpuscular volume, increases in the hematocrit and improvement of memory loss compared to parental therapy (seven injections of 1000 μg of CNCbl over a period of 1 month, followed by monthly injections) [15]. The markers of Cbl efficacy were significantly better in patients treated with oral formulations. In a randomized trial comparing oral with intramuscular CNCbl (1000 μg doses, daily for 10 days, then weekly for 4 weeks, and monthly thereafter), the two groups had similar improvements in hematologic abnormalities and Cbl levels at 90 days [16]. As a general, in severely deficient individuals who have poor absorption especially due to pernicious anemia, the use of oral Cbl in initiation of Cbl therapy is not recommended. High dose oral Cbl would be a reasonable

alternative as maintenance in patients unable to tolerate intramuscular injections [13]. Moreover, it is important to remember that data are lacking from long-term studies to assess whether oral treatment is effective when doses are administered less frequently than daily.

Low Dose Oral Treatment

Low doses oral CNCbl (50 μg) are now available and may improve serum Cbl and biochemical markers in borderline cases. Their role in the treatment of subclinical deficiency is under active research, but lacks of definitive approval. Therefore, care must be taken if low dose supplements are prescribed, as a latent and emerging pernicious anemia together with neurological impairments could be underestimated [10].

THERAPEUTIC USES

As already stated, the therapeutic approach depends on the severity of the patient's illness. In *uncomplicated pernicious anemia,* characterized by a mild or moderate anemia without leukopenia, thrombocytopenia, or neurological signs or symptoms, the administration of Cbl alone will suffice. It is important to exclude other causes of megaloblastic anemia and to provide sufficient studies of gastrointestinal function before starting therapy. In this situation, small amounts of parental Cbl (1-10 μg per day) may be useful [13]. It is important to notice that treatment of *pernicious anemia* is lifelong. This fact must be impressed on the patient and family, and a system must be established to guarantee continued monthly injections of Cbl. In patients in whom Cbl supplementation is discontinued after clinical recovery, neurologic symptoms recur within as short a period as 6 months and megaloblastic anemia recurs in several years [17]. Intramuscular injection of 100 μg of CNCbl every 4 weeks is sufficient to maintain a normal concentration of Cbl in plasma and an adequate supply for tissues [13].

Patients with severe neurological symptoms and signs may be treated with larger doses of Cbl in the period immediately after the diagnosis. Doses of 100 μg per day or several times per week may be given for several months with the hope of encouraging faster and more complete recovery [18]. Cbl must be monitored in plasma and peripheral blood counts must be obtained at intervals of 3-6 months to confirm the adequacy of therapy. These evaluations must

continue throughout the patient's life as refractoriness to therapy can develop at any time.

For those patients presenting with Cbl deficiency, but with no nervous system involvement, intranasal preparations of Cbl are suitable for maintenance of Cbl levels, following normalization [19, 20].

When neurological changes or severe leukopenia or thrombocytopenia associated with infection or bleeding are present (*Acutely Ill Patient*), emergency treatment is required. The older individual with a severe anemia (hematocrit <20%) is likely to have tissue hypoxia, cerebrovascular insufficiency and congestive heart failure. In all these cases, it is not useful to delay therapy to wait for a detailed diagnosis. Once the megaloblastic erythropoiesis has been confirmed and sufficient blood collected for later measurements of Cbl and folic acid, the patient should receive intramuscular injections of 100 μg of CNCbl several times per week for 1 to 2 weeks, together with a daily oral supplement of 1 to 2 mg of folic acid. Then therapy is administered weekly until clear improvement is shown, followed by monthly injection [13]. Because an effective increase in red-cell mass will not occur for 10-20 days, the patient with a markedly depressed hematocrit and tissue hypoxia also should receive a transfusion of 2-3 units of packed red blood cells [21]. Patients usually report an increased sense of well-being within the first 24 hours of the initiation of therapy, but full recovery of mental function may take months, or it may never occur; this is especially true for defects present for many months or years. The first objective sign of therapy efficacy is the disappearance of the megaloblastic morphology of the bone marrow. Full correction of precursor maturation in marrow with production of an increased number of reticulocytes begins about the second or third day and peaks 3-5 days later. The rate of recovery of the hematocrit is strictly related to the ability of the marrow to sustain a high rate of production [13] and to the presence of other pathological conditions.

SPECIAL RECOMMENDATION

Metformin. Treatment with metformin in type II diabetes is associated with reduced serum Cbl levels [22] as recently confirmed by a large clinical study comparing metformin-treated diabetics with diabetics not treated with metformin, or non-diabetics [23]; this effect is related to the dose and duration of treatment [24]. However, low serum Cbl levels in metformin treated patients seem not to be associated with biochemical dysbalances [25]; on the

contrary, it seems that despite a low serum Cbl, the intracellular Cbl metabolism is improved in patients taking metformin [25]. Available studies of metabolic profile in diabetics suggest that diabetics have a resistance to Cbl (that is: normal serum Cbl associated with elevation in its metabolic markers) and that metformin enhances Cbl uptake or metabolism [25].

Oral contraception and Hormone replacement therapy. Oral contraceptive use causes a reduction in serum Cbl levels [26; 27], even if the effect is not significant when 'low dose' oral contraception (20 μg ethinyl estradiol) is used [28]. The effect of *hormone replacement therapy* is not conclusive, given that both a reduction of Cbl [29] and no significant effect on Cbl levels [30] have been reported. However, a cross-sectional study of young women taking oral contraceptives did not show any biochemical evidence of impaired Cbl status despite a 25% reduction of serum Cbl [31].

Pregnancy. The interrelationship between pregnancy and Cbl is known since many years: a dietary deficiency of Cbl during pregnancy is associated with significantly smaller size-at-birth [32] and, in turn, pregnancy causes a physiological reduction of total serum Cbl (30%) by the third trimester [33]. When serum Cbl is low and strong clinical suspicion of deficiency is present, the suggestion of pernicious anemia is consistent and mother should be treated as positive, while anti-IF antibodies are checked. In order to limit extensive investigation with resultant anxiety and to treat potential fetal deficiency, three injections of HOCbl are suggested to cover the pregnancy. Serum Cbl levels will be checked 2 months post-partum to ensure resolution to normal levels [10].

CONCLUSION

Cbl has an undeserved reputation as a health tonic and has been used for a number of disease states. Effective use of the vitamin depends on accurate diagnosis and is based on the following general principles of therapy:

- The relative ease of treatment with Cbl should not prevent a full investigation of the etiology of the deficiency, which should involve studies of dietary supply, gastrointestinal absorption, and transport. An immediate therapy, without a precise diagnosis, is needed only in acutely ill elderly patients. They may not be able to tolerate the delay in the correction of a severe anemia.

- Cbl should be given prophylactically only when there is a reasonable probability that a deficiency exists or will exist as, for example, in the strict vegetarian and in gastrectomized patients. When gastrointestinal function is normal, an oral prophylactic supplement of Cbl may be indicated. Otherwise, the patient should receive monthly injections of CNCbl.
- Therapy should be as specific as possible: the available multivitamin preparations (rich of folic acid) can result in a hematologic recovery that masks continued Cbl deficiency and permit neurological damage to develop or progress.

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Chapter 9

FOOD SOURCE OF VITAMIN B_{12}

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ABSTRACT

Vitamin B_{12} (cobalamin (Cbl)) is known to be synthesized only in certain bacteria. Cbl synthesized by bacteria is concentrated and stored mainly in the body of the animals.

For this reason, the usual dietary sources of Cbl are animal-source based foods including meat, milk, eggs, fish, and shellfish. This can be a problem for some groups of people (vegetarians, vegans, elderly people, and people from underdeveloped countries) and so the need to study vegetable sources of Cbl and/or Cbl fortified foods.

Keywords: algae, Cbl content, eggs, fish, fortified food, meat, milk, vegetarians

INTRODUCTION

In USA, the intake recommendations for Cbl are provided in the "Dietary Reference Intakes" developed by the Food and Nutrition Board, Institute of

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Medicine, National Academies [1]. The Dietary Reference Intake values, which vary by age, are equal to 0.4μg/day for newborns and can reach 2.4μg/day for adults (2.6μg/day during pregnancy and 2.8μg/day for lactating woman) [1]. In Europe, the European Parliament sets the daily reference intakes for Cbl in adult at 2.5μg/day [2].

The main sources of Cbl are animal-source based foods, including meat, milk, eggs, fish, and shellfish.

The USDA National Nutrient Database for Standard Reference is realized by the U.S. Department of Agriculture [3]. It is the major source of food composition data in the United States and probably worldwide.

Data on Cbl content in foods described in this chapter are derived from USDA National Nutrient Database, unless otherwise stated. Food is described with relevant characteristics (e.g., raw, cooked etc.) since it has been demonstrated that different types of cooking methods could influence Cbl content or bioactivity. Cooking methods in the culinary arts are essentially divided into two categories: dry heat cooking, such as roasting, broiling or sautéing and moist heat cooking, like braising, steaming or poaching.

It has been demonstrated that the process of roasting and grilling had little effect on the Cbl content in the final product as compared to the raw beef meat [4]. The fried product was characterised by about a 32% lower content of Cbl than in raw meat [4].

A study on round herring's meat demonstrated that Cbl contents were significantly decreased up to ~ 62% during cooking by grilling, boiling, frying, steaming, and microwaving. There was, however, no loss of Cbl during vacuum-packed pouch cooking [5].

Another study has demonstrated that conversion of Cbl to the inactive Cbl degradation products occurs in raw beef, pork, and milk during microwave heating [6]. The latter study on Cbl degradation should be kept into consideration since the intrinsic factor-mediated gastrointestinal absorption system in humans has evolved to selectively absorb active Cbl from naturally occurring Cbl compounds, including its degradation products and inactive corrinoids that are present in daily meal foods [7]. Finally, several studies reported that pasteurization appeared to reduce Cbl levels in milk, however significant variability between studies was noted and pasteurization time could influence the result [8].

USDA National Nutrient Database shows that food with the highest concentration of Cbl is a mollusk, clam cooked with moist heat (almost 100μg of Cbl/100 g total weight) [3]. Other shellfish with elevated level of Cbl are octopus (cooked, moist heat; 36 μg/100 g); oyster (cooked, dry heat;

24µg/100g) and mussel (cooked, moist heat; 24µg/100 g) [3]. Furthermore the broth from clams is also an excellent source of Cbl that contains around 2.7-14.1µg of true Cbl/100 g [9].

The shellfishes are known to be excellent sources of Cbl since they siphon large quantities of Cbl-synthesizing microorganisms that present in water. Moreover, when corrinoid compounds were isolated and characterized in shellfish, such as oysters or mussels and short-necked clams, each corrinoid compound was identified as active Cbl [10].

It is also important to notice that shellfish can be a privileged source of Cbl for some group of populations, i.e., French elderly people in which seafood provides 56% of the French recommended dietary allowance [11].

Analyzing the meat, the organ with the highest level of Cbl is liver (around 85 µg/100 g in lamb, beef and veal) followed by kidney (80 µg/100 g in lamb) and brain (24 µg/100 g in lamb) [3]. All of these concentrations are referred to cooked organs using pan-fried or braised methods. These data confirm the prior role of liver and kidney in Cbl metabolism and the old experimental data [12].

Conversely, noblest cuts of meat present lower concentration of Cbl respect to liver and kidney. For instance, a beefsteak (boneless, outside skirt, separable lean only, trimmed to 0" fat, choice, cooked, grilled) contains “only” 6 µg/100 g [3].

USDA National Nutrient Database shows that Cbl contents of certain fishes, such as salmon, herring, mackerel (cooked, dry heat) are rather high, around 15 µg/100 g [3]. These data are very important since fish (or shellfish) contributes greatly to Cbl intake among Asians and African people and is a basic element of many diets, i.e., mediterranean diet.

Finally, we had to consider two important animal products (eggs and milk with its derivatives) that can be consumed also by some groups of vegetarian, lacto-ovo vegetarians (see below for typing vegetarian groups). Cbl content in the whole boiled egg is not neglectable, about 1µg/100 g (almost all localized in yolk). Eggs provide essential fatty acids, proteins, choline, vitamins A, selenium, and also Cbl at levels above or comparable to those found in other animal-source foods, but they are relatively more affordable [13].

Comparing Cbl content in milk from the most common domestic mammalian species it has been obtained that bovine milk ranged 0.2-0.8µg/100mL, ovine milk 0.4-0.5µg/100mL, and caprine milk 0.07-0.09µg/100mL [14].

Milk and dairy products are significant contributors of Cbl intakes. Indeed, it has demonstrated that in Dutch population dairy products

contributed around the 58% of Cbl intake in young children, and 44%-46% in adults and elderly people [15]. Moreover, milk and milk products seem to be among the lowest-cost sources of Cbl in USA (nutrient costs were calculated by dollar spent to guarantee 10% of the daily Cbl required) [16]. Interestingly, in a population-based study from Norway the level of Cbl in plasma was associated with intakes of increasing amounts of Cbl from dairy products or fish but not with intakes of Cbl from meat or eggs. This suggests that Cbl could be more bioavailable from dairy products [17]. These data were confirmed in an experimental study in pigs in which the intestinal absorption of Cbl after the ingestion of cows' milk was greater than after ingestion of equivalent amounts of cyano Cbl (CNCbl) [18].

During cheese-making procedure a percentage of Cbl contained in starting milk is lost in whey but Cbl concentration could also change during the ripening period due to its utilization and synthesis by microorganisms [19]. For this reason Cbl concentration in different types of cheese vary considerably. In fact, analysing different types of cheese it was identified that the highest concentration of Cbl was in Edam and Mozzarella (around 2.1 µg/g) and the lowest in Roquefort (around 0.39µg/g) [19].

There are several main types of vegetarian groups: (1) Lacto-ovo vegetarians that consume lacto-ovo products (milk, milk products and eggs) but no animal meats (including fish and shellfish); (2) raw vegans whose diet is mostly based on fresh fruits, vegetables, nuts, and seeds; (3) fruitarians that eat only fruits, nuts, and seeds; (4) Buddhist vegetarians that exclude from their diet all animal products and some vegetables of Allium family; (5) macrobiotics who ban from their diet processed foods and most animal products; and (6) Jain vegetarians that consume dairy products, but exclude eggs and honey as well as root vegetables [20]. Vegetarians, especially the most restrictive groups, are considered a high-risk population for Cbl-deficiency and for them it is necessary to identify plant-derived foods that contain high levels of Cbl or adopt Cbl-enriched foods in order to meet the daily recommended Cbl intake. However, since vegetarian diets are rich in folic acid, Cbl deficiency may be masked until severe health problems occur.

Trace amounts of Cbl (< 0.1 µg/100 g of wet weight edible portion) were found in broccoli, asparagus, Japanese butterbur, mung bean sprouts, tassa jute, and water shield probably due to their ability to take up Cbl from certain organic fertilizer [10]. Furthermore, it has been demonstrated that adding an organic fertilizer, such as animal manure, significantly increased the Cbl content in vegetables, although the consumption of several hundred grams of

these vegetables would be insufficient to meet the recommended dietary allowances [20].

The Cbl contents of soybeans are low or undetectable but a fermented soybean-based food called tempe contains a considerable amount of Cbl (0.7-8.0μg/100 g) [10] due to microbial activity during fermentation [21].

Cbl is also found in various types of tea leaves (approximately 0.1-1.2μg Cbl per 100 g dry weight) and black tea seems to have the highest concentration [10, 20]. An experimental study demonstrated that Cbl in fermented black tea is bioavailable in rats (measured as decreased level of urinary methylmalonic acid excretion in Cbl-deficient (Cbl-D) rats treated with black tea) [22].

The fruiting bodies of black trumpet (Craterellus cornucopioides), golden chanterelle (Cantharellus cibarius) and dried shiitake mushroom (Lentinula edodes) contain relatively high levels of Cbl (1.09-2.65μg/100 g dry weight for black trumpet and golden canterelle and 5.61μg/100 g dry weight for shiitake mushrooms) [7, 20]. The form of Cbl in mushrooms was found to be the same form found in beef, liver, and fish, suggesting that it is highly bioavailable [23]. However, in order to take the recommended dietary allowances for this nutrient (2.4μg/day) we should daily consume very large amounts of these mushrooms (approximately 50 g of dried shiitake and 100 g of black trumpet or golden chanterelle) [7, 23].

Various types of edible algae are consumed worldwide as food sources, but only dried green laver (Enteromorpha sp.) and purple laver (Porphyra sp.) contain substantial amounts of Cbl (approximately 63.6μg/100 g dry weight and32.3μg/100 g dry weight, respectively) and inactive corrinoid compounds are not present [19, 24].

However, it is largely debated if Cbl in alga is biovailable or not. A study from Dagnelie et al. reported that in children treated with plant Cbl sources (spirulina and nori alga) the blood Cbl level was increased, but the mean corpuscular volume deteriorated [25].

Vice versa, studies from Takenaka et al. demonstrated that Cbl-D rats supplemented with dried purple laver decrease the urinary methylmalonic acid excretion, until undetectable level, and significantly increase the hepatic Cbl levels [26].

See Figure 1 for a summary of the Cbl content in food.

Recent studies suggest that Cbl-fortified food (fortification refers to “the practice of deliberately increasing the content of an essential micronutrient in food product in order to improve the nutritional quality of the food and thereby

providing a public health benefit with minimal health risk ") may be an alternative source of Cbl.

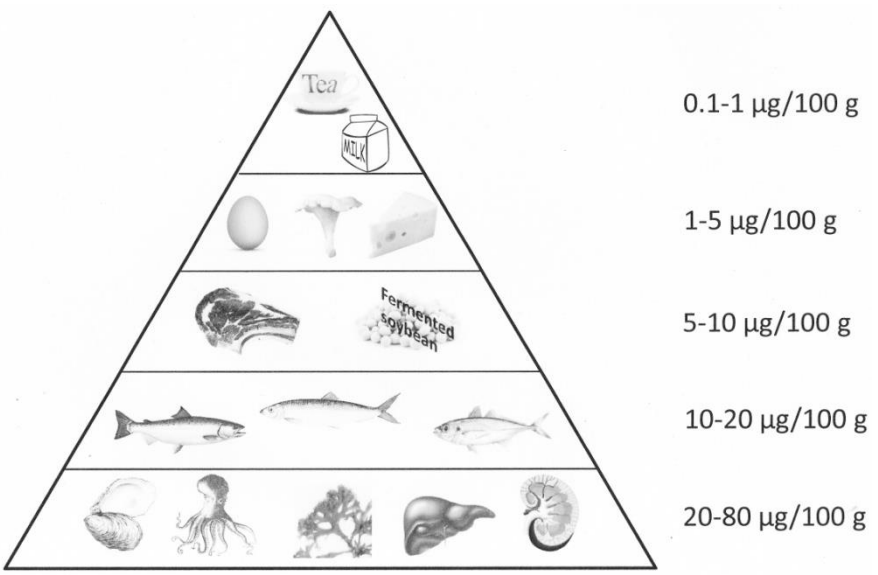

Figure 1. *Cbl content in food.* Cbl quantity in the most representative types of food.

In fortification programs, the goal is to provide a considerable dose of the specific micronutrient to - ideally - the entire population. The suggested additional intake of Cbl through Cbl-fortified food is 1.0µg/day regardless of the age group. Accordingly, the amount of Cbl added to the fortified vehicle would be determined by average consumption patterns [27].

In some countries, as United States [28] and United Kingdom, a large percentage of Cbl was intaken from fortified food and there are many vegan foods fortified with Cbl (i.e., non-dairy milks, meat substitutes, breakfast cereals, nutritional yeast).

In particular, cereals fortified with Cbl comprise a high proportion of the dietary Cbl intake and may become a particularly valuable source of Cbl for the vegetarian groups that accepted processed foods and elderly people [10, 29].

It is important to mention that Selhub et al. recommended that Cbl food fortification should accompany any folic acid fortification in order to avoid the exacerbation of both the biochemical and clinical status of Cbl deficiency sometimes associated to high plasma folate [30].

Despite the fact that Cbl fortification appears to be a good alternative source, we associate to other authors on the need to safety evaluations of long-term exposure to high-dose intake of Cbl fortified food [27].

Finally, *Spirulina platensis* is a cyanobacteria largely used in the food industry. Tablets containing *Spirulina platensis* are sold as dietary supplement, since it is known to contain a large amount of Cbl.

Watanabe et al. [7] found that commercially available spirulina tablets contained a huge amount of Cbl (127-244 µg per 100 g weight), but only 17% was real Cbl and the major compounds (83%) were identified as pseudo vitamin Cbl that could be not so bioavailable in mammals [7, 31]. The datum was confirmed by a study that determined 35-38 µg methyl Cbl/ 100 g dry biomass of *Spirulina platensis* [32].

However, a recent study on Indian pregnant and anemic women demonstrated that combined supplementation of iron-folic acid-Cbl and spirulina produce a higher increase of blood hemoglobin level than an iron-folic acid-Cbl supplementation alone [33].

If eating habits, also supported by fortified foods and supplementation, are not enough to ensure a proper intake of Cbl or other causes of deficiency are present is necessary to take up a drug therapy, in which Cbl is usually present as CNCbl (for details see the Chapter "Pharmacological use of vitamin B_{12} in therapy").

CONCLUSION

Food gives our bodies the nutrients needed to function. For many people, changing eating habits is very hard or sometimes impossible due to economical, social or cultural reasons.

So it is very important a deep knowledge of all Cbl-containing foods, their Cbl concentrations and forms. At the same time, implement the study of alternative solutions (vegetarian or fortified food) target to people with different eating habits is a priority. It is also important to know the correct conservation and cooking techniques to prevent was ting food nutritional properties.

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